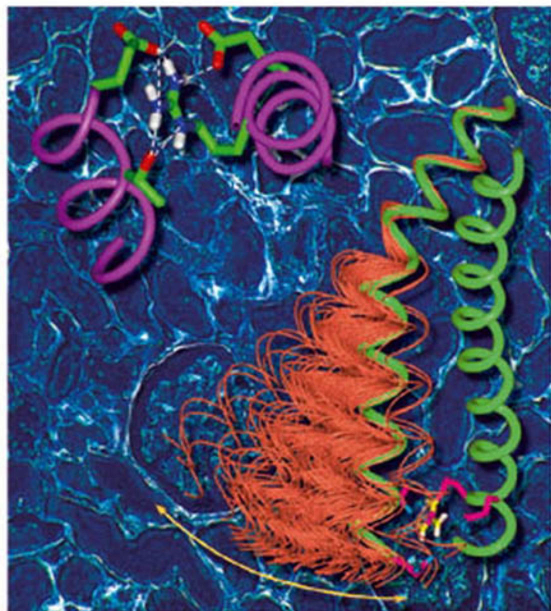
Methods and Principles in Medicinal Chemistry

Edited by
Roland Seifert, Thomas Wieland

G Protein-Coupled Receptors as Drug Targets

Analysis of Activation and Constitutive Activity



Volume 24

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Preface

Why do we address G protein coupled receptors (GPCRs) as drug targets and emphasize their constitutive activity in our series on „Methods and Principles in Medicinal Chemistry“? Taking into account that a large variety of currently used drugs are either agonists or antagonists at GPCRs, the broad interest of medicinal chemists in GPCRs is obvious. But why do we highlight the constitutive activity of GPCRs? When this phenomen was described first in the 1980's it was regarded with high scepticism and often attributed to experimental artefacts. Nevertheless, during the last two decades the phenomenon has gained more and more scientific attention and thus constitutive GPCR activity has been included in the theoretical and molecular models of GPCR activation. Consequently, GPCR ligands are today subgrouped into full agonists, partial agonists, neutral antagonist and inverse agonists. Interestingly, many of the clinically used „GPCR-blockers“ turned out to be not neutral antagonists but inverse agonists at their respective receptors.

Therefore, the present book comprehensively discusses an important biological process that has not yet been covered in such depth in any other existing books on GPCRs. In the first part the international team of authors addresses in detail current models and concepts to introduce medicinal chemists, physiologists, pharmacologists and medical researchers into the advances in the understanding of GPCR activation and constitutive activity. In addition, the book provides a chapter with an overview on methods of investigating constitutive GPCR activity on a cellular and subcellular level. In the second part of the book, the knowledge on constitutive activity of selected important GPCR systems is described in more detail. This includes consequences of constitutive activity for drug action and side effects. Most important, one chapter of the book is attributed to the major unresolved issue of constitutive GPCR activity, i.e. its physiological, pathophysiological and therapeutic relevance.

The series editors believe that this book adds a fascinating facet to the series which is unique in its topic and presentation. We are indebted to the international consortium of highly distinguished authors for their contributions which reflect today's situation in biosciences, i.e., that scientists from many disciplines have to work together closely to advance our knowledge on such important but complex issues. We would like to thank Roland Seifert and Thomas Wieland for their enthusiasm to organize this volume. We also want to express our gratitude to Frank Weinreich from Wiley-VCH for his valuable contributions to this project.

May 2005

Raimund Mannhold, Düsseldorf
Hugo Kubinyi, Weisenheim am Sand
Gerd Folkers, Zürich

A Personal Foreword

When the first observations of constitutive (i.e., agonist-independent) activity of G protein-coupled receptors (GPCRs) were made in the mid-to-late 1980s, probably nobody expected that 15 years later this would be a central theme in the biomedical sciences. Indeed, it is now clear that a large fraction of wild-type GPCRs exhibit different degrees of constitutive activity. In addition, most GPCR antagonists known so far have actually turned out to be inverse agonists, and furthermore, mutations in GPCRs can result in exaggerated constitutive activity and severe human diseases. Analysis of constitutive GPCR activity has also given rise to profound insights into the molecular mechanisms of GPCR activation and is now even exploited for drug development, including ligand identification for orphan GPCRs. During the past 15 years, sophisticated models of constitutive GPCR activity have been developed, and are being continuously refined. We now have in hand a broad spectrum of sensitive experimental methods to study constitutive activity, and many of them can be implemented in most research laboratories. Despite all the progress in the field, a major unresolved question remains. What is the (patho)physiological and therapeutic relevance of constitutive GPCR activity?

Given the complexity of the field, it is not surprising that scientists from many disciplines – classic and molecular pharmacologists, molecular biologists, theoretical biochemists, physiologists, biophysicist, immunologists, neuroscientists, medicinal chemists, and clinical scientists – have made important contributions to the field. Several review articles on different aspects of constitutive GPCR activity are available, but given the multitude of aspects of the field, it is impossible for an individual scientist to write "the" ultimate in-depth review on this topic.

Bearing those thoughts in mind and considering the broad relevance of constitutive GPCR activity to many biomedical disciplines, we developed the idea of putting together a book that covers important aspects of the field. We are very happy that we were successful in motivating many key investigators in the field to contribute to this project. Intentionally, we do not seek to be comprehensive but rather to cover seminal aspects of the field without duplicating existing reviews.

While, of course, each author has her or his own point of view and interpretation of data, there is now general consensus in the scientific community that GPCRs exist in at least one inactive (R) and one active (R*) state. Throughout the book we have tried our best to ensure that consistent IUPHAR nomenclature of GPCRs, pharmacological

terms, and designation of amino acid mutations and the positions of amino acids in transmembrane domains are used in order to avoid confusion, and have also integrated cross-references between chapters to connect different aspects. You can start reading the book wherever you want. Each chapter has its own introduction and stands by itself as an entity. If you are interested in a particular GPCR, we refer you to Table 1.1 in Chapter 1, which will then guide you to the chapter(s) in which your GPCR of interest is discussed.

We are grateful to the authors of this book for their dedication, time, and willingness to consider our critique, suggestions, and formal requests. We are also thankful to editors of the book series and Dr. Frank Weinreich from WILEY-VCH for their advice in the planning stage of the book and to Irene Rupprecht and the staff of WILEY-VCH for bringing all the contributions into a suitable form. We hope that the book will be of use for basic and clinical scientists, experts and non-experts, seasoned scientists, undergraduate students, and graduate students and will serve as a starting point for solving the remaining problems in the field of constitutive GPCR activity.

Mannheim and Lawrence
May 2005 *Roland Seifert and Thomas Wieland*

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Abbreviations and Terminology

α-adrenoceptor	αAR
α-adrenoceptor, subtype 1	α_1AR, α_{1A}AR, α_{1B}AR, α_{1D}AR
α-adrenoceptor, subtype 2	α_2AR, α_{2A}AR, α_{2B}AR, α_{2D}AR
(*S*)-(+)-α-fluoromethylhistidine	α-FMH
β-adrenoceptor	βAR
β-adrenoceptor, subtype 1	β_1AR
wild-type β_1AR	β_1AR_{wt}
β-adrenoceptor, subtype 2	β_2AR
constitutively active β_2AR mutant	β_2AR_{CAM}
wild-type β_2AR	β_2AR_{wt}
β_3-adrenoceptor	β_3AR
κB *cis*-enhancer element	κB
arachidonic acid	AA
(4'-[3-((3*R*)-3-dimethylaminopyrrolidin-1-yl) propoxy]biphenyl-4-carbonitrile	A-331440
adenylyl cyclase	AC
AC isoforms I to IX	AC I to IX
adenosine deaminases	ADAR1 and ADAR2
attention-deficit hyperactivity disorder	ADHD
4-*n*-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine hydrogen chloride	AC-42
agouti-related protein	AgRP
(–)-alprenolol	ALP
adapter protein	AP
activator protein 1	AP1
activator protein 1/tetradecanoyl phorbol acetate-response element	AP-1/TRE
adrenoceptor	AR
arginine	Arg
aspartate	Asp

angiotensin II type 1 receptor	AT_1R
angiotensin II subtype 1A receptor	$AT_{1A}R$
leukotriene B_4-receptor	BLTR
2-bromolysergic acid diethylamide	BOL
bradykinin receptor	BR
bradykinin B2-receptor	B_2R
bioluminescence resonance energy transfer	BRET
complement C5a receptor	C5aR
constitutively active mutant	CAM
cyclic AMP	cAMP
cAMP enzyme immunoassay	cAMP-EIA
Ca^{2+}/calmodulin-dependent protein kinases	CaMKs
(*Rp*)-adenosine-3':5'-cyclic monophosphothioate triethylamine	(*Rp*)-cAMPS
capri pox virus	CaPV
calcium-sensing receptor	CaSR
calcium-permeable voltage-sensitive channel subunit 2.1	Cav2.1
cannabinoid receptor	CBR
cholecystokinin receptor	CCKR
cholecystokinin receptor subtype 2	CCK_2R
[(±)-4-(3-*tert*-butylamino-2-hydroxypropoxy) benzimidazol-2-one]	CGP 12177A
(±)-2-hydroxy-5-[2-({2-hydroxy-3-[4-(1-methoxy-4-trifluoromethyl-1*H*-imidazol-2-yl)phenoxy]propyl}amino) ethoxy]-benzamide	CGP 20712
(±)-2-hydroxy-5-[2-({2-hydroxy-3-[4-(1-methyl-4-trifluoromethyl-1*H*-imidazol-2-yl)phenoxy]propyl}amino) ethoxy]-benzamide monomethanesulfonate	CGP 20712A
nicotinoyl-Tyr-Lys(Z-Arg)-His-Pro-Ile-OH	CGP42112A
Chinese hamster ovary	CHO
Chinese hamster fibroblast	CHW
C terminus of the i3 loop	Ci3
casein kinase 2	CK2
chemokine-binding protein	CKBP
cytomegalovirus	CMV
cyclic nucleotide-gated	CNG
African green monkey kidney cells	COS-7
counts per minute	cpm
1-(3-chlorophenyl)piperazine	*m*-CPP
cAMP response element	CRE
cow pox virus	CPV
cAMP-response element binding protein	CREB
cAMP-response element binding protein/cAMP response element	CREB/CRE

cyclosporin H	CsH
carboxy-terminal	C-t
cubic ternary complex model	CTC model
cytotoxic T lymphocyte antigen 4	CTLA-4
chemokine receptors	CXCR, CCR
cysteine	Cys
DADLE	([D-Ala^2, D-Leu]enkephalin)
1,2-diacylglycerol	DAG
4-diphenylacetoxy-*N*-methylperidine.	4-DAMP
dihydroalprenolol	DHA
Dulbecco's modified Eagle medium	DMEM
4-iodo-2,5-dimethoxyphenylisopropylamine	DOI
δ-opioid receptor	DOP(δ)R
dopamine receptor	DR
dopamine receptor, subtype 1	D_1R, $D_{1A}R$
dopamine receptor, subtypes 2 and 3	D_2R, D_3R
Asp-Arg-Tyr motif	DRY
Epstein Barr virus	EBV
effective concentration 50%	EC_{50}
extracellular domain	ECD
extracellular loop	ECL
extracellular loop 2	ECL2
enhanced green fluorescent protein and the pleckstrin homology domain of the PLCδ1	EGFP-$PH_{PLC\delta}$
epidermal growth factor receptor	EGFR
equine herpes virus type 2	EHV-2
nitric oxide synthase type 3	eNOS
prostaglandin E_2 receptor	EPR
electron paramagnetic resonance spectroscopy	EPR
prostaglandin E_2 receptor, subtype 3	EP_3R or $EP_{3\gamma}R$
endoplasmic reticulum	ER
extracellular signal-related kinase	ERK
extracellular signal-regulated protein kinase 1/2	ERK1/2
Glu-Arg-Leu motif	ERL motif
extended ternary complex model	ETC model
enabled Vasp homology	EVH
Ena/VASP homology 1/Wiskott–Aldrich syndrome protein homology 1	EVH1/WH1
focal adhesion kinase	FAK
Federal Drug Administration	FDA
N-formyl-L-methionyl-L-leucyl-L-phenylalanine	fMLP

prostaglandin $F_{2\alpha}$ receptor	FPR
formyl peptide receptor	FPR1
fluorescence resonance energy transfer	FRET
follicle stimulating hormone receptor	FSHR
Fourier transform infrared	FTIR
γ-aminobutyric acid	GABA
γ-aminobutyric acid receptor, subtype B	$GABA_BR$: $GABA_{B1}R$ (GBR1) and $GABA_{B2}R$ (GBR2)
glycosaminoglycan	GAG
guanosine 5'-diphosphate	GDP
guanine nucleotide exchange factor	GEF
green fluorescent protein	GFP
G protein-coupled receptor interacting proteins (or GPCR interacting proteins)	GIPs
glutamate	Glu
gonadotropin-releasing hormone receptor	GnRHR
guinea pig	gp
G protein-coupled receptor	GPCR
guanosine 5'-[β,γ-imido]diphosphate	GppNHp
guanine nucleotide binding protein	G protein
3-[3-(dimethylamino)propyl]-4-hydroxy-*N*-[4-(4-pyridinyl)phenyl]benzamide dihydrochloride	GR-55562
G protein-coupled receptor kinase	GRK
glycogen synthase kinase 3β	GSK3β
guanosine 5'-triphosphate	GTP
guanosine 5'-[γ-thio]triphosphate	GTPγS
Hank's buffered saline solution	HBSS
human cytomegalovirus	HCMV
heptahelical domain	HD
histidine decarboxylase	HDC
human embryonic kidney	HEK
human herpes virus *n*	HHV*n*
5-hydroxyindoleacetic acid	5-HIAA
hypoxia-inducible factor 1α	HIF-1α
human immunodeficiency virus	HIV
histamine receptor	HR
histamine receptors, subtypes 1 to 4	H_1R, H_2R, H_3R, H_4R
5-hydroxytryptamine or serotonin	5-HT
5-hydroxytryptamine receptor	5-HTR
5-hydroxytryptamine receptor, subtype 1	5-HT_1R, 5-$HT_{1A}R$
5-hydroxytryptamine receptor, subtype 2	5-HT_2R, 5-$HT_{2C}R$

5-hydroxytryptamine receptor, subtypes 3 to 7	5-HT_3R, 5-HT_4R, 5-HT_5R, 5-HT_6R, 5-HT_7R
herpes virus saimiri	HVS
intracellular loop 2	i2
intracellular loop 3	i3
N,N'-dimethyl-*N*-(iodoacetyl)-*N'*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-ethylenediamide	IANBD
isobutylmethylxanthine	IBMX
inhibitor concentration 50%	IC_{50}
peak L-type Ca^{2+} current	I_{Ca}
intercellular adhesion molecule-1	I-CAM-1
((±)-1-[(7-methyl-2,3-dihydro-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol) or *erythro*-DL-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol	ICI 118551
[*N,N'*-diallyl-Tyr^1,$Aib^{2,3}$]Leu^5-enkephalin	ICI 174864
immediate-early	IE
hyperpolarization-activated current	I_f
muscarinic acetylcholine receptor-gated atrial potassium channel	I_{KACh}
"unedited" ($Ile^{156(3.54)}$, $Asn^{158(3.56)}$, $Ile^{160(3.58)}$) isoform of human brain 5-HT_{2C}R	INI
inositol phosphate	IP
inositol bisphosphate	IP_2
inositol 1,4,5-trisphosphate	IP_3 or InsP_3
(–)-isoproterenol	ISO
inosine 5'-triphosphate	ITP
International Union of Pharmacology	IUPHAR
Janus kinase/signaling transducer and activator of transcription	Jak/STAT
1-[(5-chloro-1*H*-indol-2-yl)carbonyl]-4-methylpiperazine	JNJ7777120
c-Jun amino-terminal kinase	JNK
dissociation constant	K_d
knock-out	KO
Kaposi's sarcoma	KS
Kaposi's sarcoma herpes (or "sarcoma-associated") virus	KSHV
KS-derived KSHV-negative endothelial cell line	KSIMM
luteinizing hormone	LH
luteinizing hormone receptor	LHR
littermate	LM
lysergic acid diethylamide	LSD

lumpin skin disease virus	LSDV
leukotriene B_4	LTB_4
mitogen-activated protein	MAP
mitogen-activated protein kinase	MAPK
[INLKALAALAKALL-NH_2]	Mas-7
mouse cytomegalovirus	MCMV
melanocortin receptor	MCR
melanocortin receptor, subtypes 1, 3 and 4	MC_1R, MC_3R, MC_4R
molluscum contagiosum virus	MCV
molecular dynamics	MD
1-[2-(4-fluorophenyl)ethyl]-4-piperidinemethanol	MDL100907
Marek's disease virus	MDV
metabotropic glutamate receptor	mGluR
metabotropic glutamate receptor subtype 1	$mGlu_1R$
metabotropic glutamate receptor subtype 5	$mGlu_5R$
major histocompatibility class	MHC
murine γ-herpes virus 68	MHV68
μ-opioid receptor	MOP(μ)R
muscarinic acetylcholine receptor	MR
muscarinic acetylcholine receptor, subtypes 1 to 5	M_1R, M_2R, M_3R, M_4R, M_5R
(–)-5,9α-diethyl-2-(3-furyl-methyl)-2'-hydroxy-6,7-benzomorphan	MR 2266
ethylammonium methanethiosulfonate	MTSEA
myxoma virus	MV
naloxone benzoylhydrazone	NalBzOH
Na^+/Ca^{2+}-exchanger	NCX
nuclear factor κB	NF-κB
nuclear factor κB/NF-κB cis-enhancer element	NF-κB/κB
nuclear factor of activated T cells	NFAT
N terminus of the i3 loop	Ni3
N-methyl scopolamine	NMS
nitric oxide/cGMP-dependent protein kinase	NO PKG
former acronym for nucleotide-binding protein (= G protein)	N protein
nucleoside 5'-triphosphate	NTP
8-hydroxy-2-(di-*n*-propylamino)tetralin	8-OH-DPAT
opoid receptor	OPR
"open reading frame"	ORF
((–)-2-cyano-1-methyl-3-[(2*R*,5*R*)-5-(1*H*-imidazol-4(5)-yl)tetrahydrofuran-2-ylmethyl]guanidine	OUP-16

p38-mitogen activated kinase	p38 MAPK
platelet-activating factor receptor	PAFR
1-(1-phenylcyclohexyl)piperidine	PCP
phosphodiesterase	PDE
PSD95/DLG/ZO-1	PDZ
prostaglandin $F_{2\alpha}$	$PGF_{2\alpha}$
pleckstrin homology domain of PLCδ1	$PH_{PLC\delta}$
post infection	p.i.
phosphatidylinositol 3'-kinase	PI3-Kinase, PI3K
phosphatidylinositol 4,5-bisphosphate	PIP_2
protein kinase A	PKA
protein kinase B	PKB
protein kinase C	PKC
cGMP-dependent protein kinase	PKG
phospholipase A_2	PLA_2
phospholipase C	PLC
phospholipase C-β isozymes	PLCβs
phospholipase D	PLD
proline	Pro
parathyroid hormone	PTH
parathyroid hormone receptor subtype 1	PTH1R
pertussis toxin	PTX
proline-rich kinase 2	Pyk2
quinuclidinyl benzilate	QNB
inactive form of a GPCR	R
active form of a GPCR	R*
related adhesion focal tyrosine kinase	RAFTK
ground state form of a GPCR	Rg
regulator of G protein signaling	RGS
rho guanine nucleotide exchange factor	RhoGEF
(*S*)-2-(6-chloro-5-fluoroindol-1-yl)-1-methylethylamine hydrochloride	Ro 60-0175
receptor selection and amplification technology	R-SAT
respiratory syncytial virus	RSV
ryanodine receptors	RyRs
(*N*-[1-(2,3-dihydro[1,4]dioxin-5-yl)piperidin-4-yl] indan-2-ylamine)	S18127
5-methyl-1-(3-pyridylcarbamoyl)-1,2,3,5-tetrahydropyrrolo [2,3-f]indole hydrochloride	SB-206553
1'-methyl-5-{[2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl) biphenyl-4-yl]carbonyl}-2,3,6,7-tetrahydrospiro[furo [2,3-f]indole-3,4'-piperidine] oxalate	SB-224289

1'-ethyl-5-[2'-methyl-4'-(5-methyl-1,3,4-oxadiazol-2-yl) biphenyl- 4-carbonyl]-2,3,6,7-tetrahydrospiro[furo [2,3-f]indole-3,4'-piperidine]	SB-224289
6-chloro-5-methyl-1-[6-(2-methylpyridin-3-yloxy) pyridin-3-ylcarbamoyl]indoline	SB-242084
5-methyl-1-{2-[(2-methyl-3-pyridyl)oxy]-5-pyridyl]carbamoyl}-6-trifluoromethylindoline hydrochloride	SB-243213
substituted cysteine accessibility method	SCAM
sodium dodecylsulfate	SDS
Spodoptera frugiperda	Sf9
smooth muscle cell	SMC
single nucleotide polymorphism	SNP
surface plasmon resonance	SPR
swine pox virus	SPV
N-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-pyrazole-3-carboxamide	SR 141716A
serum response element	SRE
serum response factor	SRF
somatostatin 2-receptor	$SRIF_2R$
simian virus 40	SV40
trichloroacetic acid	TCA
T cell factor	TCF
transmembrane (domain)	TM
*n*th transmembrane domain	TM*n*
receptor for thromboxane A_2	TPR
thyroid stimulating hormone	TSH
tetradecanoyl phorbol acetate-response element	TRE
thyroid stimulating hormone receptor	TSHR
thyrotropin-releasing hormone receptor	TRHR
transient receptor channels 1 and 4	TRPC1, TRPC4
5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)-6-quinoxalinamine	UK14304
vasopressin receptor	VR
vasodilator-stimulated phosphoprotein	VASP
viral Bcl-2	vBCL-2
vascular cell adhesion molecule-1	VCAM-1
vascular endothelial growth factor	VEGF
"fully edited" ($Val^{156(3.54)}$, $Gly^{158(3.56)}$, $Val^{160(3.58)}$) 5-$HT_{2C}R$ isoform identified in human brain.	VGV
viral interferon factor 1	vIRF-1
1-[5-(imidazol-4-yl)pentyl)-3-(4-chlorophenylmethyl)thiourea	VUF 4742
[5-(1*H*-imidazol-4-yl)-pentyl]-isopropyl-amine	VUF 4904
4-[3-(1*H*-imidazol-4-yl)propyl]piperidine	VUF 5681

(5-chloro-1*H*-benzo[d]imidazol-2-yl)-(4-methylpiperazin-1-yl)methanone	VUF 6002
vaccinia virus	VV
N-{2-[4-(2-methoxyphenyl)piperazin-1-yl]ethyl}-N-(2-pyridyl)-cyclohexanecarboxamide	WAY 100635
xanthosine 5'-triphosphate	XTP
neuropeptide Y receptor	YR
neuropeptide Y receptor, subtype 1, 2, and 4	Y_1R, Y_2R, Y_4R
Yaba-like disease virus	YLDV

I
General Concepts

1
Historical Background and Introduction

Richard A. Bond and Robert J. Lefkowitz

Paul Ehrlich (1854–1915) and John Newport Langley (1854–1936) are generally credited with the introduction of the concept of receptors or receptive substances to describe the interaction of drugs with cells. A few years later, Alfred J. Clarke (1885–1941) began the process of applying mathematical modeling to the ligand/receptor interaction, and could thus be said to be the father of modern receptor theory. Receptor theory was then modified and expanded by others: Ariens' concept of partial agonists, Stephenson's seminal paper on efficacy, and Furchgott's modification of Stephenson's theory to produce the system-independent concept of intrinsic efficacy. (For a detailed account of the evolution of receptor theory and references see Kenakin, 2004 [1].) These developments, along with other contributions such as the Schild regression analysis, had receptor theory firmly established by the 1960s.

However, the theory was still very much based on the 'black box' concept; many scientists were still highly dubious that receptors existed as distinct proteins or entities. By the 1980s, new discoveries had begun to change the 'black box' concept. One was the cloning of receptors and another was the clear separation of ion channel receptors from receptors coupled to the newly discovered G proteins (initially also referred to as N-proteins, as an acronym for nucleotide-binding proteins). The discovery of G proteins also produced a modification of receptor theory to include precoupled receptors in what is now termed the ternary complex model. Thanks to technological advantages, scientists working on ion channels were able to record the activity of a single ion channel and realized it had a probability of being in the open state irrespective of the presence of ligand. Ligands simply altered the probability of it being in the open state. Because of this, it was easier to comprehend that 'baseline' activity could be accounted for by the probability of the channel being in the open state. Accordingly, it appeared possible to find ligands for receptors modulating these channels not only to increase their probability of being in the open state, but also to decrease this probability. Indeed, it was in the context of the GABA–benzodiazepine–receptor complex (GABA = γ-aminobutyric acid) that the term "inverse agonist" was first used to describe the allosteric modulation of the receptor complex by the benzodiazepines in a manner opposite to the modulation produced by GABA.

For G protein-coupled receptors (GPCRs), the skepticism about their existence vanished with the cloning of the first members of the superfamily, such as the β_2-adre-

noceptor (β_2AR) [2, 3], but as far as receptor theory for GPCRs was concerned, there was no apparent need to postulate spontaneously active GPCRs. Receptor theory appeared to work quite nicely with only two classes of ligands, agonists and antagonists, and one quiescent state of the receptor.

However, evidence slowly began accumulating that, at least in reconstituted systems, empty or unliganded receptors could couple to G proteins [4]. This was followed by several studies providing more functional evidence in cell lines and membranes for direct activation or inhibition of second messenger assays by empty receptors and the notion that certain antagonists could prevent this basal activation [5–10]. Though a lot of the evidence came from studies using βARs, other GPCRs were shown to exhibit constitutive activity as well. In 1989, for example, Costa and Herz described the constitutive activity of δ-opioid receptors (DOP(δ)Rs) natively expressed in NG-108 neuroblastoma cells [5]. By substitution of potassium for sodium in the assay medium, they were able to enhance 'baseline' (unstimulated) high-affinity GTP hydrolysis used as an index of receptor–G protein coupling (see Chapter 8), thus demonstrating the constitutive activity of DOP(δ)Rs. This study also demonstrated that certain compounds termed 'negative antagonists' could decrease this raised baseline. These 'negative antagonists' had previously been classified as DOP(δ)R antagonists. However, not all opioid receptor antagonists were able to produce the decrease in baseline; some had little effect on baseline and were therefore called 'neutral antagonists'. The term 'negative antagonist' has now been largely replaced with the term 'inverse agonist', in part because of the IUPHAR Receptor Nomenclature Committee's recommendation. In 1993 and 1994, additional papers were published showing constitutive activity of other GPCRs, and compounds that behaved as inverse agonists at the receptors, most notably for 5-hydroxytryptamine receptors (5-HTRs) [11], bradykinin B2-receptors (B_2Rs) [12], and α_2-adrenoceptors (α_2Rs) [13].

At the same time, evidence started accumulating that GPCRs could also be mutated (usually in the third intracellular loop) to reveal a more robust constitutive activity, and that this spontaneous activity could again be modified by certain ligands. In fact, constitutively active mutant GPCRs were discovered serendipitously. Susanna Cotecchia, working in the Lefkowitz laboratory, had created a chimeric α_{1B}-adrenoceptor (α_{1B}AR), in which a short homologous stretch of amino acid residues from the α_{1B}AR was exchanged into the C-terminal portion of the third cytoplasmic loop of the β_2AR [14]. It was expected that this would decrease α_{1B}AR coupling to its cognate G protein, G_q. Instead, it unexpectedly led to agonist-independent – that is, constitutive – activity. Subsequently, it was demonstrated that virtually any amino acid replacement at a specific site in this region (alanine 293) resulted in graded levels of constitutive activity (see Chapter 11). This suggested that only the naturally occurring residue at this position was compatible with a completely constrained or inactive conformation of the receptor [15]. Subsequently, similar findings were published for the ß$_2$AR [16] and then the α_2AR [13]. As discussed below, these findings necessitated a rethinking of classical receptor theories, such as the ternary complex model. An extended ternary complex model (ETC model), which explains these findings, adds an explicit isomerization step regulating the formation of the so-called active or R* receptor from R, the inactive form (see Chapters 2 and 3) [20]. In this model, the elevated constitutive ac-

tivity of mutant receptors is due to an increase in the isomerization (i.e., mutant receptors are more prone to adopt the active or R* conformation spontaneously in the absence of agonists). The model also predicts the experimentally verifiable findings that both agonists and partial agonists have higher affinity for the constitutively active mutant receptors in proportion to their efficacy [16].

One of the key questions that had to be addressed in these studies was whether it truly was constitutive activity or simply contamination with endogenous hormone or neurotransmitter that was producing the activated receptors. To address this issue, it became necessary to block the effects of the inverse agonist by use of a 'neutral' antagonist. Such experiments were performed in all of the studies above except the bradykinin B_2R study, which relied on showing the absence of bradykinin in the system. The field had now generated enough interest and a review has been published [17]. However, as the title of that review – "Inverse agonism: Pharmacological curiosity or potential therapeutic strategy?" – suggested, there was still a great deal of skepticism as to the physiological relevance of the constitutive activity and inverse agonism.

Much of the skepticism about the phenomena appeared to involve the lack of physiological data in support of constitutive activity and inverse agonism. The data generated so far had all been obtained in cell lines and membranes often manipulated to include substantial overexpression of the receptors or mutated receptors. One of the first studies in a physiological system to imply constitutive activity and inverse agonism was performed in isolated guinea pig and human cardiac myocytes (see Chapter 10) [18]. In 1995, the first physiological report of the use of transgenic mice cardiac-specifically overexpressing the β_2AR was published [19]. This study showed that whether one used membranes and measured cAMP formation, or used the isolated atria and measured isometric tension, or measured an index of cardiac contractility *in vivo*, it was possible to restore the elevated levels of all three indices back to normal with the β_2AR inverse agonist ICI 118 551 (($\pm$)-1-[(7-methyl-2,3-dihydro-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol), but not with the neutral antagonist alprenolol. Furthermore, alprenolol could be used to block the inverse agonist effects of ICI 118 551.

With regard to receptor theory, the most obvious consequence of spontaneously active receptors was the need for at least two states of the receptor, and the inclusion of inverse agonists in addition to agonists and antagonists as a class of ligands. The modeling began to change because of the necessity to extend the ternary complex model. Several models were proposed, ranging in complexity from the two-state model to the ternary cubic (and extended ternary cubic) model (see Chapters 2 and 3) [16, 20, 21]. While the two-state model remains very useful in its predictive ability for many conditions, there is now considerable evidence for multiple receptor states and for the ability of ligands to enrich different states preferentially (see Chapter 9) [22–24]. Indeed, the existence of multiple receptor states produced a subtle but important shift in the concept of ligand efficacy. The notion of efficacy as being the ligand's ability to *induce* a conformational change of the receptor, through which it now gained affinity for the signaling component (usually the G protein), was replaced by the notion that the ligand simply *selected* or stabilized an already existing conformational state and thereby produced its enrichment [33].

Thus, constitutively active GPCRs and inverse agonism have evolved along the same path as most discoveries: skepticism about isolated reports, criticisms about methodologies and systems, and the physiological relevance of the findings. Many of the authors of the pioneering articles discussed so far are contributing authors to this volume. Now the focus has turned to the question of whether there is any therapeutic relevance to the difference between antagonists and inverse agonists (see Chapters 7, 12, 13, and 14). This issue is complicated by the fact that many of the drugs on the market labeled as blockers or antagonists are in fact inverse agonists. A recent publication surveyed the literature for the percentage of antagonists and inverse agonists, and out of several hundred compounds tested, the overwhelming majority are actually inverse agonists [1].

There is no doubt that, at least in theory, inverse agonists would have a distinct advantage over antagonists in treating diseases produced by constitutively active mutants (CAMs). For example, inverse agonists directed at constitutively active thyroid stimulating hormone (TSH) receptors might be used to treat 90% or more of sporadic hyperfunctioning thyroid nodules, which are due to activating mutations in the TSH receptor. Diseases caused by constitutively activating mutations have also been reported in the case of receptors for luteinizing hormone, parathyroid hormone, and a growing list of other ligands [25]. In all of these cases, inverse agonists might theoretically be of therapeutic value.

Some data can be interpreted in support of therapeutic differences between antagonists (or very weak partial agonists) and true inverse agonists in non-CAM diseases. Specifically, a study using cardiac myocytes from congestive heart failure patients revealed that both carvedilol and metoprolol behaved as inverse agonists, while bucindolol was an antagonist (on average) (see Chapter 7) [26]. This correlates with their clinical efficacy in chronic heart failure: carvedilol and metoprolol are beneficial at reducing mortality, while bucindolol is not [27–30]. However, the mechanism of the beneficial effect can also be explained by ligand-directed trafficking of the receptor [23–24]; the issue is also applicable to other disease states such as schizophrenia (see Chapter 14) [32]. Similar data have been obtained in a murine model of asthma in which chronic treatment with the inverse agonists carvedilol and nadolol produces a reduction in peak airway resistance, while the weak partial agonist alprenolol does not [31]. This study tested the hypothesis that the opposing effects of agonists and inverse agonists may also extend to their effects over time: agonists acutely increase signaling, but when given chronically may decrease signaling due to desensitization mechanisms, while inverse agonists may cause the exact opposite [31].

Over the past dozen years or so, the intimately linked concepts of constitutive activity of GPCRs and the existence of inverse agonists have been validated and have become part of the mainstream of thinking in receptor biology. As this volume demonstrates, these ideas have provided fruitful avenues for experimentation and theory in numerous areas of GPCR receptor biology. It seems likely that in the years ahead this body of experimentation and theory will ultimately give rise to novel therapeutics. All these topics will be discussed in this volume. Table 1.1 provides an overview of the various GPCRs discussed in the individual chapters so that the reader interested in a specific GPCR can easily find the desired information.

Table 1.1 Classification of the G protein coupled receptors discussed in this book. Numbers in bold indicate chapters specifically dealing with these receptors.

GPCR	IUPHAR nomenclature	Appearance in Chapter
Class A Receptors		
5-Hydroxytryptamine (serotonin) receptors	5-HTR	**14**, 1, 4
Subtype 1	$5\text{-}HT_1R$	**14**
	$5\text{-}HT_{1A}R$	**14**, 6, 7
	$5\text{-}HT_{1B}R$	**14**, 7
	$5\text{-}HT_{1D}R$	**14**, 7
Subtype 2	$5\text{-}HT_2R$	**14**
	$5\text{-}HT_{2A}R$	**14**
	$5\text{-}HT_{2C}R$	**14**, 5, 7
Subtype 3	$5\text{-}HT_3R$	**14**, 13
Subtype 4	$5\text{-}HT_4R$	**14**, 4, 5
	$5\text{-}HT_{4A}R$	4
	$5\text{-}HT_{4B}R$	4
	$5\text{-}HT_{4E,F}R$	4
Subtype 6	$5\text{-}HT_6R$	**14**
Subtype 7	$5\text{-}HT_7R$	**14**, 4
	$5\text{-}HT_{7A}R$	**14**, 4
	$5\text{-}HT_{7B}R$	4
	$5\text{-}HT_{7D}R$	4
Adrenoceptors	AR	**11**
α-Adrenoceptors	αAR	12
Subtype1	α_1AR	**11**, 7
	$\alpha_{1A}AR$	**11**
	$\alpha_{1B}AR$	**11**, 1, 2, 3, 9, 12, 13, 14
	$\alpha_{1D}AR$	**11**
Subtype 2	α_2AR	**11**, 1, 2, 7, 13
	$\alpha_{2A}AR$	6
	$\alpha_{2B}AR$	**11**
	$\alpha_{2C}AR$	**11**
β-Adrenoceptors	βAR	**9**, **10**, 2, 3, 4, 11, 12, 13
Subtype 1	β_1AR	**9**, **10**, 6, 7,11
Subtype 2	β_2AR	**9**, **10**, 1, 2, 3, 4, 6, 7, 11, 13, 14, 15
Subtype 3	β_3AR	**9**, **10**, 11
Angiotensin II receptors	AT_1R	3
Bradykinin receptors	BR	2
Subtype 2	B_2R	1, 2
Cannabinoid receptors	CBR	
Subtype 1	CB_1R	2, 9
Chemokine receptors	CXCR1	**15**
	CXCR2	**15**
	CXCR4	**15**, 7
	CCR1	**15**
	CCR5	7

Table 1.1 continued.

GPCR	IUPHAR nomenclature	Appearance in Chapter
Class A Receptors		
	CCR6	**15**
	CX_3CR1	**15**
	XCR1	**15**
Cholecystokinin receptors	CCKR	3
Subtype 2	CCK_2R	5
Complement C5a receptor	C5aR	8
Dopamine receptor	DR	
Subtype 1	D_1R	7
Subtype 2	D_2R	3, 7, 14
Subtype 3	D_3R	7
Subtype 4	D_4R	7
Subtype 5	D_5R	5
Formyl peptide receptors		
Subtype 1	FPR1	**8**, 5
Glycoprotein hormone receptors		
Follicle-stimulating hormone receptors	FSHR	5, 7
Lutenizing hormone receptors	LHR	5, 7, 14
Thyroid-stimulating hormone receptors	TSHR	1, 5, 7, 14
Gonadotrophin-releasing hormone receptors	GnRHR	3
Histamine receptors	HR	
Subtype 1	H_1R	**13**, 7
Subtype 2	H_2R	**13**, 2, 3, 7, 9, 11, 15
Subtype 3	H_3R	**13**, 7
Subtype 4	H_4R	**13**
Leukotriene B4 receptors	BLTR	**8**
M-cholinoceptors	MR	**12**, 7, 13, 14
Subtype 1	M_1R	**12**, 3, 11
Subtype 2	M_2R	**12**
Subtype 3	M_3R	**12**, 3
Subtype 4	M_4R	**12**
Subtype 5	M_5R	**12**
Melanocortin receptors	MCR	7
Subtype 1	MC_1R	5, 7
Subtype 3	MC_3R	7
Subtype 4	MC_4R	5, 7
Neuropeptide Y receptors	YR	
Subtype 1	Y_1R	2
Subtype 2	Y_2R	2
Subtype 4	Y_4R	2
Opioid receptors	OPR	
δ-Opioid receptors	DOP(δ)R	1, 2, 6, 7, 12, 13
μ-Opioid receptors	MOP(μ)R	2, 7

Table 1.1 continued.

GPCR	IUPHAR nomenclature	Appearance in Chapter
Class A Receptors		
Platelet-activating factor receptor	PAFR	8
Prostanoid receptors		4
Prostaglandin E_2 subtype 3 receptors	EP_3R	4, 5
	$EP_{3\alpha}R$	4
	$EP_{3\beta}R$	4
	$EP_{3\gamma}R$	4, 5
Prostaglandin $F_{2\alpha}$ receptors	FPR	4
	FP_AR	4
	FP_BR	4
Thromboxan receptors	TP R	4
	$TP_\alpha R$	4
	$TP_\beta R$	4
Rhodopsin		3, 4, 5, 7, 11, 12, 14, 15
Somatostatin receptors	SRIFR	2
Thyrotropin-releasing hormone receptors	TRHR	4, 3
Vasopressin (oxytocin) receptors	VR	11
Subtype 2	V_2R	
Class B receptors		
PTH receptor family		
PTH/PTH-related peptide receptors	PTH1R	5, 7, 14
Class C receptors		
γ-Aminobutyric acid receptors	GABAR	
Subtype B1	$GABA_{B1}R$	1, 3, 13
Subtype B2	$GABA_{B2}R$	3, 13
Metabotrotropic glutamate receptors	mGluR	3, 4, 11
Subtype 1	$mGlu_1R$	4, 5
	$mGlu_{1A}R$	4
	$mGlu_{1B}R$	4
	$mGlu_{1C}R$	4
	$mGlu_{1D}R$	4
Subtype 5	$mGlu_5R$	3, 4
	$mGlu_{5A}$ R	4
	$mGlu_{5B}R$	4
Calcium sensor receptor	CaSR	5, 7

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2
The Nature of Constitutive Activity and Inverse Agonism

Terry Kenakin

2.1
Historical Perspective

Receptor pharmacology is rooted in functional experimentation. Accordingly, molecules that change functional assay systems are thought to possess the property of efficacy. This term, first coined by Stephenson [1], was defined as the property of a molecule that caused that molecule to exhibit a positive physiological effect. Within this framework, molecules with efficacy are denoted as agonists. In functional studies with isolated tissues, many cases of molecule-induced depression of basal function were ascribed to toxic secondary effects (i.e., there was no systematic theoretical discussion of active depressant effects on functional basal activity).

The first receptor-mediated description of reversal of elevated basal effects was reported for benzodiazapine (γ-aminobutyric acid, $GABA_A$) receptors [2, 3]. For these effects, the term "inverse agonist" was first defined. For G protein-coupled receptors (GPCRs), the first rigorous description of inverse agonism was made by Costa and Herz [4] for the δ-opioid receptor (DOP(δ)R) antagonist [*N,N*'-diallyl-Tyr^1, $Aib^{2,3}$] Leu^5-enkephalin (ICI 174 864). In these experiments, it was observed that elevated basal high-affinity GTP hydrolysis in NG108-15 cell membranes was reduced by ICI 174 864 in a concentration-dependent manner and that this effect was competitively blocked by the DOP(δ)R antagonist (–)-5,9α-diethyl-2-(3-furyl-methyl)-2'-hydroxy-6,7-benzomorphan (MR 2266). The effect of ICI 174 864 was not due to residual amounts of opioid agonist in the medium, since MR 2266 did not depress the elevated basal response. However, MR 2266 did block the negative effects of ICI 174 864 with a potency equal to that for blockade of the positive agonist effects of the DOP(δ)R agonist [D-Ala^2, D-Leu]enkephalin (DADLE). Since a functional system with special properties (i.e., constitutive activity) was required to observe this effect, not all laboratories were able to demonstrate inverse agonism at that time and it has been regarded with some skepticism. However, as the availability of constitutively active receptor systems increased, so too did the observation of inverse agonism; a survey of research papers clearly describing inverse agonism in constitutively active receptor systems is shown in Figure 2.1. In general, the current evidence suggests that approxi-

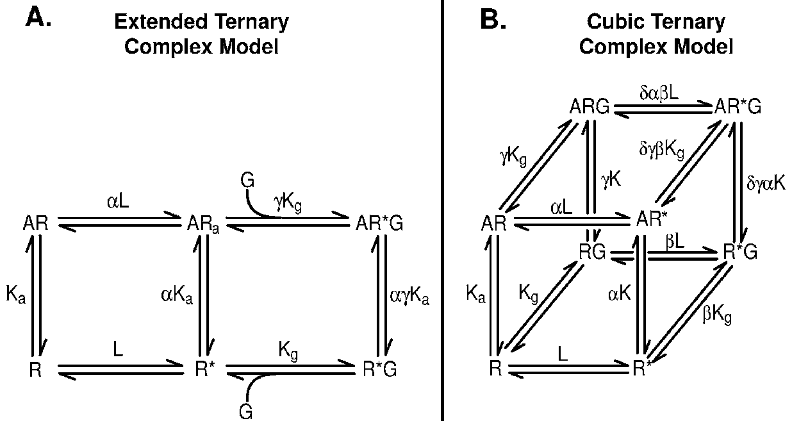

Fig. 2.1 A) Extended ternary complex (ETC) model of G protein-coupled receptor function. Receptors are assumed to exist in active R* and inactive R conformations in equilibrium driven by an allosteric constant *L*. The active state can spontaneously activate the G protein G to produce a constitutively active response-producing species R^*G. Ligands have an affinity association constant K_a for R and αK_a for R^*. G proteins have an affinity association constant K_g for R^* and γK_g for ligand-bound species AR^* (AR_a) [7]. B) Cubic ternary complex model. Similar to the ETC model, except that the inactive receptor R is allowed to interact with G protein to produce non-signaling complexes RG and ARG. There is also a tertiary interaction constant δ, denoting the unique character of the ligand-bound receptor/G protein complex. From [8–10].

mately 85 % of all competitive antagonists are inverse agonists in constitutively active systems [5].

In general, ligands are labeled for their most prominent activity: agonists produce positive physiological responses, competitive antagonists produce dextral parallel displacement of agonist dose-response curves, etc. This can be a problem when that behavior changes with the set point of the assay system and is a well known phenomenon for agonists in that many low-efficacy agonists can be full agonists in efficiently G protein-coupled systems and partial agonists in less efficiently coupled systems. This type of ambiguous labeling has especially been a problem for competitive antagonists and inverse agonists. Specifically, all inverse agonists produce simple competitive antagonism of agonist response in systems that are not constitutively active. When the assay system is constitutively active (i.e., there is an elevated basal response due to the presence of a significant concentration of receptors spontaneously present in the active state), then concentration-dependent reduction of the elevated basal response will be produced by inverse agonists in addition to a dextral displacement of agonist dose-response curves (vide infra). Thus, inverse agonism is a type of behavior observed only under the appropriate conditions.

2.2
Theoretical Basis of Inverse Agonism: Relevance of Receptor Type

A discussion of the molecular nature of constitutive activity and inverse agonism requires description of the current models for GPCR function. With the discovery of constitutive activity has come a modification of the original *Ternary Complex Model* for GPCRs (presented by De Lean and colleagues [6]) to the *Extended Ternary Complex Model* (ETC model) [7]. Experimental evidence indicated that receptors could spontaneously activate G proteins in the absence of agonist; this resulted in a modification of the ternary complex model [6]. This model incorporates aspects of a two-state theory allowing proteins to exist spontaneously in two conformations each possessing different properties with respect to ligands and other proteins. Two receptor species are described, denoted as [R*] (active state receptor capable of activating G proteins) and [R] (inactive state receptors). These coexist according to an allosteric constant L = [R*]/[R] (see Figure 2.1A).

The equilibrium equations for the various species are:

$$[AR] = [AR^*G]/\alpha\gamma L[G]K_g \quad (1)$$

$$[AR^*] = [AR^*G]/\gamma\,[G]K_g \quad (2)$$

$$[R_*] = [AR^*G]/\alpha\gamma[G]K_g[A]K_a \quad (3)$$

$$[R] = [AR^*G]/\alpha\gamma L[G]K_g[A]K_a \quad (4)$$

$$[R_*G] = [AR^*G]/\alpha\gamma[A]K_a \quad (5)$$

The conservation equation for receptor species is:

$$[R_{tot}] = [AR^*G] + [R^*G] + [AR^*] + [AR] + [R^*] + [R] \quad (6)$$

Receptor species giving rise to G protein activation (and therefore physiological response) are assumed to be complexes between the activated receptor ([R*]) and the G protein, namely [AR*G] + [R*G]. The fraction of the response producing species of the total receptor species (([AR*G] + [R*G])/R_{tot}) is denoted by ρ and is given by:

$$\rho = \frac{L[G]/K_G(1 + \alpha\gamma\ [A]/K_A)}{[A]/K_A(1 + \alpha L(1 + \gamma[G]/K_G)) + L(1 + [G]/K_G) + 1} \quad (7)$$

Further modifications have led to a more thermodynamically complete but more complex model in the *Cubic Ternary Complex Model* (CTC model [8–10]; see Figure 2.1B). This model allows both the active and inactive receptor species to interact with G proteins [8–10]. The species denoted [R*] (active state receptor capable of activating G proteins) and [R] (inactive state receptor) can form [RG] and [R*G] spontaneously

and [ARG] and [AR*G] in response to ligand binding. These species form a cube (see Figure 2.1B).

The equilibrium equations for the various species are:

$$[AR] = [AR^*G]/\alpha\gamma\delta\beta L[G]K_g \tag{8}$$

$$[AR^*] = [AR^*G]/\gamma\beta\delta\ [G]K_g \tag{9}$$

$$[R_*] = [AR^*G]/\alpha\gamma\delta\beta[G]K_g[A]K_a \tag{10}$$

$$[R] = [AR^*G]/\alpha\gamma\delta\beta L[G]K_g[A]K_a \tag{11}$$

$$[R_*G] = [AR^*G]/\alpha\gamma\delta[A]K_a \tag{12}$$

$$[RG] = [AR^*G]/\alpha\gamma\delta\beta L[A]K_a \tag{13}$$

$$[ARG] = [AR^*G]/\alpha\delta\beta L \tag{14}$$

The conservation equation for receptor species is:

$$[R_{tot}] = [AR_*G] + [ARG] + [RG] + [R_*G] + [AR^*] + [AR] + [R^*] + [R] \tag{15}$$

Receptor species giving rise to G protein activation (and therefore physiological response) are assumed to be complexes between the activated receptor ([R*]) and the G protein, namely [AR*G] + [R*G]. The fraction of the response producing species of the total receptor species $(([AR_*G] + [R^*G])/R_{tot})$ is denoted by ρ and is given by:

$$\rho = \frac{\beta L[G]/K_G(1 + \alpha\gamma\delta[A]/K_A}{[A]/K_A(1 + \alpha L + \gamma[G]/K_G(1 + \alpha\gamma\beta\delta\ L)) + [G]/K_G(1 + \beta L) + L + 1} \tag{16}$$

For description of constitutive activity and inverse agonism, the ETC model is more illustrative; the CTC model offers subtle variations on the main ideas but is less experimentally verifiable.

The ETC model describes a receptor that can exist in an inactive form (denoted [R] with respect to not being able to activate G proteins and elicit response); and an active form (denoted [R*], a form that activates G proteins to produce physiological response). The equilibrium between these two conformations is governed by the allosteric constant (denoted *L* and defined as [R*]/[R]). Therefore, the magnitude of *L* defines the ease with which a particular receptor spontaneously forms the active state (i.e., how constitutive it is). Theoretically, all receptors are potentially constitutively active in the sense that there exists a non-zero value of *L* for every receptor. However, if it is vanishingly small, then essentially very little active state may be formed spontaneously by the receptor system.

The production of signaling species ([R*G]) by spontaneous coupling of the active state receptor species ([R*]) to G protein ([G]) is shown as:

(17)

A constraint of the ETC model is that only the activated receptor can interact with G proteins. The equilibrium equations are:

$$L = [R^*]/[R] \tag{18}$$

$$K_G = [R^*G]/[R^*]\,[G] \tag{19}$$

The conservation equation for G protein is $[G_{tot}] = [G] + [R^*G]$. Under these circumstances the amount of [R*G] formed (and hence the amount of constitutive activity observed expressed as a fraction of total amount of G protein available for activation) as a function of receptors is given as:

$$\frac{[R^*G]}{[G_{tot}]} = \frac{[R]}{[R] + (K_G)/L} \qquad (20)$$ (29

where L is the allosteric constant and [R] is the amount of receptor in the inactive state

$$\text{Constitutive activity} = \frac{[R^*G]}{[G_{tot}]} = \frac{[R]}{[R] + (K_G/L)} \tag{21}$$

where K_G is the equilibrium dissociation constant of the receptor/G protein complex and L is the allosteric constant. It can be seen from Equation 21 that as $[R] \rightarrow \infty$, the ratio $[R^*G]/[G_{tot}]$ (and hence the level of constitutive activity) $\rightarrow$ 1. In contrast, if the receptor is allowed to interact with the inactive state of the receptor (as in the CTC model) such that the resulting complex does not signal, the fractional level of constitutive activity need not approach unity. In terms of the CTC model, a receptor can exist in two states: active (producing G protein activation (denoted [R*]) and inactive ([R], forming a complex that does not signal).

(22)

The equilibrium equations are:

$$[RG] = [R^*G]/\beta L \tag{23}$$

$$[G] \quad = \quad [R^*G]/\beta L K_G \, [R] \tag{24}$$

and the G protein conservation equation is:

$$[G_{total}] \quad = \quad [G] + [RG] + [R^*G] \tag{25}$$

If it is assumed that constitutive activity emanates from $[R^*G]$:

$$\text{Constitutive activity} = \frac{[R^*G]}{[G_{total}]} = \frac{\beta L \, [R]/K_G}{[R]/K_G(1+\beta L)+1} \tag{26}$$

The maximal constitutive activity is obtained as $[R] \rightarrow \infty$:

$$\text{Maximal C.A.} \quad = \quad \beta L/(1+\beta L) \tag{27}$$

where β refers to the differential multiple affinity of the active vs. inactive forms of the receptor for G proteins. It can be seen that the maximally observed constitutive activity for any expressed receptor need not be the maximal functional response of the system, but rather can be below that level (i.e., as $[R] \rightarrow \infty$, C.A. $\rightarrow \beta L/(1 + \beta L)$). In fact, if the value of β is < 1, then no constitutive activity at all may be observed for a system with over-expressed receptors. This effect would be receptor- and G protein-specific (relative magnitudes of L, K_G, and β). Figure 2.2 shows the relationship between the ratio of affinities of the active vs, inactive states of the receptor for G protein (magnitude of β) and the allosteric constant L. It can be seen that low values of β and/or L can give rise to systems of very little if any constitutive activity.

Theoretically, one method of determining whether an inactive spontaneous complex of [RG] is produced is to monitor the level of constitutive activity as a function of receptor expression. Figure 2.3 shows the constitutive activity observed for five receptor types in *Xenopus laevis* melanophores as a function of the cDNA used for expression. It can be seen that the observed constitutive activity is submaximal (relative to the maximal agonist response), thereby ostensibly supporting the notion that not all of the

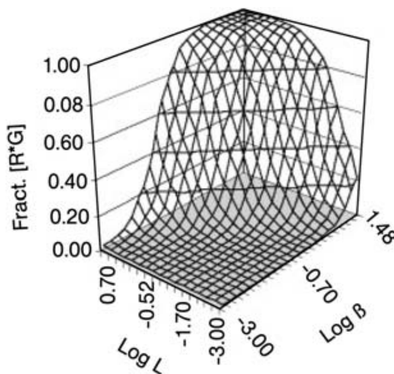

Fig. 2.2 Surface defining the constitutive activity observed in systems according to the CTC model with variation of β (the differential affinity of the active vs. inactive state of the receptor) and of the allosteric constant L. Higher values of β indicate that there is a preferential affinity of the active (over inactive) receptor state. Higher values of L indicate a naturally high propensity of the receptor to form the active state. It can be seen that there are a number of combinations of β and L that predict no observed constitutive activity either through failure spontaneously to produce significant levels of active state receptor or a sequestration of the G protein by inactive state receptor to produce non-signaling species.

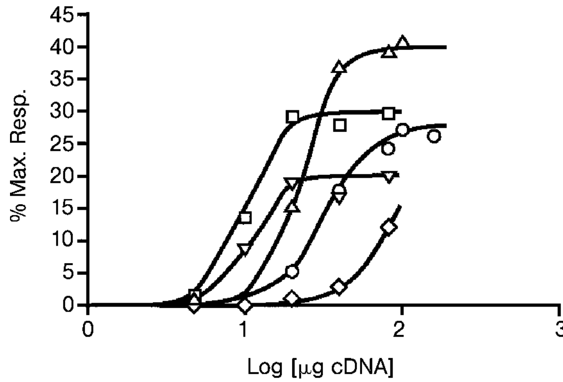

Fig. 2.3 Functional data from *Xenopus laevis* melanophores. Constitutive receptor activity (ordinates expressed as percentage of the maximal response to a full agonist for each receptor) as a function of magnitude of receptor expression (expressed as the amount of human cDNA used for transient transfection, logarithmic scale). Data shown for human chemokine CCR5 receptors (circles), chemokine CXCR4 receptors (triangles), Y_2R (diamonds), Y_2R (squares), and Y_4R (inverted triangles). Data recalculated and redrawn from [11].

receptor forms activated complexes with the G protein (compare Equations 21 and 27). In practical terms, the observation of submaximal constitutive activity in an efficiently coupled system (i.e., a system with high receptor reserve for pharmacological function) further argues for the production of low levels of [R*G] complex (i.e., if only 1 % of the receptor need be activated to achieve maximal functional response (99 % receptor reserve), then failure to observe maximal response correspondingly suggests that $< 1\%$ of the receptors form the constitutively active [R*G] complex). Figure 2.3 shows that different receptors have different propensities to produce constitutive activity. For example, neuropeptide Y type 2 receptor (Y_2R) has ten times the ability of neuropeptide Y type 1 receptor (Y_1R) to produce constitutive activity; presumably the value of L for Y_2R is 10 times that for Y_1R.

Failure to achieve constitutive activity equal to the maximal response of the system theoretically could suggest that the CTC model is operative in the particular system being observed (i.e., that the receptor spontaneously forms inactive G protein complexes that limit the production of the [R*G] complex). However, another possibility is that the failure to achieve maximal effect is not due to formation of [RG] but rather to limiting values of L for the particular receptors chosen for experimentation. An experimental approach that obviates this potential ambiguity is to attempt to quantify the extent to which the inactive receptor forms an inactive complex with a G protein to sequester it and prevent it from interacting with other receptors. For example, in the yeast *Saccharomyces cerevisiae* system, expression of a number of transmembrane segment IV mutants of pheromone receptors (Ste2p, Ste3p) produces constitutive activity. Interestingly, this is eliminated by co-expression of the corresponding wild-type

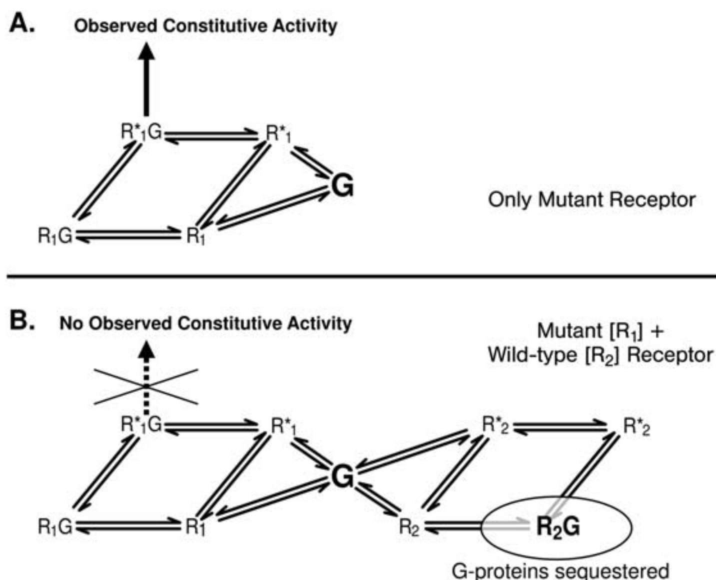

Fig. 2.4 Sequestration of G protein to a non-signaling species R_2G by a receptor R_2 from a constitutively active system of another receptor R_1. A) With only R_1 present, spontaneous formation of R^*_1 results in spontaneous formation of the constitutively active signaling species R^*_1G. In this case, R_1 is a constitutively active mutant receptor. B) Co-transfection of another receptor R_2 (wild-type receptor) results in formation of the non-signaling species R_2G, thereby removing G protein from the reaction with the mutant receptor R and resulting in a decrease in or elimination of mutant receptor-mediated constitutive activity.

receptor. It has been proposed that sequestration of G protein by the wild-type receptor causes this effect (see Figure 2.4, [12]). Similarly, expression of cannabinoid receptors (CB_1R) in superior cervical ganglion neurons abolishes the pertussis toxin-sensitive $G_{i/}$ G_o-inhibited Ca^{2+} current responses to norepinephrine-activation of α_2-adrenoceptors (α_2AR) and somatostatin receptors (SRIFR); this inhibition is reversed by expression of $G\alpha_{oB}$, $G\beta_1$, and $G\gamma_3$ [13]. These data show that α_2AR or somatostatin receptors (subtype not specified) are able to interact with the G protein normally utilized by CB_1R to produce constitutive activity and to sequester this G protein as an inactive complex.

2.3 The Interaction of Systems with Ligands

The interaction of ligands with affinity for synoptic receptor systems (referring to a general view of the complete system including G proteins) can produce perturbations resulting in positive agonism or inverse agonism. The ability of a ligand to cause such perturbations is dependent upon its efficacy, which, in terms of the ETC model, is

characterized by the terms α and γ (Figure 2.1A). The factor α represents the differential affinity of the ligand for the active (vs. inactive) receptor conformation. For example, a value of $\alpha = 10$ indicates that the ligand has a ten times greater affinity for [R*] than for [R]. Similarly, the factor γ defines the effect of ligand binding on the receptor and defines the subsequent affinity of the ligand-bound (vs. unbound) receptor for G proteins ($\gamma = 10$ indicates that the ligand-bound receptor has a ten times greater affinity than the unbound receptor for G proteins. Inverse agonism involves these factors, specifically favoring the inactive [R] state.

The molecular mechanism for the active alteration of receptor species with ligand binding involves conformational selection. Specifically, if two receptor conformations exist in equilibrium, then the relative amounts of the two species multiplied by the rate constant for conversion to each species yields the relative degree to which the two species exist. Any process that removes one of those species will leave a void of that species in the system and drive the reaction toward production of that same species. Therefore, if a ligand selectively binds to the inactive state of the receptor [R], then a third (ligand-bound inactive state) species will be created in the system, leaving a void of free [R]. This will drive the reaction toward the production of more [R] at the expense of [R*]; the result will be a diminution of any spontaneously formed [R*] and subsequent reduction in the constitutive activity of the system. This will be observed as inverse agonism. Therefore, α values < 1 for a ligand (the affinity of the ligand for [R] is greater than for [R*], so for any given concentration of ligand, the amount of ligand-bound [R] will exceed ligand-bound [R*]) will confer the property of inverse agonism upon that ligand, provided that the system is constitutively active.

The production of a bias in receptor conformation can be simulated with the ETC model. Specifically, Equation 7 predicts that in the absence of ligand [A], the response-producing species are given by:

$$\rho_0 = \frac{L[\mathrm{G}]/K_\mathrm{G}}{1 + L(1 + [\mathrm{G}]/K_\mathrm{G})} \tag{28}$$

In the presence of a saturating concentration of ligand the response-producing species is given as:

$$\rho_\infty = \frac{L[\mathrm{G}]/K_\mathrm{G}\ \alpha\gamma}{(1 + \alpha L(1 + \gamma[\mathrm{G}]/K_\mathrm{G}))} \tag{29}$$

Thus, the ratio of response-producing species in the presence and in the absence of ligand is given by:

$$\frac{\rho_\infty}{\rho_0} = \frac{\alpha\gamma(1 + L(1 + [\mathrm{G}]/K_\mathrm{G}))}{(1 + \alpha L(1 + \gamma[\mathrm{G}]/K_\mathrm{G}))} \tag{30}$$

It can be seen from Equation 30 that if the ligand has no selective affinity for either receptor species (i.e., $\alpha = \gamma = 1$), then the ratio $\rho/\rho_0 = 1$ (i.e., the presence of the ligand

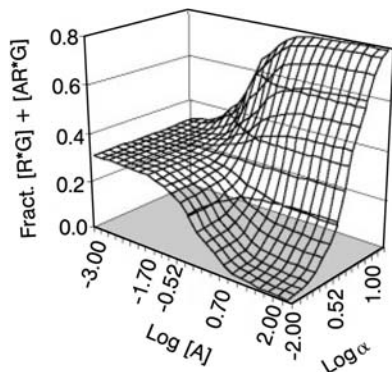

Fig. 2.5 Ligand-induced changes in levels of receptor/G protein species according to the ETC model. Ligands [A] of varying values for α are added to a moderately constitutively active synoptic receptor system of $L = 0.1$, $[G] = 5$, $\gamma = 1$. Very similar effects are seen with $\alpha = 1$ and variation of γ.

will not alter the relative proportions of the species). Alternatively, if $\alpha<1$ (for the moment γ is immaterial and may remain unity), then the ratio ρ/ρ_0 must be <1 (i.e., the amount of response-producing species will be diminished by the presence of the ligand). Thus, the ligand necessarily will be an inverse agonist. In general, Equation 30 indicates a mechanism for efficacy, either positive or negative. Any ligand with a selective affinity for a given receptor state will necessarily bias the total sum of conformations toward that state. Figure 2.5 shows the effects of different values of α on the concentration-response curve (shown as production of response-producing species [R*G] and [AR*G]) to ligands in a system with constitutive activity (note elevated basal response in the absence of ligand). It can be seen that positive agonism is produced with values of $\alpha>1$, no change for $\alpha = 1$ (neutral antagonist), and inverse agonism for $\alpha<1$.

The ETC model indicates another way in which a compound can influence the mixture of receptor conformations in a receptor system, namely through the value of γ. This value is the incremental change in affinity that the active state of the receptor ([R*]) has for G proteins when bound to the ligand. Thus, if the affinity of the active

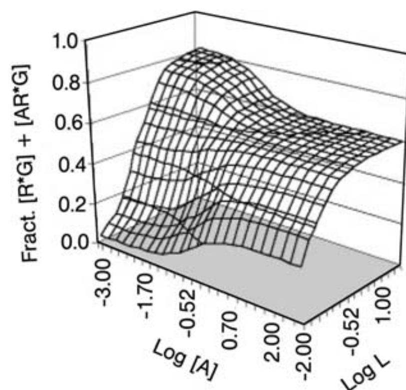

Fig. 2.6 Effects of a protean ligand ($\alpha = 30$, $\gamma = 0.3$) on systems of varying constitutive activity (varying values of L). In quiescent systems, the ligand produces increases in G protein activation (positive agonism), whereas in systems with elevated basal levels of response (constitutive activity), inverse agonism is observed.

state receptor for G proteins is given by K_g, then the affinity of this state when bound to ligand ([AR*]) is γK_g. As with α, if $\gamma < 1$, then the affinity of the receptor for the G protein, when ligand bound, is less than when it is not. The ligand thus reduces the binding of G protein and reduces basal response (i.e., the ligand is an inverse agonist). Changes in γ produce effects on concentration-response curves nearly identical to those shown in Figure 2.5.

An interesting effect of protean agonism (positive agonism in quiescent non-constitutively active systems and inverse agonism in constitutively active systems) can be produced by ligands with $\alpha > 1$ and $\gamma < 1$. Specifically, protean ligands (named after Proteus in Greek mythology, who could change his shape at will) produce receptor active states that are of lower efficacy, with respect to efficiency of G protein activation, than the naturally formed constitutively active receptor state. Under these circumstances, when there is no basal level of [R*] formed (referred to as a quiescent system), a protean ligand will produce positive agonism. Conversely, when there is a basal level of constitutive activity present due to spontaneously formed [R*], then protean ligands will produce inverse agonism by virtue of the fact that the more efficacious receptor active state will be converted into a less efficacious ligand-bound active state. Protean ligands have been predicted and defined theoretically [14–16]; this phenotype has been observed experimentally for β-adrenoceptors (βARs) [17], the α_{2A}AR [18, 19], and bradykinin receptors (BRs) [20]. The effect of a protean ligand on a series of receptor systems varying in levels of constitutive activity (varying L) is shown in Figure 2.6.

If the inactive state ligand-bound and -unbound receptors are allowed to form a non-signaling complex, a more complex synoptic system results. Thus, ligands, such as inverse agonists, which stabilize the inactive state of the receptor, have the ability to sequester G proteins and make them unavailable to other receptor systems (see Figure 2.7). In transfected CHO cells, for example, the CB_1R inverse agonist *N*-(piperidin-1-yl)-5-(4-chlorophenyl)-1-(2,4-dichlorophenyl)-4-methyl-pyrazole-3-carboxamide (SR 141716A) reduces constitutive CB_1R activity (as measured by activation of mitogen-activated protein kinase (MAPK)). However, this ligand also blocks the pertussis toxin-sensitive activation of the same MAPK by insulin and insulin-like growth factor 1 receptors [21]. CB_1R-mediated inhibition was also seen when the mastaporan analogue Mas-7 (INLKALAALAKALL-NH_2) was used to activate G_i proteins directly. In contrast, pertussis-insensitive MAPK activation through fibroblast growth factor receptors was not affected [21]. These data suggest that G_i proteins are sequestered by CB_1R in an [ARG] complex. Similarly, the H_2R inverse agonist tiotidine impedes β_2AR-mediated responses through sequestration of $G\alpha_s$ protein in U-937 promonocytes [22]. It should be pointed out that formation of a non-signaling complex of the receptor and G protein may not be the only possibility for cross-reactivity between GPCR systems. Heterologous receptor dimers have been shown to have considerably different properties from those of the reactants (see [23–25]), and it is entirely possible that direct receptor interactions, and not G protein sharing, may account from some of these phenomena.

Another provocative series of data shows that ligands that do not produce a physiological response (i.e., antagonists) do promote GTP-sensitive binding in some systems;

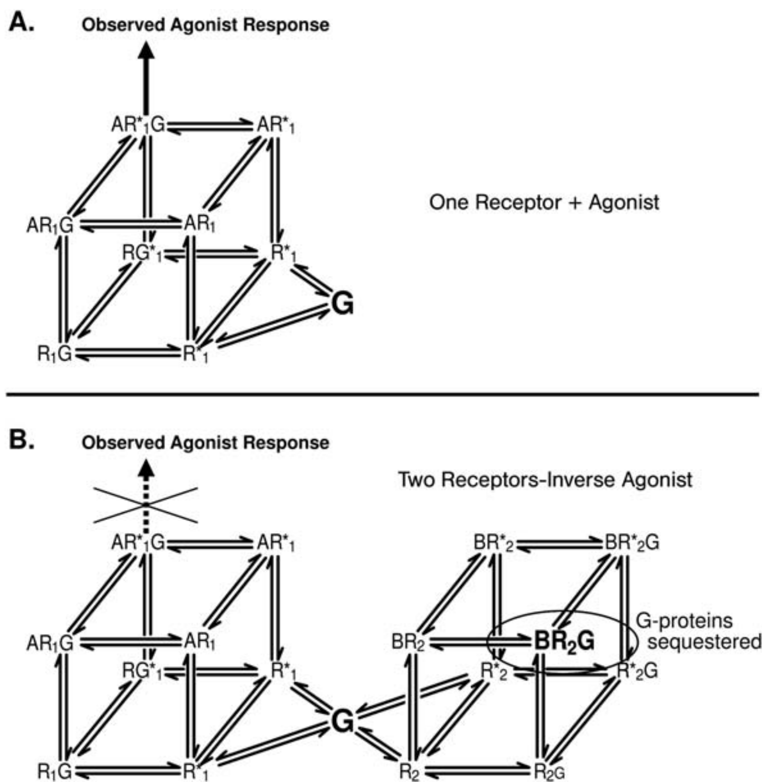

Fig. 2.7 Ligand-induced sequestration of G protein through receptor R_2 from a system mediating responses through receptor R_1. A) Agonists produce response through receptor R_1 through production of species $AR^*{}_1G$. B) Addition of an inverse agonist [B] results in formation of the non-signaling species BR_2G, thereby depleting levels of G protein needed for production of response through $AR^*{}_1G$.

this is indicative of the formation of a complex between the receptor and G proteins (see also Chapters 8 and 9). Specifically, if a receptor sequentially binds to a G protein upon ligand-binding, then the observed affinity of the ligand for the complete system will be an amalgam of the microaffinity constants of the ligand for the receptor and of the ligand-bound receptor for the G protein [26]. This can be shown by considering the following scheme. Receptor [R] binds to ligand [B] and goes on to form a ternary complex with G protein [G]:

$$B + R \overset{K_b}{\rightleftharpoons} AR + [G] \overset{K_g}{\rightleftharpoons} BRG \tag{31}$$

The equilibrium equations are:

$$K_b = [B][R]/[BR] \quad (32)$$

$$K_g = [BR][G]/[BRG] \quad (33)$$

The receptor conservation equation is:

$$[R_{tot}] = [R] + [BR] + [BRG] \quad (34)$$

Through conversion of association into dissociation constants (i.e., $1/K_b = K_B$):

$$\frac{[BRG]}{[R_{tot}]} = \frac{([B]/K_A)([G]/K_G)}{[B]/K_A(1 + ([G]/K_G) + 1} \quad (35) \quad (35)$$

Thus, for a system in which a ligand [B] interacts with a receptor, equilibrium dissociation constants of a ligand–receptor complex (K_B), which then can go on to interact with a G protein (of equilibrium dissociation constant of ligand-bound receptor/ G protein complex K_G), the observed affinity of ligand [B] for the synoptic system will be:

$$K_{obs} = \frac{K_B}{1 + ([G]/K_G)} \quad (36)$$

It can be seen that for non-zero positive values of $[G]/K_G$, the affinity of the ligand for the system will be greater than for the uncomplexed receptor. Under these circumstances, an intervention that cancels the complexation of [BR] to [BRG] (such as the addition of excess levels of GTP to the system) will produce a corresponding reduction in the observed affinity of the ligand for the system. This is a common finding for agonists where it is known that G protein complexation occurs to yield a signaling ternary complex. However, if such effects occur for antagonists, then the formation of a non-signaling complex is implied. This has been observed in stably transfected CHO cells for the MOP(μ)R receptor inverse agonist naloxone benzoylhydrazone (NalBzOH). Interestingly, binding of [^{3}H]-NalBzOH demonstrates biphasic kinetics indicative of two affinity states; this biphasic profile is eliminated by the stable GTP analogue guanosine 5'-[β,γ]imidotriphosphate, which blocks the interaction of receptors with G_i/G_o proteins. This indicates complexation of the ligand–receptor species with a G protein [27]. Similar effects are seen with pertussis toxin treatment. These data are consistent with the production of a non-signaling ternary [ARG] complex for this antagonist. Similarly, dual binding affinity states are observed for the inverse histamine H_2R inverse agonist tiotidine; the high-affinity state is eliminated by the stable GTP analogue guanosine 5'-[γ-thio]triphosphate, thereby implicating complexation with G protein. However, no G protein signaling is observed with this inverse agonist, suggesting the formation of an [ARG] complex [22].

2.4
Inverse Agonism as a Phenotypic Behavior

While it is well known that an inverse agonist reverses constitutive activity in a constitutively active receptor system, it should also be noted that an inverse agonist will produce simple competitive antagonism in a non-constitutively active receptor system (i.e., the system must possess constitutive activity to demonstrate the inverse agonist properties of the inverse agonist). Therefore, absence of observation of inverse agonism does not necessarily constitute evidence that a given antagonist is not an inverse agonist; the system may simply be incapable of revealing that particular property of the ligand. Unveilings of hitherto unknown inverse agonist properties can be dramatic, as in the testing of 23 different α_{1B}AR antagonists in a constitutively active receptor system, indicating that *all* antagonists were actually inverse agonists [28].

Another important consideration in the detection and quantification of inverse agonism is the sensitivity of the readout system. Specifically, the sensitivity of functional receptor systems is greater for the production of constitutive activity and demonstration of inverse agonism.

From Equation 26, the observed midpoint of the receptor-based curve is:

$$K_{obs} = K_G/(1+\beta L) \tag{37}$$

Let the observed constitutive response be a hyperbolic function of $[R_*G]/[G_{total}]$ with a coupling constant of ϕ:

$$\text{Constitutive activity} = \frac{[R^*G]/[G_{total}]}{([R^*G]/[G_{total}) + \varphi} \tag{38}$$

This results in the following equality:

$$\text{Constitutive activity} = \frac{\beta L[R]/K_G}{\beta L[R]/K_G(1+\varphi(1+\beta L)) + \varphi} \tag{39}$$

The midpoint for this function is:

$$K_{obs} = \frac{\varphi K_G}{1+\varphi(1+\beta L)} \tag{40}$$

The ratio of midpoints of the functional curve (K'_{obs}) and the receptor-based curve (K_{obs}) is given as :

$$\frac{K'_{obs}}{K_{obs}} = \frac{\varphi(1+\beta L)}{1+\varphi(1+\beta L)} \tag{41}$$

It can be seen from Equation 41 that for all non-zero positive values of ϕ (1 + βL), $K'_{obs}/K_{obs}<1$ (i.e., the functional curve will always lie to the left of the receptor-based curve along the receptor axis). Therefore, a functional readout of constitutive activity will always be a more sensitive indicator of constitutive activity than one utilizing receptor species. This is intuitively obvious if it is considered that a functional system with a receptor reserve for the constitutively active receptor species (e.g., 5% of the [R*G] species is sufficient to produce maximal functional response) Under these circumstances, small changes in receptor species (i.e., a low level of [R*G] converted into [AR]) could be undetectable in binding but would be relatively large in a system with such a functional receptor reserve.

2.5 Conclusion

In general, the discovery of constitutively active GPCRs has had widespread impact on receptor pharmacology and the definition of antagonism. It has also changed the receptor models used to describe receptor function. Furthermore, the therapeutic implications of inverse agonism are as yet largely unknown, and will probably be elucidated retrospectively as increasing clinical experience with inverse agonists is gained in greater therapeutic populations.

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3
Molecular Mechanisms of GPCR Activation

Søren G. F. Rasmussen and Ulrik Gether

3.1
Introduction

G protein coupled receptors (GPCRs) are integral membrane proteins that enable cells to respond to external stimuli [1–4]. They are activated upon binding of intercellular signaling molecules such as hormones, neurotransmitters, and autacoids (mediators of inflammation), or they are specialized in sensing stimuli such as light, odorants, and taste substances [1–4]. This extreme functional diversity is equally matched by the varied structures of the molecules binding to, and activating, GPCRs [1–4]. For several years, a puzzling question has been the underlying molecular mechanisms of GPCR activation. What structural changes are elicited by binding of the agonist to the receptor, and which intramolecular interactions are controlling these changes? Another key question is whether the mechanics of activation have been conserved throughout the entire GPCR superfamily and how, for example, ligand-binding sites located in the N-terminal domain of class C GPCRs compared to sites within the transmembrane domain of class A GPCRs convey the agonist binding event into G protein coupling? Other questions that have intrigued scientists include the mechanisms underlying partial agonism and, in recent years, the significance of GPCR oligomerization for receptor activation and G protein coupling. Within the last decade several of these fundamental questions have been addressed with some success by the application of both biophysical and biochemical approaches (for reviews see [4–7]). A crucial aspect for most of these studies has been the ability to interpret experimental data in the context of structural models. Firstly it became possible to generate these with the aid of the low-resolution structures of rhodopsin resolved by Schertler and co-workers [8–10]. Later, Palczewski and co-workers succeeded in generating three-dimensional crystals of rhodopsin and in solving the tertiary structure at atomic resolution (2.8 A) [11]. The availability of this high-resolution structure allowed, for the first time, detailed insight into the functions of individual side chains and pinpointed their specific role in receptor activation. In this chapter we review current knowledge regarding the molecular mechanisms responsible for activation of GPCRs, including description of agonist-induced conformational changes and the significance of receptor oligomerization.

3.2
GPCR Structure and Ligand Recognition

The rhodopsin structure, the first and so far still the only GPCR structure solved at atomic resolution [11], confirmed the counterclockwise seven transmembrane (TM) helical arrangement (as viewed from the extracellular side) originally established from the projection structure of two-dimensional crystals of rhodopsin [8–10, 12]. The high-resolution structure revealed the 11-*cis*-retinal chromophore enclosed in a binding crevice aligned by helices three through seven, in full agreement with predictions from earlier mutagenesis studies [11, 13]. A general question is to what extent other GPCRs structurally resemble rhodopsin. Importantly, a thorough comparison of the rhodopsin structure with data obtained from systematic application of the substituted cysteine accessibility method (SCAM) to the dopamine D_2 receptor (D_2R) was carried out [14]. In these studies, performed by Javitch and co-workers, each residue in all seven TMs of the D_2R was substituted one by one by cysteine [15]. The comparison indicated a remarkable structural similarity between the D_2R and rhodopsin in the transmembrane domains [14]. The amino acid residues, inferred from the SCAM analysis to form the water-accessible binding crevice between the transmembrane helices, were almost completely consistent with the predictions from the rhodopsin structure [14].

A remarkable finding in the rhodopsin crystal structure was the involvement of the second extracellular loop 2 (ECL2) in forming the binding pocket of 11-*cis*-retinal by folding into the binding crevice and lining the chromophore with one of its beta strands. Whether this finding can be generalized to other GPCRs is still uncertain. The ECL2 sequence is rather poorly conserved over the different receptors, although it does contain one of the two highly conserved cysteines known to form a disulfide bond between the top of TM3 and ECL2 [4]. It is interesting, however, that several studies have suggested that the loop region may be functionally important. In the α_{1B}-adrenoceptor ($\alpha_{1B}AR$) it was found that residues in the loop are critical for the pharmacological specificity of some adrenergic ligands, consistently with the notion that small molecule ligands other than 11-*cis*-retinal might also form contacts with residues in this loop [16]. Furthermore, previous studies of the β_2-adrenoceptor (β_2AR) with use of a fluorescent ligand have demonstrated that the ligand-binding site is completely inaccessible to aqueous quenchers [17], an observation consistent with the presence of a 'plug' in the binding crevice. Finally, a role of the loop in ligand binding to the D_2R was strongly supported by scanning of ECL2 by application of SCAM [18].

While the receptor structure traversing the bilayer membrane might be highly conserved throughout evolution, it must be emphasized that an extensive amount of evidence suggests that there is no common 'lock' for all agonist 'keys' [4, 19]. Consequently, it is not required for receptor activation that all agonist molecules form a specific set of contact points in the receptor structure to promote receptor activation. Even agonists for the same receptor may not necessarily have to share the same binding site, and it does not seem to be a specific requirement for receptor activation that the agonist ligand be docked in the transmembrane binding crevice (for reviews see [2, 4]). In other words, the mode of interaction of a given agonist depends on its individual che-

mical structure, and whether it is an agonist or antagonist is determined by the mode of interaction and by whether this mode preferentially stabilizes an inactive (R) or an active (R*) receptor state (see also Chapters 2 and 9) [2, 4]. Peptide hormones, for example, have major points of interactions in the extracellular loops and in the amino termini [2, 4], whereas small molecule ligands such as biogenic amines acting on class A GPCRs bind deeply in-between the transmembrane helices. A special case are the class C GPCRs, including the metabotropic glutamate receptors (mGluRs) and γ-aminobutyric acid receptors ($GABA_B$Rs), which are characterized by containing their 'small molecule' ligand-binding site in their large amino termini [2, 4].

3.3 Conformational Changes in the GPCR Activation Process

Despite the extreme diversity in GPCR ligands and that in the GPCRs themselves, it is highly conceivable that the fundamental activation mechanism and associated conformationals changes in the receptor structure have been conserved throughout evolution. This is at least indirectly supported by the simple fact that all GPCRs are capable of activating the same intracellular signaling pathways through the same classes of G proteins. To gain insight into the structural changes responsible for GPCR activation, one approach is obviously to achieve high-resolution crystal structures of both the inactive and the ligand-activated receptor states. However, because of difficulties in obtaining sufficient material for X-ray crystallography and the presumed more unstable nature of the activated receptor state [20–22], only one high-resolution structure, that of the inactive dark state of rhodopsin, is currently available [11]. Accordingly, our current insight into the receptor activation mechanism is based on the application of a variety of biophysical and biochemical techniques, especially to class A rhodopsin-like GPCRs [5, 6, 23, 24]. These approaches have offered the advantage of allowing insight not only into the conformational changes but also into the dynamics of receptor activation. This is in contrast to X-ray crystallography, which can only provide single 'frozen' pictures of particular receptor states.

The inherent stability of rhodopsin and abundant natural sources paved the way for an array of biophysical studies, including Fourier transform infrared resonance spectroscopy (FTIR) [25, 26], surface plasmon resonance (SPR) spectroscopy [27], tryptophan UV-absorbance spectroscopy [28], and electron paramagnetic resonance spectroscopy (EPR) [29–32]. Interestingly, the first direct evidence for structural rearrangements of TM3 and TM6 upon photoactivation was provided by Lin and Sakmar through tryptophan UV-absorbance spectroscopy [28].

Further insight into the nature of the conformational changes involved in the photoactivation of rhodopsin was provided by studies applying site-directed spin labeling and EPR spectroscopy (reviewed in [6]). Thiol-reactive nitroxide spin-labels incorporated onto cysteines site-specifically introduced on the cytoplasmic sides of the transmembrane helices reported movements in this area and in particular of the cytoplasmic end of TM6 upon light-induced activation of rhodopsin [29–34]. Subsequently, Hubbell, Khorana, and co-workers measured the distances between two cysteine-re-

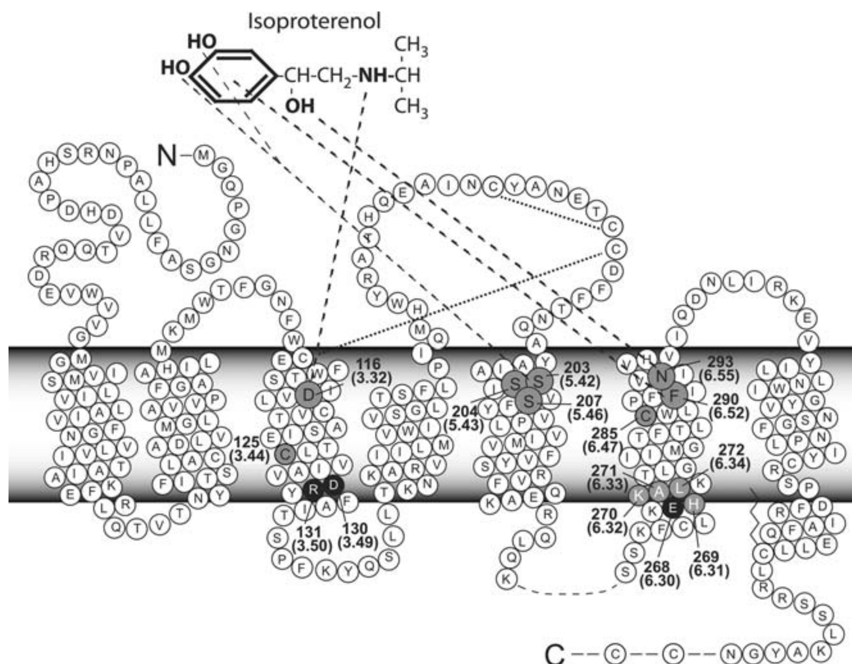


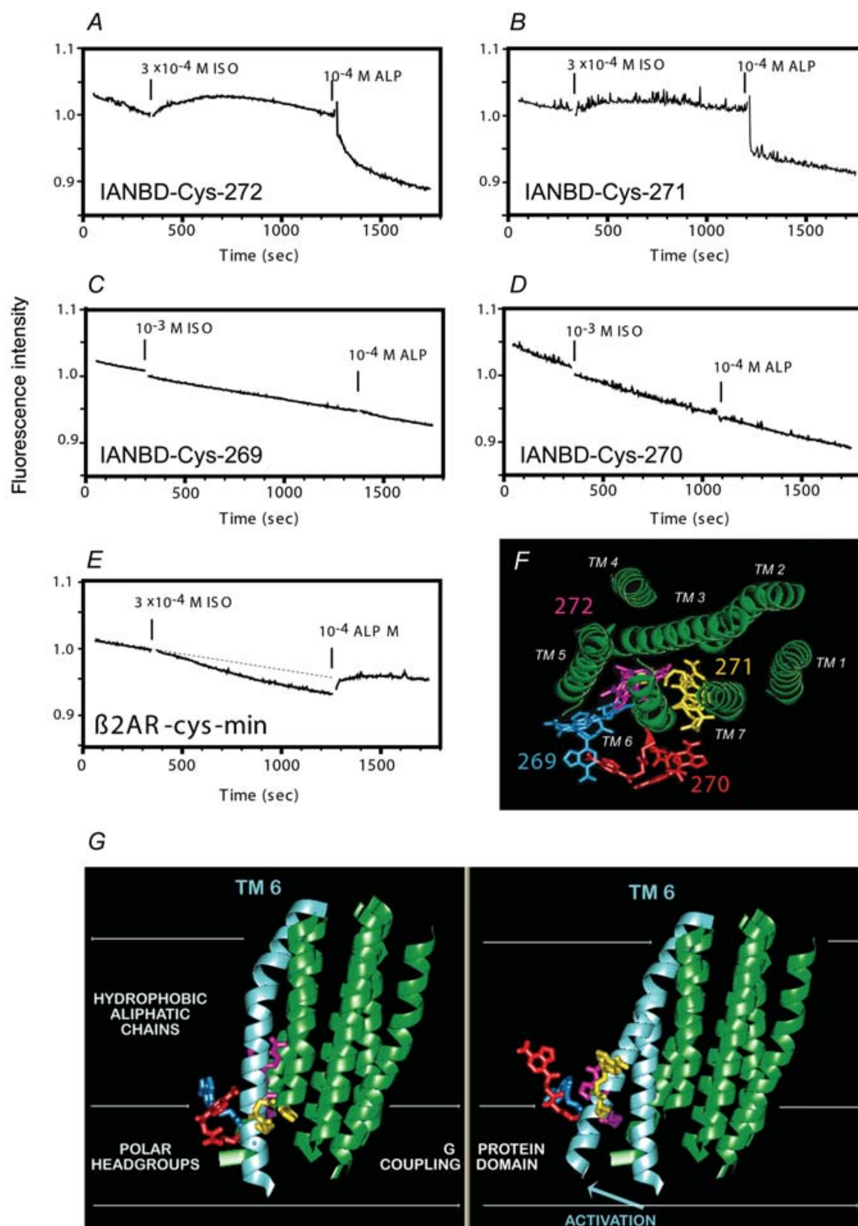
Fig. 3.1 Two-dimensional serpentine of the β_2-adrenoceptor. The amino acids interacting with catecholamines (black letters in enlarged gray circles) represented here by ISO have been identified as $Asp^{113(3.32)}$ [95], the serine residues $Ser^{203(5.42)}$, $Ser^{204(5.43)}$, and $Ser^{207(5.46)}$ [95, 96], $Phe^{290(6.52)}$ [95], and $Asn^{293(6.55)}$ [97]. $Cys^{125(3.44)}$ and $Cys^{(285)6.47}$ (black letters in gray circles) were identified as major labeling sites for the environmentally sensitive cysteine-reactive fluorophore IANBD reporting agonist-induced conformational changes [38]. $His^{269(6.31)}$, $Lys^{270(6.32)}$, $Ala^{271(6.33)}$, and $Leu^{272(6.34)}$ (white letters in gray circles) were mutated one by one to cysteines in a background mutant with a reduced number of reactive cysteines (β_2AR-Cys-min) [39]. Subsequent fluorescent labeling of these residues allowed identification of conformational changes at the cytoplasmic side of TM6 [39]. $Asp^{130(3.49)}$, $Arg^{131(3.50)}$, and $Glu^{268(6.30)}$ (white letters in black circles) are believed to form an ionic lock that is disrupted during receptor activation [63]. The positions of highlighted residues are indicated by their generic number followed by their number according to the Ballesteros–Weinstein nomenclature [42]. In this scheme the most conserved residue in each helix is given the number 50, and each residue is numbered according to its position relative to this conserved residue. For example, 3.49 (in superscript and brackets) indicate a residue in TM3, one residue amino terminal to $Arg^{(3.50)}$, the most conserved residue in this helix.

active nitroxide spin labels incorporated into a series of rhodopsin mutants each containing a cysteine in TM3 as a reference point and a cysteine at five different positions in TM6 [33]. From the GPCR structure models available at that time [35], a clockwise rotation (as seen from the intracellular side) and a large-scale outwardly directed rigid-body movement of the cytoplasmic end of TM6 relative to TM3 (and the remaining helical bundle) were inferred [33]. Moreover, these movements of TM6 in rhodopsin upon photoactivation were later supported by site-directed fluorescence labeling of single cysteines inserted at the cytoplasmic end of the helix [36].

The first direct measurement of conformational changes in a GPCR activated by a diffusible agonist was achieved in the β_2AR by fluorescence spectroscopy [20, 37–40]. The initial probing for structural changes took advantage of the sensitivities of the emissions of many fluorophores to the polarities of their molecular environments. The sulfhydryl-reactive fluorescent probe *N*,*N*'-dimethyl-*N*-(iodoacetyl)-*N*'-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)-ethylenediamide (IANBD) was covalently incorporated into the purified β_2AR. Upon exposure to the agonist (–)-isoproterenol (ISO), the IANBD-labeled receptor elicited a stereospecific, concentration-dependent, and reversible decrease in emission, consistent with movements of the fluorophore to a more hydrophilic environment [37]. Subsequent analysis of a series of mutant β_2ARs with one, two, or three of the endogenous cysteines available for fluorescent labeling showed that binding of IANBD to $Cys^{125(3.44)}$ (residues are numbered according to the Ballesteros–Weinstein nomenclature as explained in Figure 3.1) in TM3 (Figure 3.1) and to $Cys^{285(6.47)}$ in TM6 (Figure 3.1) was responsible for the observed changes in fluorescence [38], suggesting that movements of TM3 and TM6 might occur during receptor activation.

Specific movements of helix 6 have been further analyzed through a series of new mutations in which four residues at the cytoplasmic end of TM6 were substituted one by one with cysteines in the background of a mutant receptor containing as few endogenous cysteines as possible (β_2AR-Cys-min) [39] (Figure 3.1 and 3.2). Fluorescence spectroscopy analysis of the purified and site-selectively IANBD-labeled mutants at the cytoplasmic end of TM6 suggested that the covalently attached fluorophore was exposed to a less polar environment at all four positions upon agonist binding [39]. Whereas evidence for only minor changes in the molecular environment was obtained for positions $269^{(6.31)}$ and $270^{(6.32)}$, the full agonist ISO caused clear, concentration-dependent, and reversible increases in fluorescence emission at positions $271^{(6.33)}$ and $272^{(6.34)}$ (Figure 3.2A–D) [39]. Moreover, the magnitudes of the observed fluorescence changes correlated with the intrinsic biological efficacy of the agonist used, suggesting that the ligand-induced changes in fluorescence are relevant to the receptor activation mechanism [39]. Unlike the IANBD-labeled $Cys^{285(6.47)}$, which is located in a highly hydrophobic environment in the middle of the membrane, the fluorescent probes inserted at the cytoplasmic side of TM6 reside in a very complex amphipathic environment (Figures 3.1 and 3.2G). This was taken into account when the results were interpreted by use of a new computational method that incorporated the complexity of the mixed hydrophobic–hydrophilic region [41]. The outcome of simulations in this biphasic lipid–water solvent continuum model is illustrated by the most favored conformations for each of the four IANBD-derivatized cysteine residues shown in the framework of a molecular model in Figure 3.2F (viewed from the extracellular side).

A relatively small counterclockwise rotation of TM6 (as viewed from the extracellular side) was sufficient to explain the data based on IANBD-labeling of $Cys^{(285)6.47}$ [38]; however, the observation that IANBD at all four inserted cysteines residues most probably moves into a more hydrophobic environment upon agonist binding implies that the helical movement is more multifaceted and probably involves an orchestrated rotation and rigid-body movement of the cytoplasmic part of TM6 away from TM3, si-


A
3 x10-4 M ISO
10-4 M ALP
IANBD-Cys-272
B
3 x10-4 M ISO
10-4 M ALP
IANBD-Cys-271
C
10-3 M ISO
10-4 M ALP
IANBD-Cys-269
D
10-3 M ISO
10-4 M ALP
IANBD-Cys-270
E
3 x10-4 M ISO
10-4 ALP M
ß2AR-cys-min
Fluorescence intensity
Time (sec)
F
TM 4
TM 2
TM 3
272
TM 5
271
TM 1
TM 6
TM 7
269
270
G
TM 6
TM 6
HYDROPHOBIC ALIPHATIC CHAINS
POLAR HEADGROUPS
G COUPLING
PROTEIN DOMAIN
ACTIVATION

◀ **Fig. 3.2** Characterization of agonist-induced conformational changes at the cytoplasmic side of TM6 in the β_2-adrenoceptor. His$^{269(6.31)}$, Lys$^{270(6.32)}$, Ala$^{271(6.33)}$, and Leu$^{272(6.34)}$ at the bottom of TM6 (Fig. 3.1) were mutated one by one to Cys in a background mutant with a reduced number of reactive cysteines (β_2AR-Cys-min) [39]. The mutants were expressed in Sf9 insect cells, purified and labeled with the environmentally sensitive, sulfhydryl-reactive fluorophore IANBD. Panels A, B, C, D and E show time course experiments in which fluorescence intensity is measured over time in response to 3×10^{-4} M ISO followed by 10^{-4} M ALP. Excitation was at 481 nm and emission was measured at 530 nm. Fluorescence in the individual traces was normalized to the fluorescence observed just before addition of ligand [39]. In β_2AR-Cys-min, ISO causes a decrease in fluorescence that can be reversed by ALP. This decrease is reported by IANBD bound to Cys$^{125(3.44)}$ and Cys$^{285(6.47)}$ [38]. In IANBD-Cys$^{271(6.33)}$ and IANBD-Cys$^{272(6.34)}$, ISO causes ALP-reversible increases in fluorescence intensity consistent with movement of the fluorophore to a more hydrophobic environment. In IANBD-Cys$^{269(6.31)}$ and IANBD-Cys$^{270(6.32)}$, ISO causes no apparent change in fluorescence intensity. A likely interpretation is that ISO induces an increase that counterbalances the decrease observed in the control (β_2AR-Cys-min). Panel F shows an extracellular view of a receptor model with an illustrative set of the preferred conformations of the IANBD side chain covalently attached to the four substituted cysteines (color-coded). Note that IANBD attached to Cys$^{271(6.33)}$ (yellow) and Cys$^{272(6.34)}$ (purple) are facing the interior of the TM helix bundle, while IANBD attached to Cys$^{269(6.31)}$ (blue) and Cys$^{270(6.32)}$ (red) are oriented towards the lipid membrane. The preferred conformations of IANBD were determined from computational simulations as described [39]. Panel G shows the proposed conformations of the inactive and active states of the β_2AR. The inactive conformation of the receptor (left panel) is characterized by a highly kinked TM6 helix (blue) with the cytoplasmic end in close proximity to TM3 and the helix bundle. An illustrative set of the preferred conformations of the IANBD moiety covalently attached to the four substituted cysteines at the cytoplasmic side of TM6 is shown. The hypothetical active conformation (right panel) of the receptor, in which the cytoplasmic side of TM6 is moved away arbitrarily from the helix bundle and upwards towards the hydrophobic region, is marked by straight lines. This putative rearrangement of TM6 moves all four IANBD-labeled residues upwards and outwards, allowing them to penetrate further into the more hydrophobic region of the membrane/detergent micelles and away from the more hydrophilic polar headgroups, as well as from the predicted more hydrophilic interior of the receptor protein. The movement can explain the observed shift for all four IANBD-labeled cysteines towards a less polar environment upon receptor activation [39]. Note that the movement of the cytoplasmic part of TM6 is shown to occur around the conserved Pro kink but could also involve a rigid-body movement of the entire helix. However, our previous simulation of the TM6 helix indicated the possibility that the kink in the TM6 helix induced by Pro$^{287(6.50)}$ could behave as a flexible hinge, capable of modulating the movement of the cytoplasmic side of the TM6 helix relative to the extracellular region [38]. (Figure modified from Jensen et al., *J. Biol. Chem.* **2001**, *276*, 9279-9290).

milarly to what was found for rhodopsin [33]. The presence of a highly conserved Pro$^{288(6.50)}$ residing one helical turn above Cys$^{285(6.47)}$ in TM6 is predicted to produce a kinked α helix [42], as illustrated by the side view in Figure 3.2G. The high-resolution structure of the crystallized inactive state of rhodopsin [11] indicates that the cytoplasmic part of TM6 below the Pro kink is almost perpendicular to the plane of the membrane, whereas the part above the Pro kink is tilted by approximately 25° [10]. A rigid-body movement of the cytoplasmic part of TM6 away from TM3, and thus the receptor core, will result in large changes in the axial positioning of all four IANBD-modified cysteines. In the inactive conformation the IANBD moieties would be predicted to reside in the polar head group region (Figure 3.2G). However, if the cytoplasmic part of TM6 is moved away from the receptor core, all four IANBD-labeled residues are brought upwards and outwards, allowing them to penetrate further into the more hydrophobic regions of the membrane/detergent micelles and away from the more

hydrophilic polar head groups as well as from the predicted more hydrophilic interior of the receptor protein (illustrated by the hypothetical active structure in Figure 3.2G, right panel, where the cytoplasmic part of TM6 with the IANBD moieties attached is tilted arbitrarily away from the receptor core).

The nature of the conformational changes of TM6 was found to be somewhat different in another fluorescence spectroscopy study on the β_2AR in which an endogenous $Cys^{265(6.27)}$ at the cytoplasmic extension of TM6 was site-specifically labeled with fluorescein [40]. In addition to a rotation and/or tilting of TM6, it was suggested that the agonist-induced conformational change moves the cytoplasmic ends of TM6 and TM5 closer together, as the emission was decreased by moving into the vicinity of a fluorescence-quenching molecule incorporated at the cytoplasmic end of TM5 [40]. This type of movement was not predicted by the simulation of the four IANBD probes in the β_2AR, although a movement of TM6 into the lipid/detergent bilayer slightly towards TM5 could satisfy both predictions [39].

Besides the biophysical studies performed on rhodopsin and the β_2AR, biochemical evidence for the importance of movement of TM6 relative to TM3 was obtained in rhodopsin by generation of bis-His metal ion-binding sites between the cytoplasmic ends of these TM domains [43]. The sensitivity towards Zn^{2+} could be transferred both to the β_2AR and to the class B parathyroid hormone (PTH) receptor by engineering bis-His metal ion-binding sites in the analogous positions. This provides further support for an evolutionarily conserved requirement for conformational changes in at least TM6 for receptor activation [44]. The application of a disulfide cross-linking strategy to the muscarinic acetylcholine subtype 3 receptor (M_3R), another class A 6 PCR member, has moreover suggested that significant movements occur in the G protein coupling domain at the cytoplasmic side of TM6 (see also Chapter 12) [45]. Additionally, major changes in the secondary structure at the cytoplasmic end of TM6 and a movement towards the cytoplasmic end of TM5 were proposed to occur in response to agonist binding [45].

Movements of domains other than TM6 might also be a critical part of the receptor activation mechanism, as is clear from EPR spectroscopy studies in rhodopsin. It has been suggested that movements of the cytoplasmic portion of TM7 relative to TM1 and of TM2 relative to the so-called intracellular helix 8 (the horizontal extension of TM7) also occur in response to photoactivation [46, 47]. The possible importance of TM7 in receptor activation is indirectly supported by the finding that activating metal ion-binding sites can be generated between TM3 and 7 both in the β_2AR and in the neurokinin-1 receptor [48, 49]. Furthermore, in the angiotensin II type 1 receptor (AT_1R), application of SCAM in the wild-type and in a constitutively activated receptor revealed activation-dependent helical movements of TM2 [50] and TM7 [51].

3.4 Conversion to the Active Receptor State Involves Release of Stabilizing Intramolecular Interactions

It is now well established that intramolecular interactions constrain the unliganded receptor preferentially in an inactive (R) state and that release of these intramolecular constraints allows transition of the receptor from its inactive to its active (R*) state (see also Chapter 2) [52, 53]. For this perception it was particularly important that many GPCRs were found to possess a constitutive agonist-independent receptor activity even in the absence of ligand [54–56] and that specific mutations were capable of substantially increasing this activity [55, 57–60]. Even in the initial studies it was proposed that the constraining of intramolecular interactions had been conserved during evolution to maintain the receptor preferentially in an inactive conformation in the absence of agonist [55, 57–60]. Conceivably, these inactivating constraints could be released as a part of the receptor-activation mechanism, either following agonist binding or due to specific mutations, causing key sequences to be exposed to G protein. Later studies provided more direct structural evidence for this hypothesis. For example, it has been observed that constitutively activated β_2AR and histamine H_2-receptor (H_2R) mutants are characterized by marked structural instability [20–22] as well as by enhanced conformational flexibility [20]. These data imply that the mutational changes have disrupted important stabilizing intramolecular interactions in the tertiary structure, allowing the receptor to undergo conversion more readily between its inactive and active state.

It has still not been fully clarified how release of intramolecular constraining interactions is initiated after agonist binding to the receptor molecule (see also Section 3.5). Not even the availability of a high-resolution structure has provided a final answer to this question. Nonetheless, substantial evidence suggests that at least one of the key events in the activation process in family A GPCRs involves protonation of the aspartate or glutamate in the highly conserved Glu/AspArgTyr motif at the cytoplasmic side of TM3 (Figure 3.1) (see also Chapter 11). In rhodopsin, the most direct evidence for protonation of Glu/Asp$^{(3.49)}$ was obtained by flash photolysis, allowing simultaneous measurement of photoproduct formation and rates of pH changes [61]. Lowering of the pH has also been shown to facilitate transition of the receptor to the activated state in the β_2AR [62].

According to the rhodopsin structure, the ionic counterpart of Glu$^{(3.49)}$ in the inactive state is the adjacent Arg$^{(3.50)}$. In addition to interaction with Glu$^{(3.49)}$, the rhodopsin structure also suggested an interaction between Arg$^{(3.50)}$ and a conserved glutamate in TM6: Glu$^{(6.30)}$ (Figure 3.3A). We consequently proposed that similar interactions occurred in the β_2AR [63], although this was in contrast to previous predictions for the α_{1B}AR suggesting that the Arg in the inactive state interacts with the conserved Asp in TM2 (Asp$^{(2.50)}$; Asp^{79} in the β_2AR) [60]. We moreover hypothesized that the interactions formed between Asp$^{(3.49)}$, Arg$^{(3.50)}$, and Glu$^{(6.30)}$ constituted an ionic switch that controlled transition of the receptor between its inactive and active state (Figure 3.3A and 3B). Thus, we envisioned that agonist binding would result in protonation of Asp$^{(3.49)}$ and concomitant release of interaction between Asp$^{(3.49)}$, Arg$^{(3.50)}$, and Glu$^{(6.30)}$ and thereby receptor activation. The hypothesis has been supported by

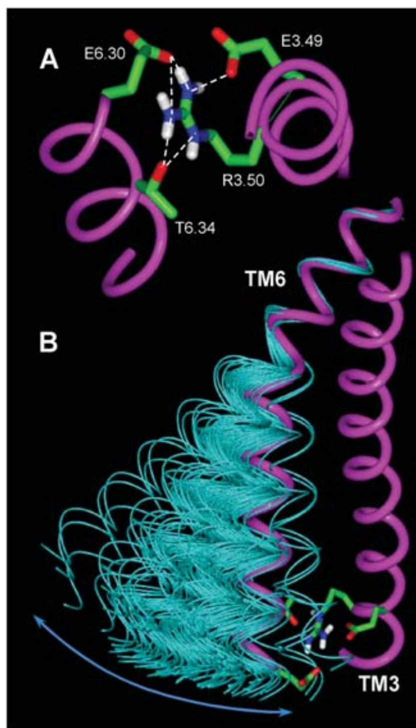


Fig. 3.3 Activation of the β_2-adrenoceptor involves disruption of an ionic lock between TM3 and TM6. A) An extracellular view of the high-resolution structure of rhodopsin showing the interaction between residues at the cytoplasmic ends of TM3 and TM6 [11]. B) The Cα ribbons of TM3 and TM6 of rhodopsin are shown in purple. The simulated structures (light blue ribbons) are superimposed from positions 1 to 17 onto the corresponding parts of TM6 (6.30 to 6.46) [63]. The blue arrow indicates the conformational space that a Pro-kink can assume relative to TM3 (figure modified from Ballesteros et al., *J. Biol. Chem.* **2001**, *276*, 29171-29177).

several lines of evidence. Firstly, charge-neutralizing mutations mimicking the protonated state of the Glu/Asp$^{(3.49)}$ cause dramatic constitutive activation of the α_{1B}AR, the β_2AR, and the H_2R [21, 22, 60, 64]. Similarly, improved coupling has been observed on mutation of the Asp$^{(3.49)}$ in the gonadotropin-releasing hormone receptor (GnRHR) [41]. Mutation of the Asp$^{(3.49)}$ residue in the muscarinic acetylcholine subtype 1 receptor (M_1R) resulted in phosphoinositide turnover responses of the mutant that were quantitatively similar to the wild-type M_1R despite markedly lowered levels of expression [65]. In parallel, constitutive activation was observed in rhodopsin after mutation of the Glu$^{(3.49)}$ found in the corresponding position in this receptor [66]. Furthermore, charge-neutralizing mutation of Glu$^{(6.30)}$ alone or in combination with Asp$^{(3.49)}$ resulted in constitutive activation and a markedly increased efficacy for the partial βAR agonist pindolol [63].

Finally, charge-neutralizing mutations of Asp$^{130(3.49)}$ and/or Glu$^{268(6.30)}$ in the β_2AR are linked to the overall conformation of the receptor [21, 63]; hence, mutation of Asp$^{130(3.49)}$ and/or Glu$^{268(6.30)}$ not only activated the receptor but also caused a cysteine in TM6 (Cys$^{285(6.47)}$) – not accessible in the wild-type receptor – to become accessible to methanethiosulfonate ethylammonium (MTSEA), a charged, sulfhydryl-reactive reagent [21, 63]. This is consistent with a counterclockwise rotation (as seen from the extracellular side) and/or tilting of TM6 in the mutant receptor consistently with the biophysical studies described above. Even more importantly, the MTSEA ex-

periments showed a tight correlation between the apparent conformational rearrangement assessed by MTSEA accessibility to $Cys^{285(6.47)}$ and the extent of constitutive activation [63].

3.5
Kinetics of Agonist Binding and Receptor Activation

Recent studies with the β_2AR [67–69] have challenged the existence of a preformed binding site for the agonist (i.e., the lock-and-key scheme where the agonist is simultaneously coordinated by the residues involved in binding the ligand). Instead, it has been suggested that the binding of the agonist and subsequent receptor activation follow a sequential process [4, 68–70]. The sequential binding model operates with several intermediate conformational states between the inactive and the fully active receptor state [68, 70]. It has been suggested that these intermediate states constitute progressively 'locking parts of the key', meaning that the chemical groups in the agonist interact with their respective amino acid counterparts in a successive manner, resulting in receptor states with increased agonist affinity [68, 70]. Through study of a purified and fluorescently labeled β_2AR, evidence for these intermediate states was obtained in the form of the appearance of two kinetically distinguishable conformational states after binding of catecholamine agonists [68]. Through the use of chemically related derivatives of catechol it was found that the catechol and amine group were required for the rapid component ($t_{1/2} \sim 2$–5 sec) whereas the amine substituent (e.g., *N*-methyl) and the chiral β-hydroxy group were necessary for the slow component ($t_{1/2} \sim 2$–3 min) [68] (see Figure 3.1). The chemical groups responsible for rapid conformational change were also shown to be sufficient for activation of the G_s protein, whereas agonist-induced internalization was to a large extent dependent on the slow conformational change [68].

In the earlier studies on the purified and fluorescently labeled β_2AR, only the agonist-induced slow conformational change of TM6 ($t_{1/2} \sim 2$–3 min) was evident [37–39]. Conceivably, the explanation for why the rapid conformational change escaped detection in these studies is most likely to be reflected by the choice of fluorescent probe and the positions labeled in the receptor. Notably, the rapid conformational change was detected from a position ($Cys^{265(6.27)}$) in the β_2AR that might detect conformational changes both in the intracellular loop 3 and in TM6, in contrast with our previous studies in which detection of conformational changes might be restricted to TM6 [37–39].

Increasing evidence also suggests a more complex mode of action for partial agonists than predicted by the extended ternary complex model (for a review see [4]; see also Chapters 2 and 9). Analysis of fusion proteins between wild-type β_2AR and $G\alpha_s$ and a constitutively activated β_2AR and $G\alpha_s$ mutant, for example, have shown an interesting discrepancy between the efficacy of ligands in stimulating GTPase activity and in their ability to stabilize the ternary complex (i.e., high-affinity, GTP-sensitive agonist binding) [71]. These data suggest that partial agonism is, at least in some cases, explained by the ability of ligands to stabilize the ternary complex strongly, resulting in a

reduced rate of G protein turnover relative to the full agonist [71] (see also Chapter 8 for illustration). Further support for a complex action of partial agonists has been obtained in the β_2AR, again by application of fluorescent techniques. These studies included the application of both single-molecule fluorescence analysis and fluorescence lifetime analysis to a purified preparation of the receptor covalently labeled with a fluorescence reporter molecule [67, 72]. The data provided clear evidence for the existence of different conformational substates of the β_2AR that are differentially modulated by different agonists, thus suggesting that different agonists stabilize distinct conformational states of the receptor molecule [67, 72] (see also Chapter 9).

3.6 GPCR Activation in an Oligomeric Context

Despite healthy initial skepticism, it is now widely accepted that many, if not all, GPCRs can form homodimeric or even heterodimeric complexes (for reviews see [7, 73]). The formation of homodimers between class A GPCRs in living cells has in particular been supported by measurements of bioluminescence resonance energy transfer (BRET) or fluorescence resonance energy transfer (FRET) through the use of fusion constructs between a GPCR and luciferase and/or spectral variants of the green fluorescent protein, respectively [7]. Recent studies have also offered information about the putative dimeric interfaces. In the D_2R the fourth transmembrane segment was identified as the symmetrical dimer interface by cross-linking of an engineered Cys at the extracellular end of TM4 [74]. The dimeric interface of rhodopsin in native membranes was also suggested by atomic force microscopy to involve TM4 in addition to TM5 [75]. In remarkable agreement, a model involving TM4 and 5 as an important interface was suggested for the complement C5a receptor (C5aR) homodimer following symmetrical cross-linking of the second intracellular loop [76]. In another study, TM1 and TM2 were suggested to mediate oligomerization of a yeast GPCR [77].

In several cases it has been reported that agonists cause alterations in the degree of dimerization as reflected in changes in BRET or FRET. However, while the agonist induced increase in energy transfer in the β_2AR and in the thyrotropin-releasing hormone receptor (TRHR) [78, 79], a decrease was observed for the cholecystokinin receptor (CCKR) [80]. In contrast, other studies have found homodimerization or heterodimerization to be constitutive processes not modulated by ligand binding [81–83]. Although these obvious discrepancies may actually reflect the real behavior of the different receptors studied, it cannot be ruled out that difficulties in interpretation of the experimental data may have resulted in erroneous conclusions. The changes in FRET or BRET upon binding of a ligand, for example, could in principle also be explained in terms of changes in the relative orientation of the donor and acceptor molecules due to conformational changes rather than being the result of dissociation or association of the dimeric complex [73]. However, this does not exclude the possibility that oligomerization might indeed be important for receptor activation if recent observation for class C GPCRs can also be extended to class A GPCRs (see below).

The strongest evidence for unequivocal roles of dimerization has been obtained for the class C GPCRs. The crystal structure of the extracellular domain (ECD) of the mGluR found not only that the receptor exists as a dimeric protein, but also that dimerization of the receptor plays a key role in the receptor activation mechanism [84]. Moreover, studies of the metabotropic $GABA_B$ receptors ($GABA_B$Rs), which also belong to class C, showed yet another function of dimerization. Thus, formation of a heterodimeric complex between the $GABA_B$R1 (GBR1) and $GABA_B$R2 (GBR2) was found to be required for surface expression of a functional receptor [85–90]. Specifically, GBR1 requires GBR2 for proper targeting to the surface, whereas GBR2 by itself is surface expressed but does not bind GABA on its own [7]. Interestingly, this heterodimerization may also be critical for receptor activation and G protein coupling: experimental support has been obtained for a remarkable model in which GABA binds to the ECD of GBR1 but transmission of the signal to the G protein occurs solely via GBR2 [91, 92]. Most significantly, mutations known to impair G protein coupling in other class C GPCRs had no effect when introduced into GBR1, but prevented G protein coupling when introduced into GBR2 [91]. Moreover, a systematic mutagenesis study ruled out the possibility that GBR agonists can bind to the ECD of GBR2 [93]. In the related metabotropic glutamate receptor subtype 5 ($mGlu_5R$), it has similarly been found that binding of a single agonist molecule to one ECD is sufficient to cause receptor activation [94]; however, full activation was only obtained if a second agonist molecule was bound to the associated subunit. Additionally, evidence was obtained that G protein activation can occur through both receptor subunits [94]. Whether a similar remarkable scenario is relevant for other GPCRs, including class A receptors, still awaits clarification.

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4
Molecular and Cellular Determinants of GPCR Splice Variant Constitutive Activity

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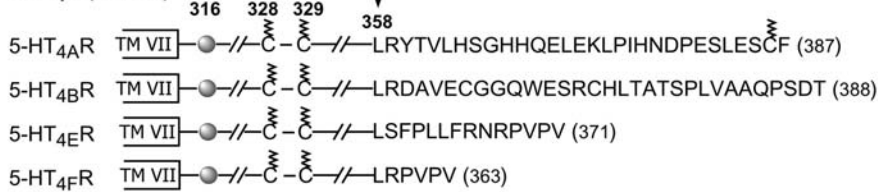
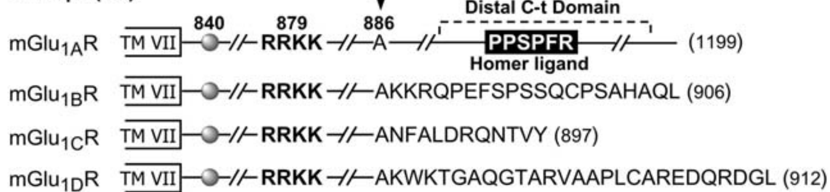
4.1
Introduction

Multicellular organisms are characterized by dense networks of cell–cell communication. Of all the proteins of the human genome, G protein-coupled receptors (GPCRs) are the most versatile. As stated in previous reviews, GPCRs are the typical example of how "molecular tinkering" has occurred during evolution, creating diversity through the copying of one ancestral gene and the production of more than one thousand receptors that can transduce signals triggered by light, smell, taste, nucleotides, lipids, peptides, and proteins [1–3]. Although the first GPCR neurotransmitter cloned, the β_2-adrenoceptor (β_2AR) [4], has no introns, it is clear that the existence of introns in many GPCRs provides scope for an additional level of diversity by virtue of alternative splicing [5]. One of the most complex genes is certainly the 5-hydroxytryptamine type 4 receptor (5-HT$_4$R) gene, which contains 38 exons in 700 Kb and generates eight carboxy-terminal (C-t) variants [6]. As in the case of 5-HT$_4$R, many other GPCR variants are generated by splicing of sites, resulting in sequence variations at their C-ts. What could be the reasons for the creation of such diversity in this domain? It is now widely accepted that the C-ts (the "magic tails") of GPCRs regulate most GPCR functions, both through their post-translational modifications (phosphorylation, palmitoylation) and through their interactions with a large number (over 50) of associated proteins called GPCR interacting proteins (GIPs) [7]. These functions include targeting, trafficking, clustering, desensitization, endocytosis, recycling, and, importantly, fine-tuning of GPCR signaling [7, 8]. Among these functions, this review concentrates on the modulation of GPCR constitutive activities (i.e., activity of the receptor in the absence of ligand) by the C-ts of some GPCR splice variants. Two types of constitutive activities are considered: the first is the spontaneous coupling of the receptor to G proteins, resulting in constitutive production of second messengers, while the second is the constitutive internalization of GPCRs that does not necessarily depend on G protein activation. We analyze only the C-ts of GPCR splice variants in the control of such constitutive activities, because there is no clear evidence that other structural domains generate such activities. The GPCR splice variants for which the largest amounts of





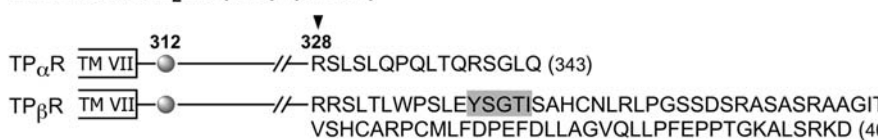


Fig. 4.1 Sequences of 5-HT_4R, 5-HT_7R, $mGlu_1$R, and prostanoid receptor (TPR, FPR, EP_3R) C-t splice variants.

data are available today are the 5-HT_4R, the metabotropic glutamate 1 and 5 receptors (mGlu$_1$R, mGlu$_5$R), the prostanoid receptors for thromboxane (TPRs), the prostaglandin $F_{2\alpha}$ receptor (FPR), and the prostaglandin E2 receptor (EPR). This review focuses on these receptors, a schematic representation of which is presented in Figure 4.1.

4.2 Constitutive Activation of Second Messenger Production by C-Terminal Splice Variants of GPCRs

Over the past 15 years, our representation of how GPCRs are activated has been reassessed on the basis of the pioneering work of Costa and Hertz, as well as that of Lefkowitz and co-workers [9, 10]. These workers demonstrated that a GPCR can isomerize from an inactive (R) to an active form (R*) in the absence of agonist, the R* receptor being "constitutively active". GPCRs are now regarded as allosteric molecules that are stabilized in the R* form by agonists (higher affinity for R* than for R), in the R form by inverse agonists (higher affinity for R than for R*), and in a constant R*/R ratio by antagonists (similar affinity for R and R*). However, it is likely that several other states also exist (multiple state model of receptor activation, see also Chapter 9). For several years, a third fundamental but very silent state called the ground state (Rg) has been proposed to exist in the case of the light receptor rhodopsin. This state can "only" be stabilized by the inverse agonist *cis*-retinal [11, 12]. The empty opsin (R) is slightly active, and the fully active state (R*) is obtained when the *cis*-retinal isomerizes to *trans*-retinal. The Rg state with bound *cis*-retinal has been crystallized [13]. Recently, further experimental evidence has established the existence of Rg states for the 5-HT_4R [14] and mGluRs [15].

The role of the C-ts in the control of constitutive activity of GPCRs has long been recognized; it has been proposed that the last twelve residues of bovine rhodopsin operate as a negative regulator of GDP/GTP exchange [16–18] and the inhibitory action of the C-t is abolished when the receptor is depalmitoylated [19]. Similarly, removal of the extended C-t of the avian βAR increases its constitutive activity [20]. Truncation of the last 59 residues of the thyrotropin-releasing hormone receptor (TRHR) causes constitutive activity of the receptor [21]. Therefore, it is not surprising that GPCR splice variants with different C-t sequences have been found to exhibit different constitutive activities.

4.2.1 The Constitutive Activities of C-Terminal 5-HT_4 Receptor Splice Variants: the Shortest, the Strongest

Eight human ($_{A,B,C,D,E,F,G,N}$), four mouse ($_{A,B,E,F}$), and three rat 5-HT_4R splice variants differing in their C-t sequences have been cloned [22–30]. Whatever the species, alignment of the putative C-t amino acid sequences predicted from their respective cDNA revealed that the divergence in their sequence starts after their last common amino

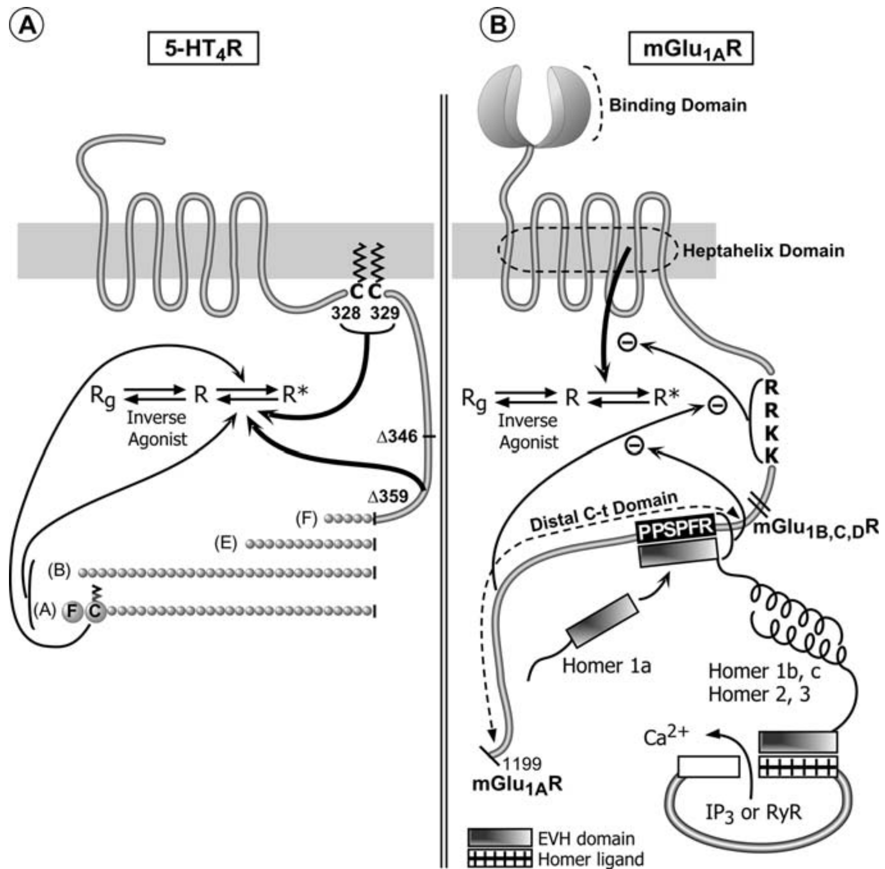


Fig. 4.2 Influence of the C-t internal sequences on the allosteric transition between different states, based on studies with the light receptor rhodopsin: the inactive state (R), the active state (R*), and a particularly "silent" one (Rg: ground state). R exhibits a very low constitutive activity, the Rg is stabilized by inverse agonists, and agonists stabilize the R* state.

A) Several C-terminal sequences stabilize the R form, including:
- the specific C-terminal sequences (following Leu^{358}) of $5\text{-}HT_{4A,B}R$ [24],
- the Ser/Thr-rich sequence between residues 346 and 359 [24].

Palmitoylation of Cys^{328} and Cys^{329} is also important to maintain the receptor in the R form [34]. Palmitoylation of the Cys localized at the very end of $5\text{-}HT_{4A}R$ has the opposite role to palmitoylation of Cys^{328} and Cys^{329} [34].

B) $mGlu_{1A}R$, but not $mGlu_{1B,C,D}R$, has a constitutive activity. The heptahelical domain (HD) of $mGlu_{1A}R$ (circled) deleted from the binding domain and the C-t is also constitutively active [15]. The -ArgArgLysLys- sequence within the C-t inhibits this constitutive activity [36]. The C-t (after this -ArgArgLysLys- sequence) suppresses the effect of the ArgArgLysLys sequence, thus reintroducing a constitutive activity. However, this can also be suppressed by the binding of coiled-coil Homer proteins capable of making a connection between the ProProSerProArg sequence of $mGlu_{1A}R$ and downstream proteins such as the inositol trisphosphate (IP_3) and the ryanodine receptors (RyRs) [41]. Production of Homer1a (an immediate early gene), following neuronal activation, which is characterized by its absence of coiled-coil domain, acts as a dominant negative protein. Homer1a disconnects $mGlu_{1A}R$ from the downstream proteins and shifts the receptor to an active state [41]. The $mGlu_{1B,C,D}Rs$, which have C-ts ending after the ArgArgLysLys sequence, have no constitutive activity. Suppression of the ArgArgLysLys sequence reintroduces a constitutive activity of the HD [36].

acid at the position Leu[358]. Figure 4.1 illustrates the divergent sequences at this position for the four mouse splice variants, which have been studied for their constitutive activity in detail (coupling to G_s, activation of adenylyl cyclase; see also Chapter 8).

At low receptor density, comparable to that found in the brain (150–200 fmol mg^{-1}), we found that the long C-t form of 5-HT_{4A}R generated twice as much cAMP as mock-transfected cells; similar results were obtained in LLC-PK1 cells [24]. This indicated a high capacity of 5-HT_4R to isomerize from R to R* in the absence of agonist. In comparison, human β_2AR did not modify cAMP production when expressed at low density [24, 31]. In COS-7 cells, an increase in cAMP production by less than two-fold was obtained when the β_2ARs were expressed at a density of 2 pmol mg^{-1} [31]. The 5-HT_4R constitutive activity could be inhibited by inverse agonists, but not by neutral antagonists [32]. 5-HT_{4A}R and 5-HT_{4B}R splice variants had similar constitutive activities four times higher than the mock activities at 800 fmol mg^{-1}. The shortest splice variants (5-$HT_{4E,F}$R) (Figure 4.1) exhibited constitutive activities over twice as high as those of the longest 5-$HT_{4A,B}$R variants [24].

We then analyzed the roles of different sequences within the C-ts of 5-HT_4Rs in more detail in relation to the R to R* isomerization [24, 33]. The Δ359 truncated mutant (Figure 4.2A) had a constitutive activity similar to those of the shortest splice variants (5-$HT_{4E,F}$R), indicating that the specific sequences of the shortest splice variants were not responsible for receptor constitutive activity in relation to those of the longest splice variants (5-$HT_{4A,B}$R) which were neutral. In contrast, we can say that the specific C-t sequences of 5-HT_{4A}R and 5-HT_{4B}R reduce the R to R* isomerization [24] (Figure 4.2A). A sequence upstream of Leu[358] between residues 359 and 346 is very important in the reduction of constitutive activity (Figure 4.2A). The Δ346 truncated mutant was highly active (ten times mock activity at 500 fmol mg^{-1}). Interestingly, this sequence (13 residues) contains six Ser and Thr residues that are potential phosphorylation sites. Further truncation of the C-t (Δ327) did not result in further constitutive activation.

However, we know that the two palmitoylated cysteines at the end of helix 8 (Cys[328] and Cys[329]) are also important for the inhibition of isomerization, because their mutation into Ser results in constitutive activation of the 5-HT_{4A}R (Figure 4.2A) [34]. The Cys located at the very end of the 5-HT_{4A}R splice variant in the Post-synaptic density Disc large Zonula occludens (PDZ) ligand type I sequence (-SerCysPhe) is also a potentially palmitoylated site. Mutation of this Cys into a Ser decreases constitutive activity in the Cys[328]Ser/Cys[329]Ser 5-HT_{4A}R mutants [34] (Figure 4.2A). Differences in the level of constitutive activity between several human 5-HT_4R splice variants with different C-t sequences have also been observed in stably transfected cell lines [27–29].

To date, we know nothing about the mechanisms by which these different sequences – within the common part of the C-t up to Leu[358] or within the specific sequences to each splice variant – modulate receptor constitutive activity. A structural role is possible, as are roles of putative post-translational modifications of these sequences (phosphorylation but also palmitoylation/depalmitoylation) or interaction with specific GIPs. Unfortunately, we have no proof that such regulations modulate 5-HT_4R constitutive activity or if expression of constitutive 5-HT_4R splice variants exist in native tissues.

4.2.2
The Constitutive Activities of $mGlu_1R$ and $mGlu_5R$ C-t Splice Variants: a Case for which a Physiological Control does exist

A further level of complexity is reached when we consider the constitutive activities of $mGlu_1R$ splice variants (Figure 4.2B). In this case, comparison between the results obtained in heterologous expression systems and native cells (neurons) generates a new concept which can be formulated as follows: "a GPCR can be activated independently by extracellular ligands and GIPs".

4.2.2.1 What has been Observed in Heterologous Expression Systems?

In both LLC-PK1 and HEK-293 cell lines, the long $mGlu_{1A}R$ C-t splice variant (Figure 4.1), which contains more than 350 residues, exhibited constitutive activity, as opposed to the shorter splice variants ($mGlu_{1B,C,D}R$) [35]. In a further analysis of the roles of the different domains of the long C-t, we observed that the absence of constitutive activity of the shorter variants was in fact due to the presence of a polybasic sequence (-ArgArgLysLys-) that inhibited receptor constitutive activity [36]. Mutation of this -ArgArgLysLys- sequence, or its truncation upstream, revealed receptor constitutive activity. In fact, Pin's group recently demonstrated that the core domain of $mGlu_{5A}R$ alone (without the C-t and with or without the N-terminal fly-trap binding domain) was constitutively active [15]. Interestingly, positive allosteric agonists that bound to the core domain became full agonists of the constitutively active truncated mutant [15].

Therefore, these experiments suggest that the core domain is constitutively active, and that the -ArgArgLysLys- sequence inhibits this constitutive activity (Figure 4.2B). Why, then, is the long C-terminal splice variant, which also contains the -ArgArgLysLys- sequence, constitutively active? Probably because the distal C-t domain (a domain including the Homer ligand sequence -ProProSerProPheArg- but also many other proline clusters located downstream of this sequence) neutralizes the inhibitory effect of the -ArgArgLysLys- sequence (Figure 4.2B). The relationship between the possible role of the -ArgArgLysLys- sequence as an endoplasmic reticulum (ER) retention sequence and its constitutive activity modulatory role is unknown. Note that the two long C-t splice variants of $mGlu_{5A,B}R$ also exhibited constitutive activity when transfected in cell lines [37].

4.2.2.2 What has been Observed in Neurons? Homer Proteins come into the Game

The first Homer cDNA (Homer1a) was isolated as an immediate early gene that is upregulated after electroconvulsive seizures and long-term potentiation (for a review on Homer see [38]). It contains an Ena/VASP homology 1/Wiskott–Aldrich syndrome protein homology 1 (EVH1/WH1) domain found in the Ena/vasodilator-stimulated phosphoprotein (VASP) family. Other Homer genes (*Homer1b, 1c, Homer2* and *3*) encode proteins with a homologous EVH1 domain, and with a C-t coiled-coil domain that confers the property of self- and hetero-multimerization to Homer. This coiled-coil domain is not present in Homer1a. The EVH1 domain in a Homer protein binds

to a specific polyproline sequence (-ProProXXPheArg-; Homer ligand) present in the C-ts of the $mGlu_{1A}R$ splice variant and $mGlu_5R$ but absent in the C-ts of the shortest $mGlu_{1A}R$ splice variants (Figures 4.1, 4.2B). By multimerization, the coiled-coil-containing Homer proteins create physical links between several proteins containing the consensus sequence -ProProXXPheArg-, therefore physically linking $mGlu_{1A}R$ and $mGlu_5R$, but not $mGlu_{1B,C,D}R$, to Ca^{2+}-permeable inositol trisphosphate (IP_3) and ryanodine receptor channels, store-operated transient receptor channels 1 and 4 (TRPC1, TRPC4), the $Ca_v2.1$ subunit of P/Q channels, Dynamin III, the post-synaptic scaffolding SH3-ankyrin repeat (Shank) protein family, Shank proteins, and phosphoinositide 3 kinase (PI3K) enhancer (Figure 4.2B) (for reviews see [39, 40]). Homer1a binds to the same -ProProXXPheArg- sequence but does not possess the coiled-coil sequence and therefore behaves as a dominant negative mutant (Figure 4.2B).

In contrast to what was observed in HEK-293 cells, native $mGlu_{1A}R$ or recombinant $mGlu_{5A,B}R$ display no constitutive activity in cerebellar granule cells. The $mGlu_1R$-specific inverse agonist BAY36-7620 ((3a*S*,6a*S*)-6a-naphthalen-2-ylmethyl-5-methylidene-hexahydro-cyclopenta[c]furan-1-on) has no effect on the activity of these receptors as determined by their ability to activate a large conductance Ca^{2+}-dependent K^+ channel (BKCa) through the release of intracellular Ca^{2+}. However, the constitutive activity of these receptors was revealed to be induced in these neurons when the link between the $mGlu_{1A}R$ or $mGlu_5R$ and Homer3 is disrupted by induction of endogenous Homer1a, by mutation of the $mGlu_5R$ C-t domain, or by knockdown of the endogenous coiled-coil Homer3 by antisense transfection [41]. Interestingly, the physical disruption between the TRPC channel and Homers also resulted in the expression of a constitutively active TRPC channel [42].

The most likely interpretation of these results is that the physical link between the -ProProXXPheArg- sequence and the coiled-coil Homer provides inhibitory control over the distal C-t domain of $mGlu_{1A}R$ (Figure 4.2B). Thus, the interplay of positive and negative regulation of the constitutive activity by the different sequences of the C-t of $mGlu_{1A}R$ and Homer is as follows (Figure 4.2B):

- The core domain is by itself constitutively active.
- The -ArgArgLysLys- sequence inhibits this constitutive activity, so the short $mGlu_{1B,C,D}R$ splice variants, which contain only this sequence, are not constitutively active.
- The distal C-t domain of mGluR1a inhibits the inhibitory effect of -ArgArgLysLys-, so the $mGlu_{1A}R$ is constitutively active in recombinant cell lines.
- The association between the -ProProXXPheArg- ligand-binding sequence of the $mGlu_{1A}R$ and coiled-coil Homer proteins inhibits the effect of the distal C-t domain of the receptor by increasing physical constraints on the C-t of the receptor. The $mGlu_{1A}R$ associated with coiled-coil Homer is no longer constitutively active. This only occurs in neurons because of the presence of Homer proteins.
- In active neurons, Homer1a is induced and competes with coiled-coil Homer proteins. This disrupts the link between $mGlu_{1A}R$ and the coiled-coil Homer complex, thus rendering the $mGlu_{1A}R$ constitutively active.

In conclusion, this series of experiments highlights the important roles of splice variants with different C-t sequences. It also suggests a new concept, proposing that a GPCR can be activated allosterically both by external ligands and by modulation of their interactions with GIPs and associated networks. Future studies should tell us whether other sequences of the $mGlu_1R$ known to control the constitutive activity of the receptor (such as the -ArgArgLysLys- motif) are subject to regulation through interaction with GIPs. Negative control of GIPs over GPCR constitutive activity may explain why it is far easier to find constitutive activity in recombinant cells (in which specific interacting GIPs are probably not expressed) than in native cells.

4.2.3
Other Examples of GPCR C-t Splice Variants with Different Constitutive Activities

Among the human $5\text{-}HT_7R$ splice variants three ($5\text{-}HT_{7A}R$, $5\text{-}HT_{7B}R$, and $5\text{-}HT_{7D}R$) differ after their last common residue (Leu^{432}) (Figure 4.1). The constitutive activity of the $5\text{-}HT_{7B}R$ coupled to G_s (stimulation of adenylyl cyclase) was significantly higher than those of the $5\text{-}HT_{7A}R$ and $5\text{-}HT_{7D}R$ splice variants [43].

The EP_3R C-t splice variants ($EP_{3\alpha}R$ and $EP_{3\beta}R$), which mediate the action of prostaglandin E_2, differ after the last common residue (Glu^{335}) [44] and in their ability to activate G_I proteins in an agonist-independent (constitutive) manner. The third splice variant $EP_{3\gamma}R$ is coupled both to G_s and G_i, but showed mostly full constitutive G_i coupling and no constitutive G_s coupling [45]. This indicates that the R* state for G_i and G_s coupling are certainly different. Multistate models of GPCR activation are discussed in Chapters 2, 3, and 9. Finally, the two prostaglandin $F_{2\alpha}$ receptor splice variants FP_AR and FP_BR (Figure 4.1) displayed significant constitutive activity, while the FP_BR splice variant displayed twice the constitutive activity of the FP_AR splice variant when expressed in COS-7 cells [46].

4.3
Differential Constitutive Internalization of C-t GPCR Splice Variants

Agonist stimulation of GPCRs can result in their internalization into intracellular vesicles followed by their recycling to the cell surface or further transport to lysosomes for possible degradation [47, 48]. Receptor internalization pathways include clathrin-coated vesicles, caveolae, and non-coated vesicles. Several non-GPCR receptors, such as the transferrin receptor, constitutively recycle through clathrin-coated vesicles. Constitutive internalization of prostanoid receptor subtypes has recently been described. Interestingly, in both cases, this constitutive internalization is observed for only one thromboxane A_2 receptor splice variant (the $TP_\beta R$) and one prostaglandin $F_{2\alpha}$receptor splice variant (the FP_BR) (Figures 4.1 and 4.3).

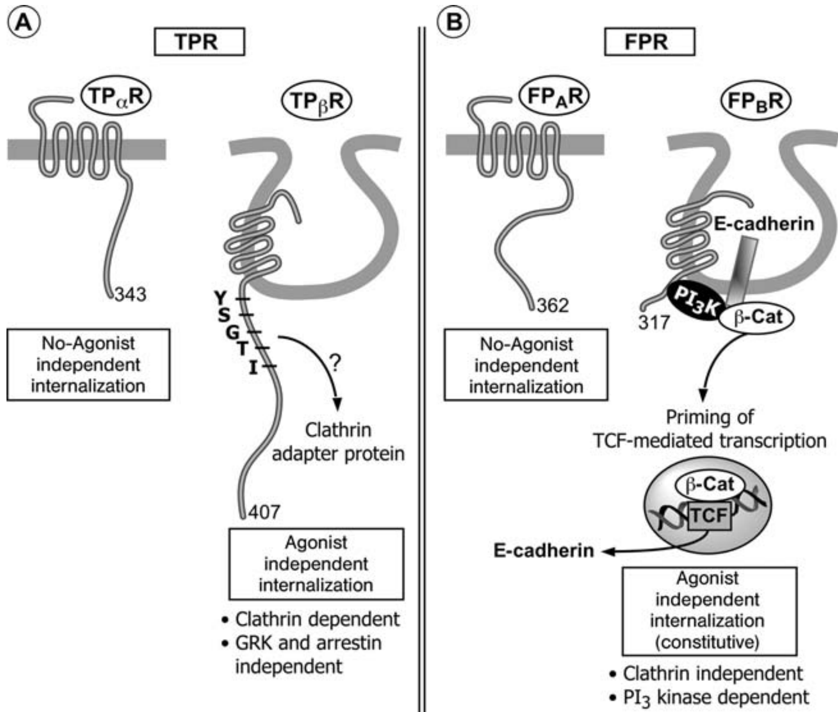


Fig. 4.3 Constitutive internalization of splice variants of TP and FPR. A) The $TP_{\beta}R$ variant, but not the $TP_{\alpha}R$, is internalized in the absence of agonists (constitutive internalization). $TP_{\beta}R$ internalization requires the presence of the -TyrSerGlyThrIle- sequence, which may link the receptor to clathrin-adapter proteins (APs). B) The $FP_{B}R$ variant, but not the $FP_{A}R$, is constitutively internalized. $FP_{B}R$ internalization is clathrin-independent, but is dependent on its interaction with PI3 kinase. The internalization also involves interactions with E-cadherin and β-catenine (βCat). The increase in cytoplasmic βCat produces a "priming" affect on TCF-mediated transcription (with synthesis of E-cadherin). This "primed" pathway is fully activated by receptor activation.

4.3.1
The Thromboxane A2 Receptor $TP_{\beta}R$, but not the $TP_{\alpha}R$ Splice Variant, is Constitutively Internalized by Clathrin-dependent, GRK- and Arrestin-independent Mechanisms

TPRs are expressed as two alternative splice variants: $TP_{\alpha}R$ (343 residues) and $TP_{\beta}R$ (407 residues), which differ after Arg^{328} in their C termini (Figure 4.1). $TP_{\alpha}R$, but not $TP_{\beta}R$, undergoes desensitization mediated by protein kinase A (PKA) (phosphorylation of Ser^{329}) [49] and nitric oxide/ cGMP-dependent protein kinase (PKG) (phosphorylation of Ser^{331}) [50]. In contrast, $TP_{\beta}R$, but not $TP_{\alpha}R$, undergoes agonist-induced desensitization and internalization in HEK-293 cells. This process involves clathrin-coated pits, GRKs, and arrestins [51]. This difference may just reflect a greater sensitivity of $TP_{\alpha}R$ to arrestin, since over-expression of arrestin-3 allowed recovery of an agonist-dependent internalization for $TP_{\alpha}R$. In addition, $TP_{\beta}R$, but not $TP_{\alpha}R$, un-

dergoes constitutive internalization. This constitutive internalization was dynamin-dependent and involved clathrin-coated pits, but was independent of GRK (G Protein Receptor Kinase) and arrestins [52]. In contrast, this process involved a specific sequence (-TyrSerGluThrIle-) present in $TP_{\beta}R$ but not $TP_{\alpha}R$ receptor splice variants (Figure 4.1) [52]. Clathrin and its adapter proteins (APs) are major structural proteins in the endocytic machinery and play a central role in clathrin-coated pit assembly. Several studies have demonstrated direct interaction between similar motifs and the μ chain of the clathrin adapter proteins AP1, AP2, and AP3 (see refs in [52]). The -TyrXXThr motif has been demonstrated to be present and in CI mannose-6-phosphate receptor, the epidermal growth factor receptor (EGFR), and the cytotoxic T lymphocyte antigen 4 (CTLA-4) receptors.

4.3.2 The Prostaglandin $F_{2\alpha}$ Receptor FP_BR, but not the FP_AR C-Terminal Splice Variant, is Constitutively Internalized by a Clathrin-independent, PI3 Kinase-dependent Mechanism

FPRs are expressed as two splice variants: FP_AR and the FP_BR. The FP_BR isoform is basically a truncated version of the FP_AR isoform, lacking the last 46 carboxyl-terminal residues (Figure 4.1).

It was first observed that stimulation of both isoforms with $PGF_{2\alpha}$ resulted in cell rounding through the activation of the Rho signaling pathway [53]. Once the agonist was removed, reversal of cell rounding appeared in cells expressing FP_AR, whereas in FP_BR-expressing cells the reversal of cell rounding did not occur [54]. The mechanism of this differential reversal after the removal of $PGF_{2\alpha}$ involved specific activation of the TCF-β-catenin (TCF = T-cell factor) signaling pathway in FP_BR-expressing cells; this does not occur in FP_AR-expressing cells [55, 56]. Another difference was found in the signaling of these two splice variants. The FP_BR variant, but not its FP_AR counterpart, displays a constitutively active internalization (Figure 4.3) [56]. Interestingly, this constitutive internalization involves a new mechanism: the interaction with phosphatidylinositol 3'-kinase (PI3 kinase) associated with its activation [57]. E-Cadherin and β-catenin are also internalized with the FP_BR (Figure 4.3) [57]. This constitutive internalization is clathrin-independent and determines the TCF-β-catenin signaling induced by the FP_BR isoform but not by FP_AR [57]. Upon $PGF_{2\alpha}$ stimulation of cells expressing FP_BR, there is a large increase in β-catenin in the cytoplasm and a strong activation of TCF signaling. This may result in cyclooxygenase 2 transcription and be related to colon cancer, since it is known that inhibition of cyclooxygenase enzymes is protective against this pathology.

4.4 Conclusion

GPCRs possess “magic tails“ that are obviously very important for most of their functions, including constitutive activation of G proteins, production of second messengers, and constitutive internalization. Here we have reviewed examples in which different C terminus sequences generated differentially by alternative splicing from a given GPCR gene can modulate these constitutive functions. Whether such sequences indeed play important roles under physiological conditions remains to be established, and this will certainly be one of the most difficult challenges over the next few years. However, the modulation of the constitutive activity of the $mGlu_{1A}R$ variant by Homer proteins should encourage work in this direction.

Acknowledgments

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5
Naturally Occurring Constitutively Active Receptors: Physiological and Pharmacological Implications

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5.1
Introduction

G protein-coupled receptors (GPCRs) exist in equilibrium between inactive (R) and active (R*) states. Active receptor conformations, whether ligand-induced or ligand-independent, trigger second messenger signaling through heterotrimeric G proteins. GPCRs that signal in the absence of ligand are, by definition, "constitutively active". Over the past 15 years it has become evident that wild-type, polymorphic, and mutant receptors may demonstrate elevations in basal signaling [1, 2].

Constitutive activity can most readily be assessed by use of expression systems (e.g., COS-7, HEK-293, *Xenopus* melanophore, or insect Sf9 cells) that result in a high density of recombinant GPCRs. Under these experimental conditions (as well as with selected receptors in native cells), signaling (e.g., cAMP or inositol phosphate production, luciferase reporter gene activity, [^{35}S]GTPγS binding) can be measured both in the presence and in the absence of ligand (see also Chapter 8). It is estimated that as many of 40% of wild-type GPCRs exhibit constitutive activity [1].

Table 5.1 Naturally occurring receptor variants that may be constitutively active.

	References
Wild-type GPCRs	
Interspecies variants of the same receptor subtype	4–7
Related GPCR subtypes within a single species	1, 10–13
Alternatively spliced receptors	6, 15–18
Polymorphic receptors	
Single nucleotide polymorphisms	21, 22, 28
RNA editing	23, 24
Disease-producing mutants	34–46

Accumulating evidence suggests that constitutive activity of many naturally occurring GPCR variants results in important physiologic consequences. Representative examples are presented in this chapter as illustrations. We refer the reader to recent reviews [1–3] and Table 1.1 (see Chapter 1) for a more comprehensive listing of constitutively active receptor subtypes and variants. The examples that follow (as outlined in Table 5.1) illustrate receptor variations that affect the basal level of signaling and thereby have the potential to modulate physiologic function and/or drug response, as well as to trigger disease.

5.2 Wild-type Interspecies Homologues

Differences in the basal levels of wild-type receptor signaling have been highlighted in studies comparing species homologues of GPCR subtypes (*e. g.*, selected melanocortin, prostaglandin, and gastrin receptors). For certain receptors [*e. g.*, melanocortin receptor subtype 4 (MC_4R)], the human homologue is constitutively active, whereas the equivalent rodent subtype lacks ligand-independent signaling [4, 5]. Conversely, an isoform of the mouse prostaglandin E_2 receptor subtype 3 ($EP_{3\gamma}R$) is highly constitutively active, whereas the corresponding human homologue shows no ligand-independent signaling [6]. In the case of the human and *Mastomys natalensis* (an African rodent) gastrin receptors, also referred to as the cholecystokinin receptor subtype 2 (CCK_2R), it is the rodent homologue that shows significant basal signaling [7]. This constitutive activity may, in part, explain the predisposition of *Mastomys* to the development of gastric endocrine tumors. Of note, in rats, chronic stimulation with gastrin also results in the development of gastric tumors [8, 9]. *In vitro* structure/function analysis of the human vs. the *Mastomys* recombinant receptors resulted in the identification of three amino acid differences in homologous positions (human: Val^{340} and Val^{349} in transmembrane domain 6 and Glu^{403} in the carboxy-terminal tail vs. *Mastomys*: Leu^{344}, Iso^{353}, and Asp^{407}, respectively) that underlie the variation in basal signaling. Substitution of the human amino acids with the *Mastomys* equivalents confers constitutive receptor activity; the converse substitutions result in a decrease in basal signaling by the *Mastomys* GPCR. From this study it is evident that even conservative amino acid substitutions (e.g. Val $\rightarrow$ Leu) are sufficient to alter the basal level of receptor-mediated signaling. Furthermore, this example illustrates that multiple residues may act in concert to define the basal level of GPCR activity. These observations and the principle they illustrate should inform the design of preclinical drug trials. It is important to consider the possibility that interspecies differences may translate into variations in the corresponding basal levels of signaling, which may in turn alter drug-induced GPCR mediated physiologic effects.

5.3
Wild-type Receptor Subtypes within a Given Species

A large number of receptor subtypes have also been demonstrated to show variations in basal signaling. Examples of this include subtypes of the dopamine, thyrotropin releasing hormone, and bradykinin receptors, as well as adrenoceptor subtypes [1, 10–13]. The molecular basis for these differences has in some instances been carefully explored. In both human and rat, within the dopamine 1-like receptor family, the dopamine receptor subtype 5 (D_5R) is constitutively active relative to the D_1R subtype [10]. In structure/function studies, it was shown that exchange of a single homologous amino acid (Phe^{264} vs. Ile^{288}) between the recombinant human D_1R and D_5R was sufficient to increase or decrease the basal level of signaling [14].

5.4
Wild-type Alternatively Spliced Receptors

GPCR splice variants are another natural source of homologous receptor variation in basal signaling. There are at least six isoforms of the human prostaglandin E_2 receptor, subtype 3 (EP_3R) that have been identified [6]. Each can be distinguished on the basis of differences in the carboxy terminal domain, which ranges from six to 65 amino acids. Among the splice variants examined, two are markedly constitutively active, whereas other isoforms show little or no ligand-independent signaling. Other examples in the literature in which the magnitude of ligand-independent signaling is linked to specific splice variants include isoforms of the serotonin receptor subtype 4 (5-HT_4R) [15–17] (see also Chapter 14) and the metabotropic glutamate receptor subtype 1 ($mGlu_1R$) (see also Chapter 4) [18].

5.5
Polymorphisms in GPCRs

More subtle differences in receptor amino acid sequence can also result in varying levels of ligand-independent signaling. With the completion of the human genome project, efforts have shifted toward defining how inter-individual genetic differences influence protein function. A large number of single nucleotide polymorphisms (SNPs) have been identified, and work is in progress to define how the corresponding alterations in GPCR amino acid sequences influence the pharmacology and physiology linked to these proteins [3, 19, 20]. Precedents in the literature suggest that receptor variants with as few as one to three amino acid differences may have both markedly altered basal and ligand-induced signaling. Human formyl peptide receptor isoforms FPR1-26 and -98 differ at two amino acid positions. Both the efficiency of FPR1-26 in coupling to G_i and the corresponding basal level of receptor signaling markedly exceed those of FPR1-98. It is postulated that functional differences in these FPR1 isoforms correlate with susceptibility to selected bacterial infections [21].

Receptor SNPs that influence basal signaling also have the potential to alter physiologic function as well as the response to GPCR selective drugs. A polymorphism in the human serotonin receptor (5-HT_{2C}R) occurring with a frequency of 0.13 % in the Caucasian population has been described (Cys^{23}Ser). This single amino acid alteration results in an elevation in 5-HT_{2C}R basal signaling, which may in turn influence mood, feeding behavior, and/or the response to antidepressant drugs [22]. Additional variation in the 5HT_{2C}R may occur through a process of RNA editing in which genomically encoded adenosine residues are converted into inosine residues by adenosine desaminase. These alterations may result in variation in the amino acid sequence of the second intracellular loop of the 5HT_{2C}R, resulting in a decrease in basal signaling with a corresponding reduction in drug response [12, 23, 24] (see also Chapter 14). The extent to which RNA editing is a source of heterogeneity for other GPCRs remains to be determined.

Melanocortin receptors provide clear precedents for how GPCR polymorphisms may give rise to variable phenotypes. The melanocortin 1 receptor subtype 1 (MC_1R) is a well established regulator of melanogenesis [25–27]. Single amino acid substitutions in the MC_1R can alter basal and/or ligand-induced signaling that can in turn result in variations in hair color. In mice, constitutively active MC_1Rs confer black coat color [28]. Although no activating mutations have been identified in humans, MC_1R variants with reduced signaling may result in red hair as well as increased sun-sensitivity and susceptibility to skin cancers [25].

The MC_4R provides an additional example of how polymorphisms/mutations can influence the basal level of GPCR function [29, 30]. This receptor is of particular interest due to the observation that the wild-type isoform is constitutively active [31]. Targeted disruption of the mouse MC_4R gene (–/–) results in an obese phenotype. Heterozygotes (+/–) show gene dosage effects with body weights that are intermediate between those of wild-type (+/+) and knockout (–/–) animals [32]. The endogenous peptides α-melanocyte-stimulating hormone and agouti-related protein are MC_4R agonist and inverse agonist, respectively. As such, these hormones increase or decrease, respectively, the basal level of receptor signaling, thereby contributing to the regulation of body weight. In humans, MC_4R polymorphisms are the most frequent monogenic cause of obesity [29, 33], accounting for approximately 2–6 % of cases. Receptor variants with a selective loss of constitutive activity but normal agonist-induced function and expression levels have been shown to predispose to obesity [31]. Other polymorphisms/mutations give rise to impaired MC_4R signaling due to a combination of defects in ligand binding, signal transduction, and/or receptor trafficking [30]. Diminution in basal and/or ligand-stimulated levels of MC_4R cellular signaling results in increased food intake and the development of obesity.

5.6
GPCR Mutation-induced Disease

Another group of naturally occurring constitutively active receptors includes those in which point mutations have been shown to enhance the basal level of signaling and thereby cause human disease (see also Chapters 8 and 15). Well established examples occur in class A [e.g., basal thyroid stimulating hormone receptor (TSHR) activity resulting in hyperthyroidism or adenomas], class B [e.g., basal parathyroid hormone receptor subtype 1 (PTH1R) signaling resulting in chondrodysplasia], and class C receptors [basal calcium-sensing receptor (CaSR) activity resulting in hypocalcemia]. These, as well as other examples are summarized in Table 5.2.

As discussed above, a wide range of GPCR alterations are sufficient to shift basal levels of receptor signaling. The spatial distributions of known activating mutations in representative receptors (e.g., TSHR, LHR, and CaSR) suggest that single amino acid substitutions in virtually any receptor domain (i.e., N terminus, extracellular loops, transmembrane domains, intracellular loops and C terminus) can result in an alteration in basal signaling [2, 47]. The consequences of this variability may include a change in ligand-independent signaling, endogenous hormone activity, and/or the efficacy or potency of drugs. Alone or in combination, these pharmacologic changes have the potential to enhance or diminish susceptibility to disease.

Table 5.2 Mutation-induced GPCR constitutive activity results in disease/altered phenotype.

Receptor	Disease	Reference
TSHR	thyroid adenoma	34–36
TSHR	hyperthyroidism	35
LHR	precocious puberty	37–39
PTH1R	chondrodysplasia	40
CaSR	hypocalcemia	41–44
Rhodopsin	congenital night blindness	45
FSHR	normal semen despite undetectable serum FSH	46

Abbreviations: TSHR: thyroid stimulating hormone receptor. LHR: luteinizing hormone receptor. PTH1R: parathyroid hormone receptor subtype 1. CaSR: calcium-sensing receptor. FSHR: follicle stimulating

5.7
Future Challenges

With the completion of the human genome project, it is evident that there are approximately 360 non-olfactory GPCRs [48, 49]. For many of these proteins, multiple polymorphic variants that alter the protein-coding region of the receptor have been, and will continue to be, identified [19, 20]. Pharmacologic effects of these single amino acid changes include alterations in receptor expression and basal signaling, as well as in the affinities, efficacies and/or potencies of cognate ligands [3]. The extent to which variant GPCRs have altered pharmacologic properties remains to be determined. One of the challenges going forward is to characterize these receptors fully and to compare them with corresponding wild-type isoforms. This will then set the stage to explore how sequence/pharmacologic alterations can result in phenotypic attributes, disease, and/or ligand/drug sensitivity. The identification and characterization of constitutively active polymorphic GPCRs will be an important part of this ongoing initiative.

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6
The Impact of G Proteins on Constitutive GPCR Activity

Graeme Milligan

6.1
Introduction

Expression of the vast majority of G protein-coupled receptors (GPCRs) in heterologous cell lines results in an enhanced level of G protein-mediated signal transduction being produced even in the absence of ligands that act as agonists at the receptor. Such constitutive activity is generally observed to increase in a linear fashion with GPCR expression levels. As this has been convincingly demonstrated in cell systems in which expression of the GPCR is under the control of an inducible promoter it is clear that it reflects an intrinsic ability of the GPCR to produce G protein activation. Such results are explained both in the two-state and in more complex models of GPCR function by an inherent capacity of the GPCR to flicker between active and inactive conformations (see also Chapters 2 and 3). Agonists function by stabilization or enrichment of the proportion of the GPCR in the active (R*) state. The identification of compounds that reduce receptor constitutive activity provided a degree of symmetry and resulted in the terminology of 'inverse agonist' for compounds that selectively enrich or favor an inactive (R) state [1–3]. Many GPCRs display relatively low levels of constitutive activity and thus require the presence of agonist to generate a substantive signal. For many GPCRs the level of constitutive activity can be enhanced by judicious mutation. Although 'hot spots', such as the region close to the junction of the third intracellular loop and transmembrane helix VI, have attracted significant attention, random mutagenesis studies [4–6] have demonstrated that the constitutive activity of many GPCRs can be modified by alterations at wide ranging locations in their sequence. This has resulted in the view that many GPCRs have evolved to be constrained in largely inactive states and that these constraints are relaxed by agonist binding. Many of these issues are addressed by other contributors to this volume and so are not dealt with here.

6.2
The Contribution of G proteins to Constitutive Activity

6.2.1
Basic Features

Heterotrimeric G proteins involved in signal transduction from GPCR to effector function as molecular switches controlled by the nature of the guanine nucleotide bound to the G protein α subunit. As with the GPCRs, G proteins must have an inherent capacity to adopt the active conformation, by binding GTP, and to return to the inactive conformation of the α subunit after hydrolysis of the terminal phosphate of GTP by the GTPase activity intrinsic to the G protein (see also Chapters 8 and 9). Different G protein α subunits display markedly different capabilities to exchange GTP for GDP and subsequently to hydrolyze the GTP. In general, at least when purified and reconstituted, the pertussis toxin-sensitive members of the G_i subfamily display markedly greater basal guanine nucleotide processing rates than the G_q/G_{11} and G_{12}/G_{13} G protein subfamilies. This may be to enhance sensitivity in their roles in regulation of ion channel function. Even in the G_i subfamily, the rate of GTP hydrolysis by purified protein is very low given expectations from the temporal aspects of signal transduction. This, as with the monomeric, small molecular mass GTP binding proteins, resulted in searches for, and identification of, GTPase-activating proteins. A family of GTPase-activating proteins for the heterotrimeric G proteins is described generically as Regulators of G protein Signaling (RGS) proteins [7–9].

Because it is a bimolecular interaction, then, as with overexpression of GPCRs, enhanced expression of appropriate G proteins would be expected to enhance agonist-independent signal transduction. This has indeed been observed [10–14] and shows selectivity for the G proteins that are cognate targets for the GPCR [13–14]. Interestingly, overexpression of some, but not all, G protein β and γ subunit pairings has been reported to increase GPCR-mediated constitutive activity [13–14]. Equally, increasing of G protein levels by expression of $G\alpha_s$, β_1, and γ_2 subunits in Sf9 insect cells resulted in a decreased number of β_2-adrenoceptor (β_2AR) binding sites as monitored by use of radiolabeled inverse agonist drugs without a decrease in amount of the receptor as monitored in immunoblot studies [15]. Agonist ligands frequently detect less high-affinity binding sites when GPCR–G protein contacts are disrupted, so the loss of high-affinity inverse agonist binding sites is consistent with the spontaneous formation of a greater fraction of β_2AR–G protein complexes with higher G protein expression levels [15]. Furthermore, it has long been appreciated that it is common for pertussis toxin treatment of cells to result in lower measured GTPase activity in membranes prepared from the cells [16]. This is consistent with uncoupling of the constitutive activity of GPCRs from activation of the G_i family G proteins that are the targets for the ADP-ribosyltransferase activity of this toxin. It is stated routinely (but incorrectly) that pertussis toxin-catalyzed ADP-ribosylation 'inactivates' G_i family G proteins. In fact, the addition of ADP-ribose to a key GPCR contact domain of these G proteins just prevents effective GPCR-mediated activation of the G protein. Addition of the relatively large and negatively charged ADP-ribose moiety simply

disallows productive GPCR–G protein interactions. Thus, loss of agonist-independent activity after pertussis toxin treatment provides rather a good first indication of there having previously been constitutive information transfer and signal transduction. More subtle modifications than ADP-ribosylation to the extreme C termini of G protein α subunits have been extremely useful in aiding understanding of some aspects of the impact of G proteins on constitutive activity (see Section 6.3.2).

6.2.2
The Distribution of G Proteins in the Plasma Membrane

Measurements of the absolute levels of expression of GPCRs and G proteins invariably conclude that the G proteins are in considerable molar excess with regard to any specific GPCR [17–18]. This poses certain problems in pharmacology, because models of GPCR–G protein function that are both robust and predictive in nature derive from the premise that [GPCR] $\geq$ [G protein]. Furthermore, the distribution of GPCRs and G proteins in the plasma membrane is non-random. Although the literature on the distribution of GPCRs is both large and complex, the equivalent literature for G proteins is relatively straightforward. Rather than being equally and randomly distributed, a substantial proportion of cellular G protein is targeted to, and located in, lipid 'rafts'. These are specialized structures enriched in cholesterol and sphingolipids that are both relatively resistant to dissolution by treatment with detergents and have low buoyant density [19]. This has allowed enrichment of such fractions on sucrose and other density gradients as 'detergent-insensitive' or 'detergent-resistant' membrane domains that contain only a small fraction of total membrane protein. They tend to be heavily enriched in G proteins because anchorage of G protein α subunits to the plasma membrane is, at least in part, determined by the covalent attachment of a pair of fatty acids to sites near the G protein N terminus [20–21] and such modified proteins partition selectively into such rafts.

A clear understanding of the plasma membrane distribution of GPCRs is therefore required in order to appreciate effects of G proteins on GPCR constitutive activity, because raft-associated GPCRs would be expected to be in a G protein-enriched environment in relation to a raft-excluded GPCR. There are limited direct data on this issue. However, it is intriguing that the β_2AR is generally accepted as displaying greater constitutive activity that the closely related β_1AR (see Chapter 9 for further details). In cardiomyocytes it has been reported that the β_2AR is more obviously raft-delineated than the β_1AR [22–23]. Is the accompanying higher concentration of the stimulatory G protein G_s, then, an explanation for this? One effort to address this issue was to build GPCR–G protein fusion proteins between the long isoform of $G\alpha_s$ and both the β_1AR and the β_2AR. These constructs define a 1:1 GPCR to G protein stoichiometry, and when these were expressed similar levels of constitutive activity were noted [24]. Although indirect, such studies are consistent with the idea that higher levels of constitutive activity of the β_2AR are indeed due to cellular compartmentation into rafts that are heavily enriched with G proteins.

6.3 GPCR–G Protein Fusion Proteins

6.3.1 Basic Features

Although artificial by design, constructs in which the N terminus of a G protein α subunit is linked to the C-terminal tail of a GPCR to generate a fusion protein containing the sequences and functionalities of both polypeptides have become widely used tools in studies of interactions between these protein classes [25–27]. Key issues analyzed through the use of such fusion products include the selectivity of interactions of different G proteins with the same GPCR [28–29], the role of posttranslational acylation in information transfer between GPCR and G protein [30–33], and the ability of GPCRs to exist as both homo- and heterooligomers [34]. The 1:1 stoichiometry between GPCR and G protein defined by the fusion strategy has been of particular use in analysis of the effects of point mutations either in GPCR or in G protein. This reflects that, even if such alterations alter the absolute expression of the construct, they do not alter the ratio of GPCR to G protein. Such fusion proteins have thus been used to examine many aspects of GPCR constitutive activity [35–38] and have been invaluable in studies on the impact of the G protein on constitutive activity.

6.3.2 Modulation of the GPCR–G Protein Interface Alters Constitutive Activity

Spiperone is a well characterized and studied inverse agonist at the serotonin 5-HT_{1A}R. Following expression of a human 5-HT_{1A}R-Gα_{i1} fusion protein in human embryonic kidney (HEK) 293 cells, spiperone was able to reduce basal high-affinity GTPase activity in membranes of these cells in a concentration-dependent manner [39–40]. Spiperone was essentially a full inverse agonist, because at maximally effective concentrations this ligand reduced the basal GTPase activity to almost the same level as seen in prior pertussis toxin treatment of the cells, while after pertussis toxin treatment spiperone was without effect. Thus, modification of the GPCR–G protein interface by ADP-ribosylation of the receptor-attached Gα_{i1} obliterated constitutive activity [39]. Confirmation of these conclusions was provided by examination of a 5-HT_{1A}R-Gα_{i1} fusion protein in which the pertussis toxin-sensitive Cys^{351} of the G protein was converted into Ile to render the protein insensitive to ADP-ribosylation. The basal GTPase activity of membranes expressing this construct was unaffected by pertussis toxin treatment, but spiperone was an effective inverse agonist in membranes from both untreated and pertussis toxin-treated cells [39]. These results confirmed that the effect of spiperone reflected reduction of constitutive information transfer from the 5-HT_{1A}R to the fused G protein rather than to endogenously expressed forms of G_i. Most interestingly, although a 5-HT_{1A}R-Gα_{i1} fusion protein in which Cys^{351} of the G protein had been converted into Gly responded to agonist ligands including 5-hydroxytryptamine (5-HT) and 8-hydroxy-2-(di-*n*-propylamino)tetralin (8-

OH-DPAT), spiperone did not act as an inverse agonist in either pertussis toxin-treated or untreated cells [39]. Thus, even when the ratio of GPCR to G protein and their proximity was defined, single amino acid alterations in the G protein altered measurable constitutive activity of the receptor–G protein fusion. As the extreme C terminus of the G protein α subunit is a key contact point for GPCRs, the molecular detail of the G protein can define constitutive activity of the GPCR. An explanation of such results can be provided from analysis of ligand-binding data and the ternary complex model of agonist–GPCR–G protein interactions. For example, specific binding of the agonist [^{3}H]D-Ala^2, D-Leu^5 enkephalin ([^{3}H]DADLE), but not of the antagonist [^{3}H]naltrindole, to the DOP opioid receptor (DOP(δ)R) is reduced by addition of increasing concentrations of GDP [41]. This is also observed when using fusion proteins between the DOP(δ)R and various pertussis toxin-insensitive point mutants of $G\alpha_{i1}$ [41]. Indeed, there is a strong correlation between the log of the effective concentration 50% (pEC_{50}) of GDP to limit [^{3}H]DADLE binding and the *n*-octanol/water partition coefficient of the amino acids used to replace the pertussis toxin-sensitive Cys [41]. Furthermore, in a comparison of the binding characteristics of [^{3}H]DADLE to DOP(δ)R-$G\alpha_{i1}$ fusion proteins with Ile or Gly replacing the pertussis toxin-sensitive Cys, the dissociation rate of the ligand was substantially greater for the Gly-containing construct and this resulted in a five times higher measured dissociation constant (K_d) for the agonist [41]. This implies a lower ability of $Gly^{351}G\alpha_{i1}$ to stabilize the agonist–GPCR–G protein ternary complex [41]. A less stable GPCR–G protein interface can be expected to result in reduced levels of constitutive activity. As well as lower constitutive activity, the capacity of DADLE to stimulate the GTPase activity of DOP(δ)R-$Gly^{351}G\alpha_{i1}$ was only 50% of that for DOP(δ)R-$Ile^{351}G\alpha_{i1}$ when measured as GTP turnover number. Although not tested directly, it might be expected that the constitutive activity of DOP(δ)R-$Ile^{351}G\alpha_{i1}$ would be inhibited by classic inverse agonists of this receptor, such as *N,N*-diallyl-Tyr-Aib-Aib-Phe-Leu (ICI 174 864), whereas this ligand would have little effect at the DOP(δ)R-$Gly^{351}G\alpha_{i1}$ fusion protein simply because this construct would display lower or undetectable levels of constitutive activity due to the poor GPCR–G protein interface.

Key characteristics often associated with GPCR mutants with enhanced levels of constitutive activity include higher relative efficacy of partial agonist ligands and a shift to the left in agonist potency and affinity (see Chapter 9). It is therefore interesting to note that such characteristics can be observed on mutation of the G protein within the C-terminal region. In the use of fusion proteins between the α_{2A}-adrenoceptor (α_{2A}AR) and forms of $G\alpha_{i1}$ with Cys, Ile, or Gly as residue^{351}, for example, the agonist 5-bromo-*N*-(4,5-dihydro-1*H*-imidazol-2-yl)-6-quinoxalinamine (UK14304) increased in efficacy relative to adrenaline with Ile $>$ Cys $>$ Gly [42]. Equally, the EC_{50} for UK14304 increased in the same order [42]. This was not restricted to UK14304, but was also the case for a considerable number of ligands generally viewed as partial agonists at this receptor [42]. Such analyses were extended further by the placing of nine different amino acids at this position and measurement of the efficacy of oxymetazoline relative to adrenaline. Oxymetazoline is generally regarded as a selective, high-affinity, but weak partial agonist at the α_{2A}AR. However, on moving from fusion proteins containing amino acids such as Arg or Gly at residue^{351} of the G protein to hydrophobic amino acids such as Ile or Leu at this position this compound displayed between 1 and 45% of the efficacy of adrenaline [25].

6.3.3
Use of G Protein Variation to Detect Ligand Efficacy

The results described above provide overwhelming evidence that alteration of aspects of the G protein is able to regulate the capacity to detect constitutive activity of a GPCR. However, as well as providing means to assess concepts in receptor and pharmacological theory, they can also provide practical benefits in ligand screening and characterization. For example, initial studies on the 5-HT_{1A} receptor (5-HT_{1A}R) selective ligand ***N***-(2-4-(2-methoxyphenyl)-1-piperazinyl)ethyl)-N-(2-pyridyl)-cyclohexanecarboxamide (WAY 100 635) indicated it to lack efficacy [43]. As noted earlier, constitutive activity of the 5-HT_{1A}R, monitored as elevated basal GTPase activity, is detected more easily when using membranes expressing the same levels of a 5-HT_{1A}R-$Ile^{351}G\alpha_{i1}$ fusion protein than with a 5-HT_{1A}R-$Gly^{351}G\alpha_{i1}$ fusion protein. This difference can be enhanced by the addition of recombinant forms of certain RGS proteins [40, 44]. RGS proteins function to enhance deactivation of G protein α subunits by increasing the rate of GTP hydrolysis [7–9]. Although it was originally believed that different RGS proteins had similar catalytic effectiveness, this picture is currently more complex. It appears that the identity of the GPCR that caused G protein activation also determines the effectiveness of RGS proteins in acting as GTPase-activating proteins [45], probably because of direct interactions between the GPCR and the RGS [46] (Figure 6.1). When recombinant RGS1 is added to membranes expressing a 5-HT_{1A}R-$Ile^{351}G\alpha_{i1}$ fusion protein, the GTPase activity measured in the presence either of 5-HT or of 8-OH-DPAT is greatly increased [40]. RGS16 also has this capacity, but equimolar levels are less effective than RGS1. This also provides a strategy to enhance detection of weak partial agonists. WAY 100 635 is clearly a partial agonist in this context and EC_{50} is easily measured [40]. In the absence of addition of RGS1, the signal produced by WAY 100 635 is small and could easily be overlooked in ligand screens. Clear constitutive activity of the 5-HT_{1A}R-$Ile^{351}G\alpha_{i1}$ fusion protein was uncovered by the addition of RGS1. Basal GTPase activity – that is, in the absence of receptor ligands – was almost doubled [40]. Even more impressive, however, were equivalent experiments performed with a 5-HT_{1A}R-$Ile^{351}G\alpha_{o1}$ fusion protein. Addition of RGS1 increased basal membrane high-affinity GTPase activity almost fivefold [40]. If this was due to the action of the RGS on the GTPase activity of the fusion protein it would be expected that an inverse agonist would block this. Spiperone did so, in a concentration-dependent fashion, and reduced GTPase activity down to the same level as it did in membranes expressing the fusion construct but in the absence of RGS1. The RGS1-enhanced GTPase activity was therefore shown to be blocked by spiperone and thus to reflect 5-HT_{1A}R-mediated constitutive activation of $Ile^{351}G\alpha_{o1}$. The very much greater activity window produced by addition of RGS1 greatly improved the precision of inhibitor concentration 50% (IC_{50}) estimates of a range of compounds displaying inverse agonism at this receptor. It might be argued that the constitutive activity of a GPCR is automatically enhanced by the proximity of a G protein provided by the fusion strategy. The ability to reduce or eliminate measured constitutive activity by single-point mutations in the G protein that provide a less ideal GPCR–G protein interface clearly argues against this. Furthermore, G protein activation is not observed by link-

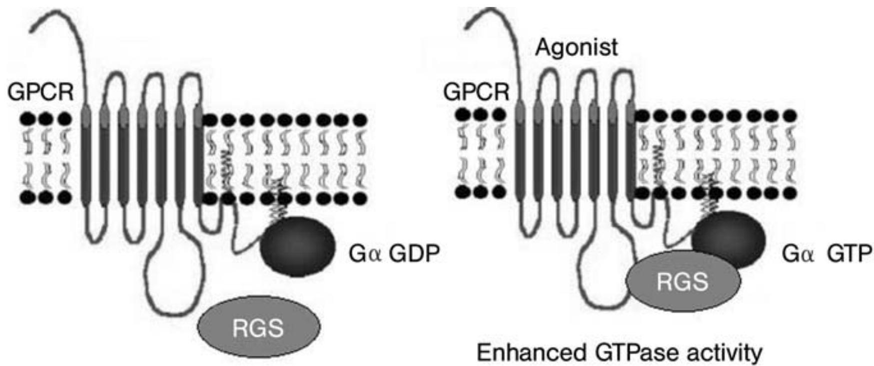

Fig 6.1 Agonist-activated GPCR–G protein α subunit fusions are regulated by RGS proteins. A GPCR–G protein α subunit fusion protein and an RGS protein are illustrated. Such GPCR–G protein fusion constructs are generally produced by the simple expedient of removing the stop codon from a cDNA encoding a GPCR and adding the sequence of a G protein α subunit in frame. See [47–48] for details on construction and use. Agonists stimulate the high-affinity GTPase activity of the GPCR–G protein fusion, and addition of a recombinantly produced RGS protein can markedly accelerate the GTPase activity. This basic concept has been used to enhance and monitor constitutive activity and to probe the effects of alteration of a key receptor interaction face in the G protein on constitutive activity [40]. Interactions between RGS4 and $G\alpha_{i1}$ have been mapped at the atomic level [49]. However, recent data indicate that as well as interacting with the G protein, RGS proteins may also make direct contacts with the receptor [45–46]. At least in certain cases this involves the third intracellular loop of the GPCR [50].

ing an inappropriate G protein to a GPCR. For example, a fusion protein in which the IP prostanoid receptor was linked to a form of $G\alpha_i$ was not activated by prostanoid agonists, whereas a fusion between this GPCR and its cognate G protein $G\alpha_s$ was [28].

6.4 Conclusions

A series of recent studies have provided clear evidence, although anticipated from 'mass-action' formalisms, for direct effects of G proteins on the ability to detect and the extent of constitutive activity of GPCRs.

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7
(Patho)physiological and Therapeutic Relevance of Constitutive Activity and Inverse Agonism at G Protein-Coupled Receptors

Lutz Hein

7.1
Introduction

Constitutive activity at G protein-coupled receptors (GPCRs) has been studied in recombinant systems with high levels of receptor expression [1–4]. However, constitutive activity (i.e., receptor signaling in the absence of endogenous agonist) may also occur in native tissues expressing physiological levels of GPCRs. According to the extended ternary complex model of receptor function (see Chapter 2) [1], GPCRs may spontaneously switch between an inactive state (R) and an active state (R*), in which they are able to interact with heterotrimeric G proteins. GPCR agonists preferentially bind to receptors in the R* state, which can interact with G proteins. This model predicted that even in the absence of agonists, a small (but measurable) fraction of receptors may assume the active conformation. Constitutive receptor activity may be physiologically relevant if basal receptor activation initiates intracellular signaling. In this case the cellular signal depends on, and may be influenced by, the density of receptor expression in a particular tissue. Constitutive activity may, in addition, acquire pathophysiological relevance if novel, activating receptor mutations are causal in inducing a disease state or may affect the progression of disease.

As a logical extension of the two-state concept, not only may ligands selectively bind to the active, G protein-coupled state of a receptor, but ligands that preferably bind to the inactive receptor state should also exist. Ligands that reduce constitutive GPCR signaling, and thus stabilize an inactive receptor conformation, are termed "inverse agonists". Their function is "inverse" relative to the signal elicited by classic receptor agonists, and they are "agonists" because the reduction of basal signaling is an active event (see also Chapter 8). Thus, the terms "constitutive activity" and "inverse agonism" are intrinsically linked with each other (see Chapter 2) and the physiological and therapeutic relevance of the phenomena should be discussed together (Figure 7.1). Fifteen years after the first description of an "inverse agonist" in a cell line [5], only a few examples of the physiological relevance of inverse agonists have been reported.

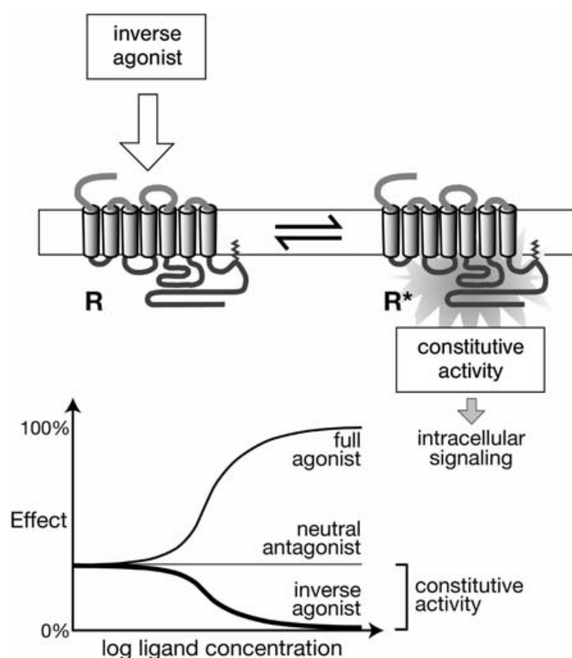


Fig. 7.1 Constitutive activity and inverse agonism. Ligands may have several effects on GPCR signaling. Full agonists induce a maximal intracellular effect, whereas neutral antagonists block the access of an agonist without eliciting a signal. "Inverse agonists" reduce constitutive receptor activity and may thereby completely switch off a GPCR.

This chapter discusses:

- the physiological relevance of constitutive receptor activity,
- disease states influenced by constitutive receptor signaling,
- endogenous ligands that have been identified as inverse agonists, and
- the therapeutic relevance of inverse agonists to suppression of constitutive activity.

Many of the examples mentioned in this review are discussed in more detail in other chapters in this book. Here, the physiological relevance and potential impact of constitutive activity and inverse agonism for drug therapy are highlighted.

7.2 Physiological Relevance of Constitutive Activity of GPCRs

Constitutive activity of GPCRs was frequently observed in cell culture systems with high levels of overexpression of recombinant receptors [6, 7]. In this case, the level of constitutive activity detectable in the absence of an agonist was correlated with receptor density. By overexpression in cells, almost all GPCRs may be forced to elicit some degree of constitutive activity [8]. Even with significant levels of overexpression, however, most GPCRs show only modest constitutive activity. Constitutive activity may differ between different families of GPCRs, but closely related receptor subtypes sometimes also differ in their constitutive activities. When expressed at similar levels,

the β_2-adrenoceptor (β_2AR) displayed higher constitutive activity than the β_1 subtype (see also Chapters 9 and 10) [9]. Relatively high levels of constitutive activity have been reported for melanocortin, sphingolipid, and cannabinoid receptors [3]. One of the first examples of a high degree of constitutive activity in a native tissue was demonstrated for the histamine H_3 receptor (H_3R) [10]. The H_3R is a presynaptic autoreceptor on histaminergic neurons in the brain and a presynaptic heteroreceptor in non-histaminergic neurons in both the central and the peripheral nervous systems. Constitutive H_3R activity suppresses release of neuronal histamine in the rodent brain *in vivo*, while inverse H_3R agonists suppressed basal receptor activity and thus *enhanced* the release of histamine *in vivo* [10]. While this may represent the most impressive example of *in vivo* constitutive activity of a GPCR, the biological relevance for the histamine system is unknown. It may, however, be speculated that changes in the presynaptic H_3R levels affect the basal rate of histamine release from neurons. Further experimental evidence is required to test this hypothesis and to evaluate the effects of H_3R inverse agonists for therapy of human attention and aging disorders (see also Chapter 13) [10].

In principle, two concepts may explain the physiological significance of constitutive receptor activity (Figure 7.1). Firstly, the presence of constitutive activity may increase the physiological spectrum of receptor regulation. Agonists would further activate intracellular signaling, and inverse agonists could reduce or even completely shut off basal receptor signaling. However, as discussed below, only very limited data exist for endogenous agonist/inverse agonist pairs (e.g., melanocortin/agouti). A second concept for regulation of signal transduction by constitutive activity is related to the control of receptor density. Similar to the situation in recombinant systems *in vitro*, the expression level of a constitutively active receptor may control the level of basal signaling. Along these lines, increased histamine H_2R expression after treatment of patients with the inverse agonists cimetidine or ranitidine has been associated with drug tolerance and increased signaling after drug withdrawal [11].

7.3 Constitutive Activity of GPCRs and Pathophysiology of Disease

Constitutive activity of GPCRs may be of relevance for disease development and progression. Firstly, mutations in GPCR genes may cause disease by inducing constitutive activity. Secondly, constitutive activity of GPCRs may be relevant for infectious disease development, as several viruses contain constitutively active receptors in their genomes (see Chapter 15) [12]. Below, a few examples should illustrate how GPCR mutations and virally encoded GPCRs may affect human pathophysiology (Figure 7.2).

During recent years, a large number of mutations and polymorphisms have been identified in human genes encoding GPCRs. However, only some of these mutations have been associated with increased basal receptor signaling [3]. The first report of a constitutively active GPCR mutant was published for a thyrotropin receptor (TSH) variant associated with hyperfunctioning thyroid adenoma [13]. In the meantime, several dozens of activating mutations have been identified in TSH receptors, which sti-

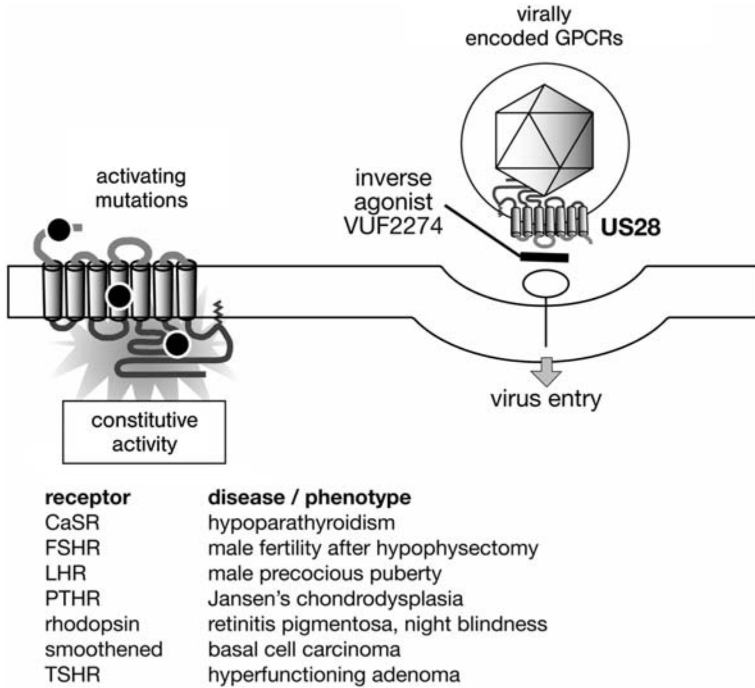


Fig. 7.2 Constitutive activity of GPCRs and pathophysiology of disease. Genetic mutations may elicit constitutive receptor activity (left) and thereby cause several diseases. Constitutive GPCR activity may also be relevant for viral diseases (right). Virally encoded GPCRs (e.g., the cytomegalovirus GPCR US28) can act as co-receptors for virus entry into host cells. The inverse agonist VUF2274 has been shown to block cellular entry of HIV viruses (for references, see text). Abbreviations: CaSR, calcium-sensing receptor.

mulate thyroid hormone secretion and thus result in hyperfunctioning adenoma with clinical symptoms of hyperthyroidism.

In the case of the luteinizing hormone (LH) receptor, activating mutations have been associated with the development of male precocious puberty [14]. Interestingly, a point mutation resulting in constitutive activity of the FSH receptor has been identified as maintaining male fertility after hypophysectomy [15]. In the visual signaling cascade, several mutations have been linked with congenital eye disease. Most importantly, constitutive activity of the light sensor rhodopsin may cause retinitis pigmentosa and night blindness [16].

The calcium-sensing receptor (CaSR) is a GPCR expressed in the PTH-producing cells (PTH = parathyroid hormone) of the parathyroid gland and in the cells lining the kidney tubule. By virtue of its ability to sense changes in circulating calcium concentration and to couple this information to intracellular signaling pathways that modify PTH secretion or renal cation handling, the CaSR plays a central role in the maintenance of calcium homeostasis in the human body. Lienhardt et al. identified activating CaSR mutations in eight (42%) out of 19 unrelated individuals with isolated hypopar-

athyroidism [17]. A constitutively active mutation in the PTH receptor was observed in patients with Jansen's chondrodysplasia [18]. A genetic mouse model with expression of this human PTH receptor variant replicated the human disease phenotype and may thus be an important model for the development and screening of inverse agonists for the treatment of genetic chondrodysplasia.

In addition to these diseases, activating GPCR mutations may be relevant for the development of certain forms of cancer. Xie and colleagues identified activating somatic missense mutations in the human "smoothened" gene in sporadic basal cell carcinomas from three patients [19]. "Smoothened" is a GPCR that functionally interacts with the "sonic hedgehog" (ligand)–"patched" (receptor) signaling pathway (see references in [19]). In the future, gene therapy may not be the only option for treatment of these diseases, but inverse agonists may be used to dampen constitutive activity of the GPCR mutants.

Other examples of constitutively active receptors of potential relevance for disease are virally encoded GPCRs. Such GPCRs have been described in the genomes of human cytomegalovirus (CMV) and in Kaposi sarcoma herpes virus (KSHV) [12]. Human CMV carries four GPCRs, termed US27, US28, UL33, and UL78 (see also Chapter 15) [20]. Cytomegaloviruses are species-specific herpes viruses that may cause acute, persistent, and latent infections in both humans and animals. The fact that lifelong latent infections may occur implies that CMV viruses are well adapted to the physiologies of their hosts and have developed several sophisticated strategies to escape from the hosts' antiviral defense mechanisms. Some of these strategies of host adaptation may involve viral GPCRs homologous to human CCR5 and CXCR4 chemokine receptors. Thus, viruses may engage chemokine signaling pathways to interfere with the human immune system. However, only limited evidence exists about the pathophysiological relevance of viral GPCRs. The KSHV-GPCR plays a crucial role in the pathogenesis of Kaposi's sarcoma [21].

So far, no natural ligands for these virally encoded GPCRs have been identified. Experimental evidence suggests that the CMV receptors UL33 and US28 may signal in a ligand-independent fashion [22]. The US28 and US33 GPCRs may be involved in the infection of CD4-positive cell lines with HIV-1 and HIV-2 viruses. Most recently, a non-peptide ligand acting as an inverse agonist at US28 receptors has been identified [23]. Most importantly, this inverse agonist inhibited US28-mediated HIV entry into cells [23].

Another virally encoded GPCR, ORF74 (ORF = "open reading frame"), currently provides the best link between constitutive activity and viral pathogenesis. ORF74 is a GPCR encoded by a herpes simplex virus-8 associated with Kaposi's sarcoma. ORF74 is constitutively active in heterologous cells and acts as a viral oncogene and angiogenesis activator [21]. Interferon-γ-inducible protein 10 and stromal cell-derived factor 1α act as inverse agonists at ORF74 [12]. Although the precise mechanisms by which viruses benefit from expression of constitutively active GPCRs in their host cells are still unknown, inverse agonists at these receptors may provide exciting new targets for future antiviral therapy.

7.4
Physiological Relevance of Inverse Agonists

Several examples of endogenously expressed inverse agonists have been reported. The best characterized endogenous inverse agonist is retinal, which is bound to the light sensor rhodopsin [24]. Rhodopsin has evolved a unique mechanism to minimize basal receptor activity. The chromophore 11-*cis*-retinal, which acts as an inverse agonist in rhodopsin, is covalently bound to the receptor to ensure extremely low receptor signaling in the dark. Thus, 11-*cis*-retinal is responsible for the fact that the constitutive activity of rhodopsin is virtually zero (Figure 7.3).

A second receptor system with an endogenous inverse agonist is the family of melanocortin receptors (MCRs) [25]. To date, four MCR subtypes have been identified. The melanocortin receptor system represents the only example for which both an endogenous agonist (melanocortin, α-MSH) and an inverse agonist (agouti) have been identified. Melanocortin stimulates melanin pigment synthesis in skin melanophores through activation of MC_1 receptors (MC_1Rs), resulting in a dark skin or hair color. The agouti protein acts as a competitive antagonist to prevent α-MSH from binding to the receptor and suppresses basal MCR activity, resulting in a yellow hair color. The receptor subtypes MC_3R and MC_4R, which are expressed in the brain, are involved in the development of obesity and cachexia. Agouti and agouti-related protein (AgRP) act as inverse agonists at these receptors [25]. AgRP and α-MSH are expressed by distinct neurons of the arcuate nucleus of the hypothalamus. Both groups of neurons sense obesity through leptin receptors [25, 26]. Increased plasma leptin concentrations activate pro-opiomelanocortin-containing neurons, which inhibit further food intake. Conversely, low levels of circulating leptin result in increased AgRP secretion to sti-

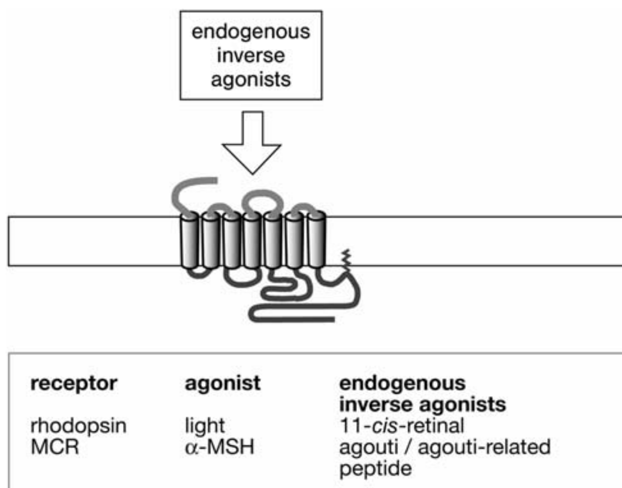


Fig. 7.3 Endogenous inverse agonists and their receptors. Two pairs of endogenous agonists/inverse agonists have been identified, modulating rhodopsin and melanocortin receptor signaling.

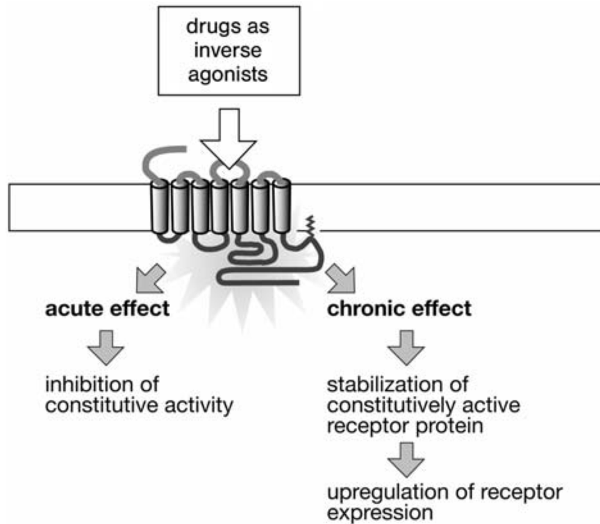
mulate food intake. Mutations of the components of this receptor system have been described in extremely obese patients [27]. Interestingly, genetic variants of the MC_4R lacking constitutive activity in heterologous expression systems have been identified in some obese patients. This finding demonstrates that constitutive activity of the MC_4R is essential for maintenance of normal body weight [25].

7.5 Inverse Agonists as Drugs

Until recently, most antagonists at GPCRs were considered to be neutral antagonists. However, further insight into the mechanisms of constitutive activity of GPCRs and more sensitive assay systems have resulted in a reclassification of GPCR antagonists [28]. A large number of drugs frequently used in human therapy have actually been determined to act as inverse agonists [2]. As discussed by Kenakin and Onaran [29], the thermodynamic receptor model predicts that affinity and efficacy of ligand receptor interactions are directly related. Thus, most ligands that interact with a receptor should somehow alter basal receptor signaling (i.e., possess negative or positive efficacy). Indeed, in a comprehensive overview of commonly used receptor antagonists compiled by Kenakin [30], the large majority of drugs were inverse rather than neutral antagonists. Out of a total of 380 antagonist–receptor pairs, 322 (85 %) were inverse agonists and only 58 ligands (15 %) could be classified as neutral antagonists. Inverse agonists were found to act at all different classes of GPCRs, and there was no association between inverse agonism and a particular G protein signaling pathway.

Inverse agonists elicit responses opposite to those of an agonist. However, for inverse agonism to become apparent, constitutive receptor activity is required. In the absence of constitutive activity, the inverse agonistic response may not be detectable and the drug will act as a neutral competitive antagonist. Inverse agonists may in principle elicit two different effects (Figure 7.4). Upon acute exposure, inverse agonists may decrease basal receptor activity and thus inhibit constitutive activity. One example is the increase in neuronal histamine release that has been observed *in vivo* with inverse histamine H_3R agonists [10]. Upon chronic exposure, inverse agonists may alter receptor expression. Indeed, several studies have demonstrated that prolonged administration of inverse agonists may increase receptor density both *in vitro* and *in vivo*. Chronic treatment with the inverse histamine H_2R agonist cimetidine resulted in upregulation of H_2R expression, associated with the development of tolerance and increased receptor sensitivity following withdrawal of the inverse agonist [11]. It remains a matter of debate whether upregulation of receptor density is due to prevention of agonist activation of the receptor or whether intrinsic activity properties are required [11]. However, evidence from cell culture and transgenic mouse studies indicates that inverse agonists may increase receptor expression by stabilization of constitutively active receptors [31–33].

One important question remaining to be addressed refers to the therapeutic relevance of inverse agonism. Antipsychotics are used to treat symptoms of schizophrenia. Frequently, antipsychotics are divided into two groups of drugs: typical and atypical



receptor	neutral antagonists	drugs as inverse agonists
α_1AR		prazosin, doxazosine, tamsulosin, terazosin
α_2AR	atipamezole	phentolamine, yohimbine
β_1AR	carvedilol	metoprolol, bisoprolol
β_2AR		pindolol, alprenolol, propranolol, timolol
D_{1A}R		clozapine, olanzapine
D_2R		haloperidol, clozapine
D_3R		haloperidol, clozapine
H_1R		cetirizine, loratadine
H_2R		cimetidine, ranitidine
H_3R		thioperamide
5-HT_{1A}R		spiperone
5-HT_{1B}R		olanzapine, clozapine
5-HT_{1D}R		ketanserin, clozapine
5-HT_{2C}R		spiperone, ketanserin, clozapine
MR		atropine, pirenzepine, N-methylscopolamine
DOP(δ)R		naloxone
MOP(μ)R		naloxone

Fig. 7.4 Drugs as inverse agonists. Most antagonists that block GPCRs have been classified as "inverse agonists". Inverse agonists may induce acute effects (inhibition of constitutive activity) or may increase receptor density by stabilization of constitutively active receptors. For reference, see Kenakin [30]. Abbreviations: α_1AR: α_1-adrenoceptor. α_2AR: α_2-adrenoceptor. β_1AR: β_1-adrenoceptor. β_2AR: β_2-adrenoceptor. D_{1A}R: dopamine D_{1A} receptor. D_2R: dopamine D_2 receptor. D_3R: dopamine D_3 receptor. H_1R: histamine H_1 receptor. H_2R: histamine H_2 receptor. H_3R: histamine H_3 receptor. 5-HTR: 5-hydroxytryptamine receptor. MR: muscarinic acetylcholine receptor. DOP(δ)R, δ-opioid receptor. MOP(μ)R, μ-opioid receptor.

antipsychotics. Whereas "typical" antipsychotics may cause unwanted extrapyramidal motor effects, "atypical" neuroleptic drugs have a lower propensity to induce these unwanted effects [34]. Although the precise mechanism of the antipsychotic action of neuroleptics is still a matter of debate, antagonism at the dopamine receptor (DR) subtypes D_2, D_3, and D_4 has been linked with therapeutic effects [34]. Interestingly, all of the antipsychotic compounds, including the atypical drugs clozapine, olanzapine, and risperidone, demonstrate inverse agonism at the D_2R and/or D_3R [35]. Thus, it may be tempting to speculate that the inverse agonism of neuroleptics is essential for the therapeutic action of these drugs (see also Chapter 14). Indeed, limited clinical data suggest that a neutral dopamine D_3R antagonist does not exhibit any antipsychotic effects in schizophrenic patients [36]. In contrast, the antipsychotic drug aripiprazole acts as a partial agonist at the D_2R [37]. Thus, inverse agonism at DR subtypes does not seem to be essential for the antipsychotic efficacy of neuroleptic drugs, but may rather be linked with the side effects of these drugs.

7.6 Conclusions

Significant advances in the identification of constitutively active receptors and ligands acting as inverse agonists at these receptors have recently been made. However, only few examples of the physiological relevance of constitutive activity of GPCRs have been published. Several genetic mutations giving rise to constitutive activity of GPCRs have been identified. An important goal for future research will be to identify drugs that may act as inverse agonists to inhibit the cause of disease. Similarly, constitutively active receptors encoded by viruses represent promising targets for the treatment of viral infections and virally induced tumors. The large number of constitutively active receptors so far identified suggests that the human body may also use endogenously expressed inverse agonists to modulate receptor signal transduction in many more aspects than is currently appreciated. Future clinical trials will be essential to determine whether established drugs that act as inverse agonists confer therapeutic advantages relative to neutral antagonists.

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8
Methodological Approaches

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8.1
Introduction

Numerous methods through which to assess constitutive GPCR activity are available [1]. Each methodological approach has its specific applications, advantages, and disadvantages and provides distinct information. In general, a combination of several methodological approaches should be used to obtain comprehensive information on constitutive activity of a given GPCR. Some methods, such as the analysis of purified GPCRs by fluorescence spectroscopy [2–5] (see Chapter 3) and the analysis of GPCRs in knock-out and transgenic animals [6–9], are limited to laboratories specifically equipped for those studies. The purpose of this chapter is to discuss some commonly employed methods for assessment of constitutive GPCR activity that can be implemented in most biochemical and pharmacological research laboratories without heavy investment in specialized instrumentation.

Methods for the assessment of constitutive GPCR activity exploit various steps of the G protein cycle. G proteins are heterotrimeric and consist of an α subunit (Gα) and a βγ complex (Gβγ) [10–12]. In the basal or *off-state*, Gα is bound to GDP. GPCR in the R* state, and this can be agonist-free or agonist-occupied GPCR, catalyzes GDP dissociation from Gα (*step 1*), which, in general, is the rate-limiting step of the G protein cycle. Subsequently, the agonist-bound receptor forms a ternary complex with the nucleotide-free Gαβγ heterotrimer (*step 2*). The ternary complex is characterized by high agonist affinity [13–15]. Both agonist-free and agonist-bound GPCR promote the binding of GTP to Gα (*step 3*). Gα bound to GTP is regarded as the active or *on-state* of the G protein. GTP-binding to Gα induces a conformational change in this subunit, resulting in dissociation of the heterotrimer into $G\alpha_{\text{-GTP}}$ and Gβγ and in GPCR uncoupling from G protein. GPCR uncoupling from G protein results in a decrease in receptor agonist affinity [13–15]. Both $G\alpha_{\text{-GTP}}$ and Gβγ increase or reduce the activity of effector systems (*step 4*). The switch from the on- to the off-states of G proteins is mediated by the GTPase of Gα, which cleaves GTP into GDP and P_i (*step 5*), followed by the reassociation of $G\alpha_{\text{-GDP}}$ and the Gβγ complex.

Pertussis toxin ADP-ribosylates a cysteine residue near the C terminus of a $G\alpha_i$, $G\alpha_o$, or $G\alpha_t$ subunit and thereby blocks the interaction of GPCRs with their cognate

G proteins [10, 11]. Historically, pertussis toxin has played a crucial role in assigning specific GPCRs to G_i/G_o protein-mediated signaling pathways [10, 11, 16]. In studies of GTPase activity or [^{35}S]GTPγS binding in membranes from pertussis toxin-treated cells, it was regularly observed that the basal signals in membranes from the toxin-treated cells were considerably smaller than in control membranes [17–19]. Initially, those data could not be easily interpreted. However, in the framework of the extended ternary complex model (see also Chapter 2), the effect of pertussis toxin is now interpreted as ADP ribosylation of $G\alpha_i/G\alpha_o$, not only blocking coupling of agonist-occupied GPCRs to G_i/G_o proteins, but also coupling of agonist-free (i.e., constitutively active) GPCRs to these G proteins [1, 20]. Thus, an inhibitory effect of pertussis toxin on basal activities, mostly prominently observed at the membrane level, is an indication of the presence of constitutively active GPCRs. However, since pertussis toxin lacks specificity between G_i/G_o-coupled GPCRs, its value as a tool for the analysis of constitutive GPCR activity is, unfortunately, limited. For G_s- and G_q-coupled GPCRs, no uncoupling toxins analogous to pertussis toxin are presently known. In this chapter we discuss methods that can be applied in broken-cell preparations (membranes) and in intact cells. The two approaches complement each other.

8.2
Analysis of Constitutive GPCR Activity in Membranes and Intact Cells

Studies with membranes have the advantage of being able to eliminate contaminating agonists that may cause an apparent constitutive GPCR activation through multiple rounds of membrane centrifugation and resuspension (see also Chapter 1). In addition, studies with membranes allow for precise control of the concentrations of GTP and ions and also of pH, all of which have an effect on constitutive GPCR activity (see Chapter 9) [21–25]. Because of the striking sensitivity of the apparent constitutive activity of GPCRs in membranes to experimental variables, one could argue that analysis of constitutive GPCR activity in membranes is artificial and that only studies with intact cells allow the drawing of conclusions with respect to the physiological relevance of constitutive activity. However, we have almost no knowledge about the precise chemical microenvironments of GPCRs and G proteins near the plasma membrane. Thus, the physiological relevance of data obtained with membranes cannot be dismissed. Conversely, a concern with intact cell studies is the fact that contamination with endogenous agonist, be it from the cell culture medium or produced within the cultured cells, may bias data analysis. While elimination of agonists from the cell culture medium is generally possible, it can be extremely difficult, if not impossible, to eliminate endogenously produced agonists stored within the cell, be they neurotransmitters or autacoids.

Studies with membranes are crucial for study of the impact of G proteins on ligand-binding properties of GPCRs [15, 26]. Studies with permeabilized cells are a valid alternative to studies with membranes [27], but permeabilized cells have not yet found broad application for analysis of constitutive GPCR activity. Furthermore, experiments with membranes allow analysis of constitutive GPCR activity directly at the G protein level (i.e., GDP/GTP exchange and steady-state GTP hydrolysis) [21, 28–30]. Some

G protein-regulated effector systems, most importantly adenylyl cyclase (AC), can also be used as read-outs for constitutive GPCR activity with membrane preparations [2, 31, 32]. With respect to membranes, in this chapter we mainly discuss data obtained with GPCRs expressed in *Spodoptera frugiperda* (Sf9) insect cells, since this system has been shown to be a highly sensitive model system for analysis of constitutive activity of many GPCRs [21, 28, 31–34]. The methods discussed here can also be applied to membranes from typical mammalian expression systems such as Human Embryonic Kidney (HEK) 293 cells and Chinese Hamster Ovary (CHO) cells and cultured mammalian cells lines such as NG 108-15 neuroblastoma x glioma cells, endogenously expressing the constitutively active δ-opioid receptor (DOP(δ)R), and HL-60 leukemia cells, endogenously expressing various constitutively active chemoattractant receptors, specifically the formyl peptide receptor (FPR1), the complement C5a receptor (C5aR), the platelet-activating factor receptor (PAFR), and the leukotriene B_4-receptor (BLTR) [35–38]. However, there can be substantial differences in the sensitivities of the various systems in detecting constitutive activity of a given GPCR [20, 21]. Additionally, obtaining large amounts of membrane protein for extensive pharmacological studies is much more difficult, expensive, and time-consuming with most mammalian systems than with insect cell expression systems.

While studies with membranes are certainly informative with respect to the *direct* analysis of GPCR/G protein interaction and AC analysis (mostly G_s-mediated AC activation), it must be clearly emphasized that for analysis of constitutive activity of GPCRs coupled to G_q proteins and phospholipase C (PLC), comprising about one third of all constitutively active GPCRs [1, 39], studies with intact cells are crucial because guanine nucleotide exchange at G_q proteins is more difficult to assess than at G_i and G_s proteins [40, 41] and because PLC studies with membranes are more difficult than with intact cells [42–44].

With respect to the analysis of G_s-coupled GPCRs mediating AC activation and G_i-coupled GPCRs mediating AC inhibition, AC assays with membranes rely on the use of [α-^{32}P]ATP as substrate [31, 32, 45]. While such studies ensure high sensitivity for G_s-coupled GPCRs, disadvantages are the use of radioactive substrate and difficulties in scaling up the AC assay to high-throughput screening settings. In addition, AC assay with membranes may not be sufficiently sensitive to analyze constitutively active G_i-coupled GPCRs in detail [46]. Unlike AC assays with membranes, cAMP accumulation assays with intact cells do not necessarily rely on the use of radioactive reagents and can be more easily scaled up to high-throughput screening settings geared towards the identification of inverse agonists (see Section 8.3.1.) [31, 47]. cAMP accumulation assays using intact cells are also quite popular for the analysis of constitutively active G_i-coupled GPCRs, measuring *increases* in forskolin-stimulated cAMP accumulation by inverse agonists [1, 48, 49].

More recent developments in the analysis of constitutive GPCR activity in intact cells are reporter gene assays applied to G_s-, G_i- and G_q-coupled GPCRs [50–52]. Such reporter gene assays are very sensitive and can be applied to high-throughput screening settings in drug discovery. Melanin dispersion in *Xenopus laevis* melanophores is regulated by G_i and G_s proteins in opposite ways, and can therefore be exploited to assess constitutive GPCR activity photometrically [53]. However, it should be kept in mind

that for the purpose of analysis of constitutive GPCR activity, gene reporter and melanin dispersion assays monitor R to R* isomerization at a very distal point, raising the possibility of multiple factors affecting the ultimate read-out. In fact, it has already been documented that the apparent constitutive activity of a GPCR may depend on which specific parameter is being analyzed. Specifically, even when determining parameters relatively proximal to the initial R to R* isomerization (i.e., GTP hydrolysis and GTP-dependent AC activation mediated by G_s-coupled GPCRs), the efficacies of inverse agonists may be quite different, with AC probably being the rate-limiting factor in the signaling process [22, 54].

It should be emphasized that the majority of studies on constitutive GPCR activity have so far been conducted with recombinant mammalian or insect cell expression systems, ensuring high GPCR expression levels and, accordingly, high sensitivity for the analysis of constitutive activity [1, 55, 56]. Another advantage of the use of expression systems is the fact that commonly used cell lines (i.e., Sf9 cells, HEK293 cells, and CHO cells) do not express significant levels of endogenous constitutively active GPCRs that could generate undesired background noise. In contrast, the presence of multiple endogenous constitutively active GPCRs in HL-60 leukemia cells constituted a major obstacle in the analysis of individual receptors with use of the native cell line [19, 20, 24, 57]. Thus, for mechanistic analyses, studies with recombinant cell lines expressing a single defined GPCR at high levels are crucial, ensuring precise determination of the potencies and efficacies of inverse agonists, particularly of partial inverse agonists [22, 31]. However, with respect to physiological relevance, one should also aim at conducting experiments with low (i.e., physiological) GPCR expression levels whenever possible. Such studies have been successfully conducted for several GPCRs [58–62], but it will be difficult to achieve this goal for all GPCRs, because many available techniques are still not sensitive enough to detect constitutive GPCR activity at low GPCR expression levels, particularly in the presence of multiple constitutively active GPCRs.

Below we provide detailed experimental procedures for the analysis of constitutive activity that work reliably for the systems available in our laboratories. While the principles of the procedures can be translated to numerous systems, the procedures provided are actually only starting points for systems other than those discussed here. For each system, all experimental variables have to be carefully varied and optimized to obtain maximal sensitivity for the analysis of constitutive GPCR activity.

8.2.1 Procedure for Sf9 Cell Culture and Membrane Preparation

Sf9 cells are cultured in 125–500 mL disposable Erlenmeyer flasks at 28 °C with rotation at 150 rpm in SF 900 II medium (Invitrogen, Carlsbad, CA, USA) supplemented with 5 % (vol./vol.) fetal bovine serum (Bio Whittaker, Walkersville, MD, USA) and 0.1 mg mL^{-1} gentamicin (Roche, Indianapolis, IN, USA). Although fetal bovine serum is not absolutely necessary, Sf9 cells grow better and show higher GPCR expression levels in the presence of serum. Cells are maintained at a density of $1.0–6.0 \times 10^6$

cells mL^{-1} and are suspended in fresh medium at each passage (three times weekly) and for each infection. Recombinant baculoviruses encoding FLAG epitope-tagged GPCRs, G protein α and βγ subunits, and GPCR-Gα fusion proteins are generated in Sf9 cells by use of the BaculoGOLD transfection kit (Pharmingen, San Diego, CA, USA) following the manufacturer's instructions. Detailed polymerase chain reaction and cloning procedures are described elsewhere [21, 63, 64]. We have also used other methods, involving less cloning, to generate baculoviruses but have found the BaculoGOLD system to yield the best GPCR and G protein expression levels. After transfection of Sf9 cells with pVL-1392 plasmids encoding for the various signaling proteins and culture for 7 days, virus work stocks are generated by two sequential amplifications. In the first amplification, cells are seeded at 2.0×10^6 cells mL^{-1} and infected with a 1:100 dilution of the supernatant of the initial culture following transfection. Cells are cultured for 7 days, resulting in death of virtually the entire cell population. The supernatant from this infection is harvested by a 10 min centrifugation at $3000 \times g$ and stored under light protection at 4 °C. In a second amplification, cells are seeded at 3.0×10^6 cells mL^{-1} and infected with a 1:20 dilution of the supernatant from the initial amplification. Cells are cultured for 48 h, and the supernatant is harvested. After the 48 h culture, the majority of cells show signs of infection (e.g., altered morphology and viral inclusion bodies), but most are still intact. The supernatant from the second amplification constitutes the routine virus stock for infection of cells to prepare membranes. For membrane preparation, cells are seeded at a density of 3.0×10^6 cell mL^{-1} in 50–125 mL cultures and infected with 1:100 dilutions of the second amplification baculovirus stocks. Infected cells are cultured for 48 h before membrane preparation. From a typical 100 mL culture of baculovirus-infected Sf9 cells ~35–55 mg of membrane protein are harvested. In order to obtain different expression levels of GPCRs and G proteins, virus dilutions and infection times can be varied, but it is difficult to control protein expression levels precisely by changing those parameters [41, 65]. We found that suspending Sf9 cells in fresh medium at each passage and infection is more important than the actual virus dilution or the age of virus stock for efficient protein expression. In fact, we have used viruses stored at 4 °C with protection from light for up to 5 years without apparent loss in potency in inducing protein expression.

All membrane preparation steps are performed at 4 °C. Sf9 cells are harvested, checked for signs of infection (only a few cells are dead under the chosen conditions), centrifuged for 10 min at $1000 \times g$, and washed once by a 10 min centrifugation at $1000 \times g$ with 50 mL of a buffer consisting of 137 mM NaCl, 2.6 mM KCl, 0.5 mM $MgCl_2$, 0.9 mM $CaCl_2$, 1.5 mM KH_2PO_4, and 0.8 mM Na_2HPO_4, pH 7.4. Cells are lysed in 15 mL of a buffer containing 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride (Sigma, St. Louis, MO, USA), 10 μg mL^{-1} benzamidine (Sigma), and 10 μg mL^{-1} leupeptin (Roche) in 10 mM Tris/HCl, pH 7.4, with 25 strokes in a Dounce homogenizer. Nuclei and unbroken cells are removed by centrifugation for 5 min at $500 \times g$. The supernatant suspension is removed and centrifuged for 20 min at $40\,000 \times g$. The resultant pellet is resuspended in 20 mL of lysis buffer and re-centrifuged. Membranes are suspended at 0.5–1.5 mg of protein mL^{-1} in binding buffer (12.5 mM $MgCl_2$ and 1 mM EDTA in 75 mM Tris/HCl, pH 7.4). Protein concentration is determined by use

of the Bio-Rad DC protein assay kit (Bio-Rad, Hercules, CA, USA) by following the manufacturer's instructions and with use of bovine serum albumin as standard protein. Sf9 membranes are stored in 1 mL aliquots at concentrations of 0.9–1.6 mg of protein mL^{-1} at –80 °C until use. When stored under these conditions, GPCRs and G proteins remain structurally and functionally intact for periods of up to 4 years (longer periods of time were not studied). Expression of FLAG epitope-tagged GPCRs and G protein subunits is confirmed by SDS-polyacrylamide electrophoresis and immunoblotting with specific antibodies as described [21, 32].

8.2.2 GPCR Radioligand Binding Studies

Good starting points for analysis of constitutive GPCR activity by radioligand binding studies are the availability of: i) an affordable and stable high-affinity tritiated radioligand, and ii) various GPCR isoforms with different constitutive activities. The radioligand should preferably be a neutral antagonist, since the binding of neutral antagonists per se is not changed by G proteins [23, 32], allowing for the analysis of the impact of G proteins on the binding of both inverse agonists and agonists in an unbiased manner (see Chapters 2 and 3).

[^{3}H]Dihydroalprenolol ([^{3}H]DHA) is available at a reasonable price and with good specific activity (85–90 Ci $mmol^{-1}$) from various sources and binds with high affinity (K_d, 0.36 nM) to the β_2-adrenoceptor (β_2AR) and β_2AR-Gα_s fusion protein expressed in Sf9 cells [32]. Alprenolol is actually a weak partial agonist at the β_2AR-Gα_s fusion protein as assessed in the GTPase and AC assay (efficacy 0.21–0.22 relative to the full agonist isoproterenol), but the binding of [^{3}H]DHA is not changed by guanine nucleotides [22, 23, 32]. Thus, [^{3}H]DHA can be used as a probe for study of G protein regulation of the binding of inverse agonists and of agonists more efficacious than the radioligand itself. The wild-type β_2AR (β_2AR_{wt}) possesses constitutive activity, but a constitutively active β_2AR mutant, also referred to as β_2AR_{CAM}, exhibits even higher constitutive activity [15, 31, 32, 66, 67]. Thus, analysis of β_2AR_{wt} and β_2AR_{CAM} with [^{3}H]DHA is a prototypical model for studying the impact of constitutive GPCR activity on ligand-binding properties. In order to enhance receptor/G protein interactions and to ensure a defined stoichiometry of the coupling partners, [^{3}H]DHA binding studies are best performed with GPCR–Gα fusion proteins [32, 64] (see also Chapters 7 and 9). Coexpression of GPCRs with specific Gα (and G$\beta\gamma$) subunits is an alternative to the fusion protein approach, but the achievement of defined GPCR/G protein stoichiometries is problematic [24, 34, 65]. In addition, interactions between agonist-occupied GPCRs and coexpressed Gα_s proteins are less efficient in terms of ternary complex formation than in fusion proteins [32, 65].

Inverse agonists exhibit high affinity for the inactive (R) state of GPCRs, whereas agonists exhibit high affinity for the active (R*) state of GPCRs (see also Chapters 2 and 3) [39, 68, 69]. In Sf9 membranes expressing β_2AR_{wt} fused to the long splice variant of Gα_s (G$\alpha_{s\text{-}L}$), the inverse agonist *erythro*-DL-1-(7-methylindan-4-yloxy)-3-isopropylamino-butan-2-ol (ICI 118551) inhibits [^{3}H]DHA binding according to a monophasic com-

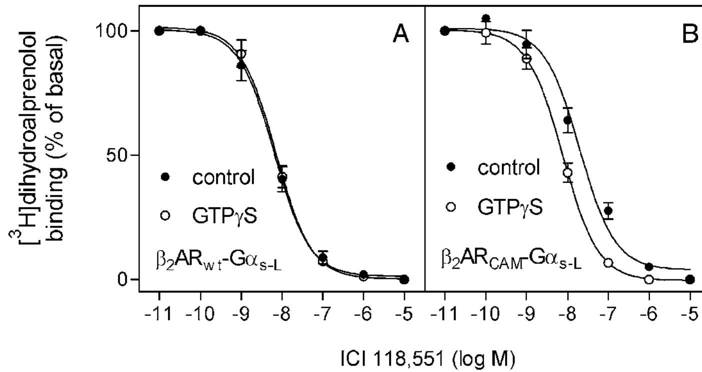

Fig. 8.1 Competition for [^{3}H]DHA binding by ICI 118551 in Sf9 membranes expressing β_2AR_{wt}-$G\alpha_{s\text{-}L}$ or β_2AR_{CAM}-$G\alpha_{s\text{-}L}$: effect of GTPγS. [^{3}H]DHA binding was performed as described in Section 8.2.2. Reaction mixtures contained Sf9 membranes (15–40 μg of protein per tube) expressing β_2AR_{wt}-$G\alpha_{s\text{-}L}$ (3.3–7.5 pmol mg^{-1}) (A) or β_2AR_{CAM}-$G\alpha_{s\text{-}L}$ (2.5–4.9 pmol mg^{-1}) (B), 1 nM [^{3}H]DHA, and ICI 118551 at the concentrations indicated on the abscissa. Reaction mixtures additionally contained distilled water (control) or GTPγS (10 μM). Data shown are the means ± SDs of four independent experiments and were analyzed by nonlinear regression.

petition isotherm (K_i, 1.7 nM) that is not changed by the stable GTP analogue and G protein activator guanosine 5'-[γ-thio]triphosphate (GTPγS) [10, 70] (Figure 8.1A). In membranes expressing β_2AR_{CAM}-$G\alpha_{s\text{-}L}$, ICI 118551 inhibits [^{3}H]DHA binding with a lower affinity (K_i, 3.4 nM), reflecting the lower abundance of R in β_2AR_{CAM} than in β_2AR_{wt}. Nucleotide-free G proteins stabilize the R* state and increase the affinity of agonists for GPCRs [13, 14, 68]. Conversely, disruption of GPCR/G protein interaction by GTP analogues such as GTPγS should decrease the abundance of GPCRs in the R* state, increase R abundance, and, thereby, increase the affinity of inverse agonists. In fact, in Sf9 membranes expressing β_2AR_{CAM}-$G\alpha_{s\text{-}L}$, GTPγS increases the affinity of ICI 118551 for β_2AR_{CAM} from 3.4 nM to 1.4 nM (i.e., a value comparable to that observed for β_2AR_{wt}; Figure 8.1B). Similar effects of guanine nucleotides on inverse agonist binding properties of GPCRs were observed for the 5-hydroxytryptamine 2C receptor (5-HT_{2C}R) [26, 61] and the bradykinin subtype 2 receptor (B_2R) [71].

The effect of increased constitutive activity of a GPCR on agonist-binding properties can be illustrated by analysis of the partial β_2AR agonist dichloroisoproterenol. Dichloroisoproterenol is a moderately strong partial agonist (efficacy 0.49 relative to isoproterenol) at β_2AR_{wt}-$G\alpha_{s\text{-}L}$ as assessed in the steady-state GTPase assay [15]. However, in the [^{3}H]DHA competition binding assay, dichloroisoproterenol inhibits [^{3}H]DHA binding according to a monophasic competition isotherm that is only slightly shifted to the right by GTPγS (K_i 96 nM versus 140 nM) (Figure 8.2A). In membranes expressing β_2AR_{CAM}-$G\alpha_{s\text{-}L}$, the efficacy of dichloroisoproterenol in the GTPase assay is increased from 0.49 to 0.58, and its potency in activating GTP hydrolysis increases from 29 nM to 17 nM [15]. These moderate changes in potency in the GTPase assay are accompanied

by profound alterations in the binding properties of dichloroisoproterenol at β_2AR_{CAM}-$G\alpha_{s\text{-}L}$ (Figure 8.2B). Specifically, in the absence of GTPγS, dichloroisoproterenol inhibits [^{3}H]DHA binding according to a biphasic competition isotherm (high-affinity K_i (K_{ih}), 12 nM; low-affinity K_i (K_{il}), 240 nM), with 48.8% of the β_2AR_{CAM} molecules being in the high agonist-affinity state. The high-affinity binding component of dichloroisoproterenol reflects the increased abundance of R* in β_2AR_{CAM} relative to β_2AR_{wt}, increased affinity of β_2AR_{CAM} for dichloroisoproterenol, and ternary complex formation between dichloroisoproterenol, β_2AR_{CAM}, and nucleotide-free $G\alpha_{s\text{-}L}$. The high-affinity binding component of dichloroisoproterenol to β_2AR_{CAM}-$G\alpha_{s\text{-}L}$ is completely inhibited by the addition of GTPγS. In the presence of GTPγS, the dichloroisoproterenol competition isotherm is shifted to the right, monophasic and exhibits a K_i of 470 nM. This binding isotherm reflects disruption of the ternary complex and the reduced affinity of dichloroisoproterenol to G_s protein-uncoupled β_2AR_{CAM}. Collectively, these data show that β_2AR_{CAM} fused to $G\alpha_{s\text{-}L}$ exhibits a higher affinity for dichloroisoproterenol than β_2AR_{wt} fused to $G\alpha_{s\text{-}L}$ and that ternary complex formation is greatly facilitated with the β_2AR mutant because of the increased abundance of R* relative to β_2AR_{wt}. The very large increase in ternary complex formation with dichloroisoproterenol at β_2AR_{CAM} compared to β_2AR_{wt} in relation to the relatively small increases in potency and efficacy in the GTPase assay are explained by the existence of non-signaling or frozen ternary complexes (see also Chapters 2 and 9) [15].

A procedure for the [^{3}H]DHA binding assay targeting the β_2AR in Sf9 membranes is provided below. This procedure has been successfully adapted to agonist and antagonist radioligands for several other GPCRs expressed in Sf9 cells [21, 63–65].

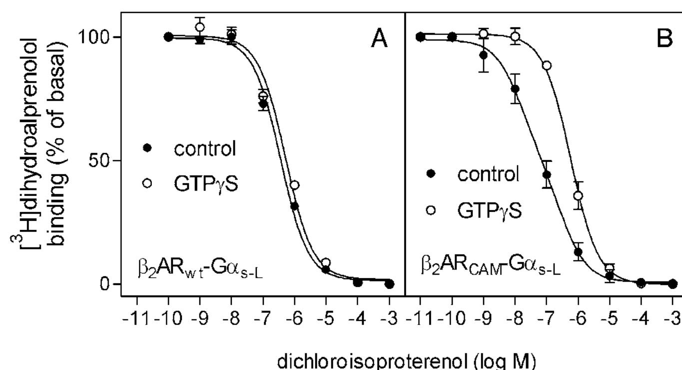

Fig. 8.2 Competition for [^{3}H]DHA binding by dichloroisoproterenol in Sf9 membranes expressing β_2AR_{wt}-$G\alpha_{s\text{-}L}$ or β_2AR_{CAM}-$G\alpha_{s\text{-}L}$: effect of GTPγS. [^{3}H]DHA binding was performed as described in Section 8.2.2. Reaction mixtures contained Sf9 membranes (15–40 μg of protein per tube) expressing β_2AR_{wt}-$G\alpha_{s\text{-}L}$ (3.3–7.5 pmol mg^{-1}) (A) or β_2AR_{CAM}-$G\alpha_{s\text{-}L}$ (2.5–4.9 pmol mg^{-1}) (B), 1 nM [^{3}H]DHA, and dichloroisoproterenol at the concentrations indicated on the abscissa. Reaction mixtures additionally contained distilled water (control) or GTPγS (10 μM). Data shown are the means ± SDs of five independent experiments and were analyzed by non-linear regression.

Before experiments, membranes are sedimented by a 15 min centrifugation at 4 °C and 15 000 × *g* and resuspended in binding buffer (12.5 mM $MgCl_2$ and 1 mM EDTA in 75 mM Tris/HCl, pH 7.4). The washing procedure is crucial in order to remove, as far as possible, endogenous guanine nucleotides, since any remaining guanine nucleotide could interfere with the analysis of the effects of exogenous guanine nucleotide on inverse agonist and agonist binding [15, 32]. Membranes are suspended at least 30 times with a tuberculin syringe. By this process, optimal membrane suspension and data quality are ensured. Binding experiments are performed in 5 mL round-bottomed culture tubes (12 × 75 mm; VWR, West Chester, PA, USA). For determination of K_d and B_{max} values, Sf9 membranes (2.5–5 μg of protein per tube) are suspended in 500 μl of binding buffer supplemented with [^{3}H]DHA (0.1–10 nM; 85–90 Ci mmol^{-1}; Amersham Pharmacia Biotech, Piscataway, NY or Perkin–Elmer, Boston, MA, USA) and 0.2% (mass/vol.) bovine serum albumin. Bovine serum albumin is added to [^{3}H]DHA saturation binding experiments to prevent absorption of membranes to tubes. We have stored [^{3}H]DHA stock solutions for more than 2 years at –20 °C without loss of data quality. We recommend the use of low amounts of membrane protein in [^{3}H]DHA saturation binding studies to verify that no more than 10% of the total amount of [^{3}H]DHA added is bound to the filter even with the lowest radioligand concentration employed. Nonspecific binding is determined in the presence of [^{3}H]DHA (0.1–10 nM) plus 10 μM (±)-alprenolol (Sigma). Even with the highest [^{3}H]DHA concentration, nonspecific binding is $<$20–30% of total binding.

To set up the saturation binding assay, we first add 50 μl of [^{3}H]DHA to the tubes to give the desired final concentration, followed by the addition of 100 μl of distilled water or (±)-alprenolol. Thereafter, 300 μl of binding buffer is added, and reactions are initiated by addition of 50 μl of a mixture consisting of suspended Sf9 membranes and bovine serum albumin to tubes. Incubations are conducted for 60–90 min at 25 °C with shaking at 200–250 rpm. Bound [^{3}H]DHA is separated from free [^{3}H]DHA by filtration through GF/C filters (Schleicher and Schuell; Dassel, Germany), followed by three washes with 2 mL of binding buffer (4 °C). For processing of ≤24 tubes, a Millipore 12-well filtration manifold (Millipore, Bedford, MA, USA) is used. For processing larger number of tubes, a 48-well Brandel MB-48R harvester (Brandel, Gaithersburg, MD, USA) is used. Radioactivity is determined by liquid scintillation counting with, for example, environmentally safe Cytoscint cocktail (ICN, Irvine, CA, USA) (5 mL of cocktail in a 6 mL scintillation counting tube). Tubes are equilibrated for 3–4 h at room temperature, after which samples are counted for 1 min. Counting for longer times is only necessary if filter-bound radioactivity values are below 100 counts per minute (cpm). For fitting to a binding hyperbola, data are analyzed by non-linear regression by use of, for example, the Prism 4.0 program (Graph-Pad-Prism, San Diego, CA).

Inverse agonist and agonist competition binding experiments are carried out with 1 nM [^{3}H]DHA in the presence of unlabeled inverse agonist or agonist at various concentrations with 10 μM GTPγS (Roche) or water (control). Experiments are set up in 48-tube panels. Three to four parallel racks with 48 tubes can be readily handled by a trained person. Firstly, unlabeled ligands (50 μl) are added to tubes, followed by the addition of 400 μl of binding buffer supplemented with GTPγS or water (control).

Then, suspended membranes (25 μl per tube containing 15–40 μg of protein, depending on the expression level) are added. Reactions are initiated by the addition of [^{3}H]DHA (25 μl per tube). Tubes are mixed and incubated for 60–90 min at 25 °C with shaking at 200–250 rpm. Stopping and counting of samples is performed as described above.

We routinely perform saturation and competition binding experiments in triplicate. Binding experiments should be set up in such a way as to fit the 48-well design of the Brandel MB-48R harvester if such equipment is available. We have found that running samples in triplicate and using competitor concentrations at a log scale gives more reliable results than running samples in duplicate and incorporating more ligand concentrations (e.g., at half-log scale). The amount of membrane protein added to binding tubes is adjusted in such a way that not more than 10% of the [^{3}H]DHA added is filter-bound. For generation of ligand competition curves, the [^{3}H]DHA binding in the absence of added unlabeled competitor is set at 100%, the binding observed with 10 μM (±)-alprenolol is set at 0%, and the other values are referred to these values. Data are analyzed by non-linear regression by use of the Prism 4.0 program.

For generation of [^{3}H]DHA saturation binding curves, absolute antagonist binding (pmoles of [^{3}H]DHA bound per mg of membrane protein) is calculated as follows:

$$\text{pmol mg}^{-1} = \frac{(\text{cpm total} - \text{cpm nonspecific}) \times \text{pmol } [^3\text{H}]\text{DHA}}{\text{cpm added} \times \text{mg protein}}$$

Explanations:

cpm total:	Filter-bound radioactivity of [^{3}H]DHA from binding tubes, except from those tubes containing 10 μM (±)-alprenolol.
cpm nonspecific:	Filter-bound radioactivity of [^{3}H]DHA from binding tubes containing 10 μM (±)-alprenolol.
pmol [^{3}H]DHA:	Absolute amount of [^{3}H]DHA present in the binding tube, i.e. with 10 nM [^{3}H]DHA, 5 pmoles of [^{3}H]DHA are present in a 500 μl assay volume.
cpm total added:	The radioactivity of [^{3}H]DHA added to each tube (no filtering).
mg protein:	Absolute amount of membrane protein added per tube. In saturation binding studies, each tube contained 2.5–5.0 μg of protein. In competition binding assays, tubes contained 15–40 μg of protein.

8.2.3
GTPase Assay

Some of the seminal pioneering experiments on constitutive GPCR activity were performed by use of the steady-state GTPase assay [17, 35, 72–74], so this assay constitutes a classic approach to study of constitutive GPCR activity. An advantage of the GTPase assay for the assessment of constitutive GPCR activity is that the assay monitors the outcome of R to R* isomerization at a proximal point of the signal transduction cascade (i.e., at the G protein level) as compared to, for example, the gene expression level

in reporter gene assays (see Section 8.3.3), thereby minimizing the number of factors that may interfere with the magnitude of signals [22]. However, in some systems, constitutive activity cannot be detected without such signal amplification. Another advantage of the GTPase assay is that it constitutes a steady-state assay, with monitoring of the outcome of multiple rounds of GDP/GTP exchange and GTP hydrolysis at a G protein catalyzed by a GPCR, thereby minimizing the impact of the specific time period for which the reaction is monitored [75]. In contrast, with the popular [^{35}S]GTPγS binding assay, one has to be quite careful about choosing the time point at which the binding reaction is stopped (i.e., at short incubation times the constitutive activity of GPCRs may appear to be smaller than at long incubation times; see also Section 8.2.4.) [21, 40].

The GTPase assay can be applied to various G_i/G_o-coupled constitutively active GPCRs expressed in native cell membranes, specifically the DOP(δ)R expressed in NG 108–15 neuroblastoma x glioma cell membranes [35, 73] and chemoattractant receptors expressed in HL-60 leukemia cell membranes [17, 19, 36]. In addition, the GTPase assay can be used in recombinant mammalian and insect cell systems expressing defined GPCRs [21, 29, 76]. In the case of insect cells, coexpression of GPCRs with mammalian G_i/G_o proteins is crucial, since the insect cells do not express endogenous mammalian-type G_i/G_o proteins to which mammalian GPCRs couple efficiently [21, 24, 34, 77]. Constitutive activity of G_i/G_o-coupled GPCRs can also be efficiently monitored in the GTPase assay by use of GPCR-$G\alpha_i/G\alpha_o$ fusion proteins [29, 78].

With respect to G_s-coupled GPCRs, reconstituted systems consisting of purified GPCR and G_s are feasible for studying constitutive GPCR activity in the GTPase assay [72]. However, for extensive pharmacological studies, this is not a practical approach. Unfortunately, in mammalian cell lines, it is very difficult, if not impossible, to detect reasonable GTPase regulation by G_s-coupled GPCRs [75]. Even when G_s-coupled GPCRs and mammalian G_s proteins are coexpressed at very high levels in Sf9 insect cells, detection of decent receptor-regulated GTPase activity is almost impossible [32, 65]. A practical approach to detection of excellent GTPase stimulation by G_s-coupled GPCRs is to analyze GPCR-$G\alpha_s$ fusion proteins [63, 79]. In those fusion proteins, when expressed in Sf9 insect cells above a low background of endogenous GTPase activity, substantial (i.e., up to three- to fourfold) stimulation of GTP hydrolysis by agonists is detected (Figure 8.3A) [79]. The high sensitivity of this system also allows for the detection of inhibitory effects of inverse agonists on GTP hydrolysis [63, 79]. The reason for the highly efficient GTPase activation in GPCR-$G\alpha_s$ fusion proteins is probably that the fusion prevents $G\alpha_s$ from dissociating into the cytosol and ensures a close proximity between the coupling partners [80]. As an alternative to the GPCR-$G\alpha_s$ fusion protein approach for monitoring GTPase activity, GPCRs can be coexpressed with a tethered $G\alpha_s$ (tet-$G\alpha_s$): a $G\alpha_s$ permanently attached to the plasma membrane by an anchor consisting of the first transmembrane domain of the β_2AR [81]. This artificial anchor does not interfere with the overall function of $G\alpha_s$ [81].

Another advantage of the use of the GTPase assay with fusion proteins as a read-out for assessment of constitutive GPCR activity is the fact that various receptors can be compared with each other independently of the expression level. This is important, since some constitutively active GPCR mutants are also structurally unstable and

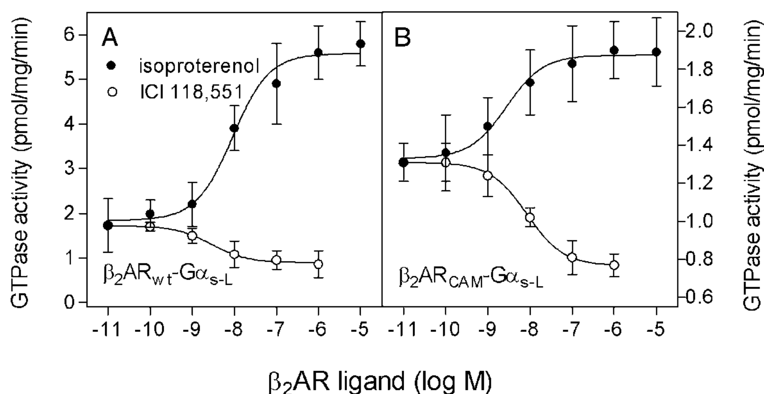

Fig. 8.3 Effects of isoproterenol and ICI 118551 on GTPase activity in Sf9 membranes expressing β_2AR_{wt}-$G\alpha_{s\text{-}L}$ or β_2AR_{CAM}-$G\alpha_{s\text{-}L}$. GTPase activity was determined as described in Section 8.2.3. The expression level of β_2AR_{wt}-$G\alpha_{s\text{-}L}$ (A) was 6.5 pmol mg^{-1}, and the expression level of β_2AR_{CAM}-$G\alpha_{s\text{-}L}$ (B) was 2.5 pmol mg^{-1}. Reaction mixtures contained 10 µg of membrane protein and isoproterenol or ICI 118551 at the concentrations indicated on the abscissa. In order to assess the inhibitory effect of ICI 118551 on GTPase activity in membranes expressing β_2AR_{wt}-$G\alpha_{s\text{-}L}$ reliably, this construct had to be expressed at higher levels than β_2AR_{CAM}-$G\alpha_{s\text{-}L}$. Accordingly, the absolute GTPase activities in (A) are considerably higher than in (B). Data shown are the means ± SDs of three independent experiments and were analyzed by non-linear regression.

are expressed at lower levels than their wild-type counterparts [15, 66, 82–84]. This issue is illustrated in Figure 8.3 for the comparison of β_2AR_{wt}-$G\alpha_{s\text{-}L}$ and β_2AR_{CAM}-$G\alpha_{s\text{-}L}$. β_2AR_{CAM}, both in the fused and in the non-fused state, expresses at considerably lower levels than β_2AR_{wt} [15, 66, 82]. For the data shown in Figure 8.3, β_2AR_{wt}-$G\alpha_{s\text{-}L}$ was expressed at 6.5 pmol mg^{-1} of membrane protein, while β_2AR_{CAM}-$G\alpha_{s\text{-}L}$ was expressed at just 2.5 pmol mg^{-1}. Accordingly, the absolute GTPase activities in Sf9 membranes expressing β_2AR_{CAM}-$G\alpha_{s\text{-}L}$ are considerably lower than in membranes expressing β_2AR_{wt}-$G\alpha_{s\text{-}L}$. However, despite the differences in absolute GTPase activities, it is nonetheless clear that the relative inhibitory effect of ICI 118551 on GTP hydrolysis in membranes expressing β_2AR_{CAM}-$G\alpha_{s\text{-}L}$ is considerably larger than in membranes expressing β_2AR_{wt}-$G\alpha_{s\text{-}L}$. Moreover, ICI 118551 is more potent at β_2AR_{wt}-$G\alpha_{s\text{-}L}$ (IC_{50} 2.8 nM) than at β_2AR_{CAM}-$G\alpha_{s\text{-}L}$ (IC_{50} 8.5 nM). These findings reflect the fact that R* is more abundant in β_2AR_{CAM} than in β_2AR_{wt} and that ICI 118551 possesses a higher affinity for the R state than for the R* state. Conversely, the agonist isoproterenol is more potent at β_2AR_{CAM}-$G\alpha_{s\text{-}L}$ (EC_{50} 2.4 nM) than at β_2AR_{wt}-$G\alpha_{s\text{-}L}$ (EC_{50} 13 nM), reflecting the greater abundance of the R* state in β_2AR_{CAM} than in β_2AR_{wt} and the higher affinity of isoproterenol for the R* state than for the R state.

With the use of GPCR-$G\alpha_s$ fusion proteins as system, the GTPase assay is highly sensitive for precise determination of the potencies and efficacies of partial agonists and inverse agonists and comparison of the constitutive activities of various related systems. Those systems include β_1-adrenoceptor (β_1AR) isoforms, β_2AR, and histamine H_2-receptor (H_2R) coupled to $G\alpha_s$ isoforms, species isoforms of the H_2R coupled

to the short splice variant of $G\alpha_s$ ($G\alpha_{s\text{-}S}$), β_2AR coupled to various classes of $G\alpha$ proteins, and the FPR1 coupled to various $G\alpha_i$ isoforms [15, 40, 54, 63, 85–88].

Under basal conditions, guanine nucleotide exchange at G_q proteins is slow, and detection of receptor-stimulated GTP hydrolysis by G_q proteins is accordingly very difficult [41, 75, 89]. However, G_q-catalyzed GTP hydrolysis is strongly enhanced by RGS (regulator of G protein signaling) proteins [90]. Taking advantage of this acceleration of GTP hydrolysis by RGS proteins, the histamine H_1-receptor (H_1R) was coexpressed with the RGS proteins RGS4 or GAIP in Sf9 cells. Under these conditions, the histamine efficiently increases GTP hydrolysis (up to 175 % stimulation) by the endogenous G_q proteins of the insect cells [41, 64]. This system enabled us to examine the constitutive activity of the H_1R. In agreement with the groups of Leurs and Weiner [50, 51, 91], we found the majority of H_1R antagonists actually to be inverse agonists [64]. However the GTPase assay is a less sensitive read-out for analysis of constitutive activity of G_q-protein-coupled receptors [64] than PLC- and reporter gene expression assays [50, 51, 91]. Differences in the apparent constitutive activity of a given GPCR by measurement through various read-outs is not without precedence [22, 32]. Possibly, in analogy with the β_2AR/G_s/AC system [22, 32, 92], effector availability limits signal transfer in the H_1R/G_q/PLC system, thereby favoring detection of inverse agonists in the PLC assay relative to the GTPase assay. Finally, coexpression of H_1R with mammalian G_q proteins in Sf9 cells was inefficient at reconstituting receptor-regulated GTP hydrolysis, despite very high $G\alpha_q$ expression levels [41]. Apparently, most of the expressed $G\alpha_q$ proteins are functionally inactive [41].

A procedure for the steady-state GTPase assay is given below. This procedure has been successfully applied to various G_s-coupled GPCRs [32, 63], G_i-coupled GPCRs [21, 24], and G_q-coupled GPCRs [41, 64] expressed in Sf9 cells.

Before the experiments, membranes are sedimented by a 15 min centrifugation at 4 °C and 15 000 × *g* and carefully resuspended in 10 mM Tris/HCl, pH 7.4. This washing step ensures removal of all endogenous agonists and guanine nucleotides. Reactions are conducted in 1.5 mL reaction tubes. Tubes are kept at 4 °C before the addition of [γ-^{32}P]GTP (6000 Ci mmol^{-1}; Perkin–Elmer, Boston, MA, USA). Firstly, inverse agonists or agonists (10 µl per tube) are added to tubes. Then, 50 µl of a reaction mixture containing (final) 0.1 µM unlabeled GTP (Roche), 1.0 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM ATP (Roche), 1 mM adenosine 5’-[β,γ-imido]triphosphate (AppNHp), (Roche), 5 mM creatine phosphate (Sigma), 40 µg creatine kinase (Sigma) and 0.2 % (mass/vol.) bovine serum albumin in 50 mM Tris/HCl, pH 7.4, are added. Suspended membranes (10 µg of protein in 20 µl) are then pipetted into tubes. Assay tubes (containing 80 µl) are placed in a heat block set at 25 °C and incubated for 3 min before the addition of 20 µl of [γ-^{32}P]GTP diluted in 20 mM Tris/HCl, pH 7.4 (0.05–1.0 µCi per tube). We recommend buffering the [γ-^{32}P]GTP work solution because [γ-^{32}P]GTP dissolved in distilled water can become unstable. Depending on the experience of the researcher, preincubation, starting, and stopping of reactions is performed at 10–20 s intervals. In order to assess non-enzymatic degradation of [γ-^{32}P]GTP, a large excess of unlabeled GTP (1 mM) is added to some tubes. Usually, the amount of [^{32}P]P_i released under these conditions is $<$1 % of the total amount of radioactivity added. In mammalian cell lines, low-affinity GTPase activity (usually assessed with 50 µM GTP) [75, 93]

should be determined. This is not necessary when studying Sf9 membranes, because absolute GTPase activities in this system are much lower than in mammalian membrane systems [32, 94, 95].

Reactions are conducted for 20 min at 25 °C and are terminated by the addition of 900 µl of slurry (4 °C) consisting of 5 % (mass/vol.) activated charcoal (Sigma) and 50 mM NaH_2PO_4, pH 2.0. The slurry does not have to be chilled during the stopping process, but stirring is essential since the charcoal sediments rather quickly. In addition, the pipette tip used for addition of the charcoal slurry should be cut wider in order to avoid clogging of the tip with charcoal particles. Charcoal-quenched reaction mixtures are centrifuged for 15 min at room temperature at 15 000 × *g*. Seven hundred µl of the supernatant fluid of reaction mixtures are carefully removed, and $[^{32}P]P_i$ is determined by liquid scintillation counting with environmentally safe Ecolume cocktail (ICN, 5 mL of cocktail in a 6 mL scintillation counting tube). Alternatively, $[^{32}P]P_i$ can be counted in water by Čerenkov radiation, but the counting efficiency is more than 50 % lower. When the above assay design is used, generally less than 10 % of the added $[\gamma\text{-}^{32}P]$GTP is degraded. However, even with up to 20 % of substrate consumption, we found ligand potencies and efficacies to be unchanged.

Although the GTPase assay in Sf9 membranes in general works very reliably, occasionally there are changes in the basal GTPase activity within one assay; this is due to non-enzymatic $[\gamma\text{-}^{32}P]$GTP degradation during the assay. Since $[\gamma\text{-}^{32}P]$GTP is inherently unstable, it is important to dilute the radiolabeled nucleotide in 20 mM Tris-HCl, pH 7.4, and to avoid frequent freeze/thaw cycles of $[\gamma\text{-}^{32}P]$GTP stock solutions. We prepare small aliquots (10–20 µl) of the original stock solution of $[\gamma\text{-}^{32}P]$GTP and keep them at –80 °C. Under these conditions, in general, $[\gamma\text{-}^{32}P]$GTP can be used for up to 4 weeks. Storage at –20 °C is not recommended because of accelerated degradation of $[\gamma\text{-}^{32}P]$GTP, resulting in high blank values (>2 %) that compromise data quality. In order to ensure maximum data accuracy, we recommend that basal GTPase activities are assessed at the beginning of each concentration-response curve for a given ligand. We routinely process 60 tubes in one assay, since this is the number of tubes that fits into the commonly employed laboratory incubators (three heat blocks with 20 wells each).

The precision and sensitivity of the GTPase assay is high enough to run samples in duplicate. Thus, by use of a 60-well incubator, 30 different sets of experimental conditions (including basal GTPase activities and the 1 mM GTP value) can be incorporated in one assay. Drugs are routinely studied at a log scale. In our experience, more detailed concentration response curves (e.g., at a half-log scale) do not significantly increase the precision for assessment of ligand potencies and efficacies as assessed by nonlinear regression analysis. Rather, we prefer to include as many ligands as possible in one assay to allow their direct comparison. Sigmoid concentration-response curves to agonists and inverse agonists are analyzed by nonlinear regression, by use of the built-in function in the Prism 4.0 program. With this program, EC_{50} values for agonists and IC_{50} values for inverse agonists can be conveniently calculated. Moreover, the program calculates the *bottom* (baseline) and *top* (plateau) values for each concentration-response curve so that the efficacies for different ligands can be precisely determined.

For generation of concentration-response curves to full agonists and strong partial agonists on GTP hydrolysis in Sf9 membranes expressing $\beta_2AR_{wt}\text{-}G\alpha_{s\text{-}L}$, low amounts

of [γ-^{32}P]GTP (0.05 μCi per tube) can be used since the background is low. However, for determination of the potencies and efficacies of weak partial agonists and inverse agonists, we recommend the use of larger amounts of [γ-^{32}P]GTP (up to 0.5 μCi per tube) to increase the sensitivity of the assay.

AppNHp is routinely used as a nucleotidase inhibitor in GTPase assays with membranes [93], but the nucleotide is quite expensive. We have examined the effect of omission of AppNHp from the reaction mixture in Sf9 membranes and have found the nucleotide to be dispensable without compromising the sensitivity of the GTPase assay. However, in mammalian cell membranes, where nucleotidase activity is higher [75, 95], omission of AppNHp can result in depletion of substrate for the high-affinity GTPase and is therefore not recommended.

Another important factor that has to be considered in elaboration of the conditions for the GTPase assay is the optimization of the $MgCl_2$ concentration. In the absence of added $MgCl_2$ and in the presence of EDTA, there is no agonist-stimulation of GTPase in Sf9 membranes expressing β_2AR_{wt}-$G\alpha_{s\text{-}L}$. With $MgCl_2$ at 0.1 mM a small stimulatory effect of agonist on GTPase is evident, and $MgCl_2$ at concentrations between 1–10 mM gives similarly high GTPase stimulations with $\beta_2ARG_{s\alpha}$. We routinely use $MgCl_2$ at a concentration of 1 mM. Finally, NaCl has an important impact on basal GTPase activity and the effects of inverse agonists [17, 73]. This topic is discussed in detail in Section 8.2.4, since NaCl addition exhibits similar effects in the GTPase- and [^{35}S]GTPγS-binding assays.

GTPase activity (pmoles of P_i released per mg of membrane protein per min) is calculated as follows:

$$\text{pmol mg}^{-1}\text{ min}^{-1} = \frac{(\text{cpm total} - \text{cpm GTP}) \times \text{pmol GTP unlabeled} \times 1.43}{\text{cpm added} \times \text{min incubation} \times \text{mg protein}}$$

Explanations:

cpm total: Radioactivity ([^{32}P]P_i) counted in the 700 μl aliquot taken from all tubes except those containing 1 mM GTP.

cpm GTP: Radioactivity ([^{32}P]P_i) counted in the 700 μl aliquot taken from tubes containing 1 mM GTP.

pmol GTP unlabeled: Absolute amount of substrate present in the tube; i.e., with 100 nM GTP, 10 pmoles of GTP are present in a 100 μl reaction volume. Since the specific activity of [γ-^{32}P]GTP is very high (6000 Ci mmol^{-1}), the radiolabeled compound does not significantly contribute to the total amount of substrate present.

1.43: Factor correcting for the fact that only 700 μl out of 1000 μl in the tube are counted.

cpm total added: The radioactivity of [γ-^{32}P]GTP added to each tube (no charcoal addition).

min incubation: Assays are routinely conducted for 20 min.

mg protein: Absolute amount of membrane protein added per tube. Each tube routinely contains 10 μg of membrane protein.

8.2.4 [^{35}S]GTPγS Binding Assay

In general, GDP dissociation is considered to be the rate-limiting step in GPCR-mediated G protein activation [10, 11]. Unfortunately, GPCR-catalyzed GDP dissociation can only be directly examined in a few specialized systems [96, 97], precluding wide use of this assay. The situation is further complicated by the fact that the GDP affinity of G proteins is actually rather low [22, 54, 98]. Accordingly, basal GDP dissociation from Gα subunits, specifically $G\alpha_i$ and $G\alpha_o$ subunits and $G\alpha_{s\text{-}L}$, can be quite substantial [37, 99, 100]. This, together with the GDP dissociation induced by constitutively active GPCRs, reduces GDP-loading of Gα to such an extent that a sensitive and widely applicable assay to determine GPCR-mediated GDP dissociation cannot be implemented. However, by loading G proteins with an excess of exogenous GDP (typically concentrations of 0.1–10 μM are used) and by adding the high-affinity G protein ligand [^{35}S]GTPγS (typically used at 0.2–1.0 nM), GPCR-mediated guanine nucleotide exchange can be monitored with high sensitivity in a convenient binding assay format since GPCRs decrease GDP affinity and increase the [^{35}S]GTPγS affinity [18, 32, 37, 99].

The [^{35}S]GTPγS binding assay can be readily applied to the analysis of constitutively active G_i/G_o-coupled GPCRs in native and recombinant systems [21, 24, 101–104]. The [^{35}S]GTPγS binding assay works equally well in GPCR-$G\alpha_i$ coexpression and fusion protein systems [21, 29]. With respect to G_s-coupled GPCRs, the application of the [^{35}S]GTPγS binding assay is very similar to the use of the GTPase assay. Specifically, in native and recombinant membrane systems expressing G_s-coupled GPCRs alone or together with $G\alpha_s$, the assay does not yield good receptor signals; only with GPCR-$G\alpha_s$ fusion proteins or GPCRs coexpressed with tet-$G\alpha_s$ is excellent receptor-stimulated [^{35}S]GTPγS binding detected [32, 40, 65, 81].

With regard to G_q proteins, in our hands it has been difficult to monitor [^{35}S]GTPγS binding in a conventional binding assay, studying mammalian and insect cell $G\alpha_q$ proteins, coexpression, and fusion protein systems [40, 41]. The reasons for this situation are the sluggish intrinsic guanine nucleotide exchange at G_q proteins and the low affinity of these proteins for GTPγS, which results in significant [^{35}S]GTPγS dissociation, thereby substantially decreasing the sensitivity of the binding assay [40, 105]. Squid retinal $G\alpha_q$ has been successfully used to reconstitute [^{35}S]GTPγS binding with the constitutively active 5-HT_{2C}R expressed in Sf9 cell membranes [33]. However, squid $G\alpha_q$ protein has not found general use for the analysis of constitutively active GPCRs, probably because of difficulties in obtaining sufficiently large amounts of the purified G protein. As an alternative to the conventional [^{35}S]GTPγS binding assay, a combination of [^{35}S]GTPγS binding and subsequent immunoprecipitation of the [^{35}S]GTPγS-bound $G\alpha_q$ proteins has been implemented [30, 106, 107]. This assay exhibits increased sensitivity in the detection of GPCR-stimulated [^{35}S]GTPγS binding relative to the conventional binding assay format. However, for large scale studies, the combined [^{35}S]GTPγS binding/immunoprecipitation assay is less convenient and more expensive than a conventional binding assay. The combined [^{35}S]GTPγS binding/immunoprecipitation assay has also been applied to $G\alpha_z$- and $G\alpha_{12/13}$-coupled GPCRs [106].

During the past 15 years, the [^{35}S]GTPγS binding assay has gained substantial popularity for examination of GPCR/G protein coupling in general and constitutive GPCR activity in particular, in relation to the GTPase assay [1]. The reasons for this development are that, in the [^{35}S]GTPγS binding assay, larger relative GPCR signals than in the GTPase assay can be obtained by loading G proteins with GDP, that [^{35}S]GTPγS has a $t_{1/2}$ approximately five times longer than and is more stable than the GTPase substrate [γ-^{32}P]GTP, and that the capacity of the binding assay is larger that

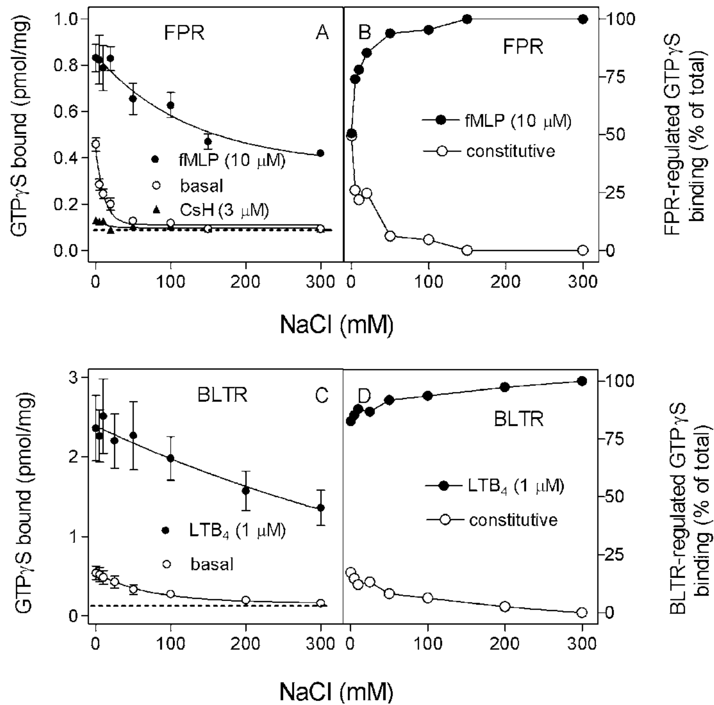

Fig. 8.4 Effects of NaCl on basal and agonist-stimulated [^{35}S]GTPγS binding in Sf9 membranes expressing chemoattractant receptors plus $G\alpha_{i2}\beta_1\gamma_2$. [^{35}S]GTPγS binding in membranes expressing the FPR1 plus G_i (A and B) or the BLTR plus G_i (C and D) was determined as described in Section 8.2.4. The GPCR expression level was ~10 pmol mg^{-1} as assessed by immunoblotting [24]. Reaction mixtures contained 15 μg of membrane protein, 0.4 nM [^{35}S]GTPγS, and 1 μM GDP, as well as distilled water (basal), agonist, or inverse agonist at a maximally effective concentration. Reaction mixtures additionally contained NaCl at the concentrations indicated on the abscissa. The dashed lines in (A) and (C) represent extrapolations of basal [^{35}S]GTPγS binding in the presence of 300 mM NaCl. These values were used to define [^{35}S]GTPγS binding stimulated by agonist-free chemoattractant receptors. Data shown are the means ± SDs of four independent experiments and were analyzed by nonlinear regression. For the data shown in (B) and (D), total chemoattractant-regulated [^{35}S]GTPγS binding was defined as the difference between maximum agonist-stimulated [^{35}S]GTPγS binding at a given NaCl concentration and the minimum [^{35}S]GTPγS binding observed in the presence of 300 mM NaCl. For each NaCl concentration, the total GPCR-regulated [^{35}S]GTPγS binding was defined as 100%, and the relative contributions of agonist-dependent and agonist-independent (constitutive) GPCR activity were calculated.

that of the GTPase assay [18, 37, 75, 108]. For various G_i- and G_s-coupled GPCR systems, however, the GTPase- and [^{35}S]GTPγS-binding assays can essentially be used interchangeably to assess the potencies and efficacies of agonists and inverse agonists [21, 22, 29, 40, 54].

Although the principle of the [^{35}S]GTPγS binding assay is straightforward, one has to pay great attention to the specific experimental conditions when analyzing constitutive GPCR activity. It has been known for almost 25 years that monovalent cations, specifically Na^+, reduce basal GTP hydrolysis and [^{35}S]GTPγS binding in membrane systems expressing G_i/G_o-coupled GPCRs [17, 35, 73, 109]. In addition, in the presence of Na^+, the inhibitory effects of inverse agonists on basal GTP hydrolysis and [^{35}S]GTPγS binding are reduced [21, 24, 73, 102, 110].

The effect of Na^+ on [^{35}S]GTPγS binding catalyzed by the FPR1 and BLTR coexpressed with the G protein $G\alpha_{i2}\beta_1\gamma_2$ in Sf9 cells is shown in Figure 8.4. The FPR1 is activated by the bacterial formyl peptide *N*-formyl-L-methionyl-L-leucyl-L-phenylalanine (fMLP), and the BLTR is activated by leukotriene B_4 (LTB_4) [16, 111]. In Sf9 membranes expressing the FPR1 and BLTR, Na^+ reduces basal [^{35}S]GTPγS binding more efficiently than agonist-stimulated [^{35}S]GTPγS binding (Figure 8.4A and 8.4C), thus enhancing relative agonist effects (Figure 8.4B and 8.4D). With Na^+ at 200–300 mM, basal [^{35}S]GTPγS binding reaches a low plateau, but Na^+ does not completely inhibit basal [^{35}S]GTPγS binding. In the absence of Na^+, the inverse FPR1 agonist cyclosporin H (CsH) [21, 29] strongly reduces basal [^{35}S]GTPγS binding in membranes expressing the FPR1 and $G\alpha_{i2}\beta_1\gamma_2$ (Figure 8.4A). However, with Na^+ at increasing concentrations, the inhibitory effect of CsH becomes smaller. At a Na^+ concentration of 50 mM, the Na^+ and CsH inhibition curves overlap (i.e., CsH does not exhibit an additional inhibitory effect on basal [^{35}S]GTPγS binding in the presence of Na^+). In other words, CsH and Na^+ exhibit similar maximum inhibitory effects on [^{35}S]GTPγS binding. These results indicate that both CsH and Na^+ stabilize the R state of the FPR1 and thereby reduce the ability of the agonist-free FPR1 in promoting [^{35}S]GTPγS binding to G_i proteins. In contrast, the ability of the agonist fMLP to stabilize the R* state of the FPR1 is largely preserved. The difference between the maximum agonist-stimulated [^{35}S]GTPγS binding for each Na^+ concentration and the minimum [^{35}S]GTPγS binding in the presence of CsH or Na^+ constitutes the total FPR1-regulated [^{35}S]GTPγS binding. In the absence of Na^+, about 50% of the FPR1-catalyzed [^{35}S]GTPγS binding is mediated by the constitutively active (agonist-free) FPR1 (Figure 8.4B). Thus, the FPR1 exhibits a rather high constitutive activity. With increasing Na^+ concentrations, constitutive [^{35}S]GTPγS binding is reduced, and agonist-stimulated [^{35}S]GTPγS binding is increased.

An important aspect regarding the effect of Na^+ on constitutive GPCR activity is that Na^+ regulation appears to encompass G_i/G_o-coupled GPCRs in general. Specifically, inhibitory effects of Na^+ on basal G protein activation hve been observed for the BLTR [24], C5aR [24], PAFR [24], DOP(δ)R [73], μ-opioid receptor (MOP(μ)R) [110], α_{2D}-adrenoceptor (α_{2D}AR) [102,112], muscarinic acetylcholine M_2 receptor (M_2R) [101, 113], and somatostatin 2-receptor ($SRIF_2$R) [114]. It is likely that interaction of Na^+ with Asp^{71} in the second transmembrane domain of the FPR1, which is highly conserved in GPCRs, plays a crucial role in constraining the GPCR in the R state [115, 116]. Thus, by the use of Na^+ as a universal inverse agonist for G_i/G_o-coupled GPCRs, one can

assess the constitutive activity of a given GPCR without the necessity to be in possession of a true inverse agonist. If we now compare the Na^+ sensitivity of [^{35}S]GTPγS binding catalyzed by the FPR1 and BLTR, it becomes evident that Na^+ exhibits a much smaller inhibitory effect on basal [^{35}S]GTPγS binding in membranes expressing the BLTR than in membranes expressing the FPR1 (Figures 8.4A and 8.4C). Specifically, basal [^{35}S]GTPγS binding in membranes expressing BLTR accounts for just 15–20% of the total BLTR-catalyzed [^{35}S]GTPγS binding (Figure 8.4D). These data show that the BLTR is much less constitutively active than the FPR1.

With use of the β_2AR_{wt}-$G\alpha_{sL}$ fusion protein as model system, both agonist-stimulated and inverse agonist-inhibited [^{35}S]GTPγS binding is detected in the absence of added GDP, indicating that a certain fraction of the $G\alpha_{s\text{-}L}$ molecules is GDP-bound even in washed membranes and that this endogenously bound GDP can be displaced by [^{35}S]GTPγS through GPCR catalysis [32]. However, because of significant GDP dissociation from $G\alpha_{s\text{-}L}$ even in the absence of agonist [23, 54], the addition of GDP enhances the relative stimulatory effects both of inverse agonist and of agonists on [^{35}S]GTPγS binding [32]. GDP reduces absolute basal [^{35}S]GTPγS binding in a concentration-dependent manner. In the presence of the agonist isoproterenol, the GDP inhibition curve is shifted to the right, reflecting the agonist-induced reduction of the GDP affinity of $G\alpha_{s\text{-}L}$ relative to the [^{35}S]GTPγS affinity of the G protein. In the presence of 1 nM [^{35}S]GTPγS, the addition of 1 μM and 10 μM GDP enhances the relative stimulatory effect of isoproterenol on [^{35}S]GTPγS binding from about 20% to 200% and 500%, respectively. In contrast to the agonist, the inverse agonist ICI 118551 shifts the GDP inhibition curve of [^{35}S]GTPγS binding to the left, indicative of an increase in the GDP affinity of $G\alpha_{s\text{-}L}$ when β_2AR_{wt} is stabilized in the R state and uncoupled from the G protein. In the absence of GDP, ICI 118551 inhibits basal [^{35}S]GTPγS binding by 25%. In the presence of GDP at 1–10 μM, the inhibitory effect of ICI 118551 increases to about 60%. Thus, in order to ensure a good signal to noise ratio for agonist and inverse agonist signals while maintaining reasonable absolute [^{35}S]GTPγS binding values, GDP should be present at a concentration about a thousand times higher than [^{35}S]GTPγS. However, it should also be noted that GDP at concentrations of 1–10 μM reduces the efficacy of partial agonist relative to full agonists at various GPCRs [40, 117]. Thus, partial agonists are less efficient than agonists at decreasing the GDP affinity of $G\alpha$.

Another important variable in designing [^{35}S]GTPγS binding experiments is the time point at which the binding reaction is stopped. In a study of β_2AR_{wt}-$G\alpha_{s\text{-}L}$ and the FPR1 coexpressed with, or fused to, $G\alpha_i$ proteins, it emerged that inverse agonist effects are smaller at early time points (5–30 min incubation) than at later time points where the binding does not further increase (60–180 min incubation) [21, 29, 40]. These data reflect the ability of agonist-free GPCR to promote [^{35}S]GTPγS binding to a large fraction of the available G proteins provided that sufficient time is allowed for GDP/[^{35}S]GTPγS exchange to take place. In view of these kinetics, the assessment of agonist and inverse agonist efficacies and potencies should be determined only at equilibrium. From a practical perspective, incubation times of 60–90 min are a good compromise between equilibrium conditions and stability of GPCRs and G proteins, which may decrease during extended binding reactions (>120 min). The lack of im-

pact of the incubation time on apparent inverse agonist efficacies is certainly an advantage of the steady-state GTPase assay (see Section 8.2.3.).

In studies with membranes, [^{35}S]GTPγS is typically used at concentrations between 0.4–1.0 nM, and GDP is used at 1 μM [18, 21, 24, 32]. These are experimental conditions that constitute a reasonable compromise between good signal to noise ratio for detection of agonist and inverse agonist effects, sufficiently high amounts of [^{35}S]GTPγS trapped on the filter, and economic use of the radioligand. However, it should be emphasized that agonists and inverse agonists may have opposite effects on the [^{35}S]GTPγS affinity of G proteins. Specifically, agonists increase the apparent [^{35}S]GTPγS affinity of G proteins, whereas inverse agonists reduce their apparent [^{35}S]GTPγS affinity [24, 40]. Thus, in order to obtain maximum inverse agonists, [^{35}S]GTPγS concentrations higher than those routinely used in [^{35}S]GTPγS binding assays (5–10 nM instead of 0.4–1 nM) have to be employed. While this is, of course, technically possible, one has to take into consideration that considerably higher amounts of [^{35}S]GTPγS have to be used for such experiments. As an alternative, [^{35}S]GTPγS can be mixed with defined concentrations of unlabeled GTPγS to obtain the desired final [^{35}S]GTPγS/GTPγS concentration [21, 24, 40]. However, a drawback of this approach is the isotopic dilution of the [^{35}S]GTPγS. Thus, if one plans to conduct such isotope dilution studies, fresh [^{35}S]GTPγS with the highest possible specific activity should be used in order to ensure that sufficient amounts of [^{35}S]GTPγS can be trapped on the filter.

A [^{35}S]GTPγS binding procedure is described below. This procedure has been successfully applied to various G_s-coupled GPCRs [40, 63] and G_i-coupled GPCRs [21, 24], and, less successfully, to various G_q-coupled GPCRs expressed in Sf9 cells [40, 41].

Before experiments, membranes are sedimented by a 15 min centrifugation at 4 °C and 15 000 × *g*. Sf9 membranes (15–25 μg of protein per tube) are carefully suspended in binding buffer (12.5 mM $MgCl_2$ and 1 mM EDTA in 75 mM Tris/HCl, pH 7.4) by use of a tuberculin syringe. Binding experiments are performed in 5 mL culture tubes. Routinely, three to four parallel racks with 48 tubes each are processed. Firstly, water or GDP (10 nM–10 μM final concentration, 50 μl) are added to the tubes, followed by the addition of 50 μl of agonist or inverse agonist and 300 μl of binding buffer supplemented with (final) 0.05 % (mass/vol.) bovine serum albumin. The suspended membranes (15 μg of protein in 50 μl) are then added. Reactions are initiated by the addition of 50 μl of [^{35}S]GTPγS (1100–1500 Ci mmol^{-1}, Perkin–Elmer, 0.1–0.25 μCi per tube, 0.4–1.0 nM final concentration) in distilled water. Tubes are mixed and incubated for 60–90 min at 25 °C with shaking at 200–250 rpm. Nonspecific binding is determined in the presence of 10 μM GTPγS and is <0.2 % of total binding, providing an excellent background for GPCR analysis. Bound [^{35}S]GTPγS is separated from free [^{35}S]GTPγS by filtration through GF/C filters, followed by three washes with 2 mL of binding buffer (4 °C) with a 48-well Brandel harvester. Radioactivity is determined by liquid scintillation counting by use of a scintillation cocktail (e.g., Cytoscint, 5 mL of cocktail in a 6 mL scintillation counting tube). Tubes are equilibrated for 2–3 h, after which the samples are counted for 1 min. With the above assay design, less than 10 % of the added [^{35}S]GTPγS is filter-bound, even in the absence of added GDP. For more technical details regarding the binding assay, see Section 8.2.2.

[^{35}S]GTPγS is commercially available at high specific activity (1100–1500 Ci mmol^{-1}) and reasonable price (e.g., from Perkin–Elmer, Boston, MA, USA). Chemically, [^{35}S]GTPγS is quite stable when stored at –80 °C, and the $t_{1/2}$ of [^{35}S] (90 days) is much longer than that of [^{32}P] (14 days). Thus, [^{35}S]GTPγS can be used for a much longer period of time than [γ-^{32}P]GTP. We prepare multiple small (10–50 µl) aliquots of freshly delivered [^{35}S]GTPγS and keep those aliquots (1 µM final [^{35}S]GTPγS concentration in 20 mM Tris/HCl, pH 7.4, supplemented with 1 mM dithiothreitol) as work stock solutions at –80 °C for up to 5 months without loss of data quality in the binding experiments.

[^{35}S]GTPγS binding (pmoles of GTPγS bound per mg of membrane protein) is calculated as follows:

$$\text{pmol mg}^{-1} = \frac{(\text{cpm total} - \text{cpm nonspecific}) \times \text{pmol } [^{35}\text{S}]\text{GTP}\gamma\text{S}}{\text{cpm added} \times \text{mg protein}}$$

Explanations:

cpm total:	Filter-bound radioactivity of [^{35}S]GTPγS from binding tubes, except from those tubes containing 10 µM GTPγS.
cpm nonspecific:	Filter-bound radioactivity of [^{35}S]GTPγS from binding tubes containing 10 µM GTPγS.
pmol [^{35}S]GTPγS:	Absolute amount of [^{35}S]GTPγS (including any unlabeled GTPγS for isotope dilution experiments) present in the tube (i.e., with 1 nM [^{35}S]GTPγS, 0.5 pmoles of [^{35}S]GTPγS are present in a 500 µl assay volume).
cpm total added:	The radioactivity of [^{35}S]GTPγS added to each tube (no filtering).
mg protein:	Absolute amount of membrane protein added per tube. Assay tubes routinely contain 15 µg of protein each.

8.2.5
Adenylyl Cyclase Assay

The AC assay with membranes and [α-^{32}P]ATP as substrate is a classic method to assess constitutive activity of G_s-coupled GPCRs [31, 58, 66, 67, 118]. In fact, without the AC assay it would have been much more difficult to establish constitutive activity of G_s-coupled GPCRs, since the GTPase- and [^{35}S]GTPγS-binding assays do not yield sufficiently large agonist and inverse agonist signals for most membrane systems expressing G_s-coupled receptors [32, 65]. Several one- and two-column AC methods are available [45, 119–121]. In our experience, the method of Alvarez and Daniels [45] is the best approach by which to generate large sets of high-quality data. The single-column AC assay method of Alvarez and Daniels [45], using alumina columns for separation of [^{32}P]cAMP from [α-^{32}P]ATP, is very robust, reliable, sensitive, and convenient to perform, and ensures extremely low blank values, which is of great importance for study of the inhibitory effects of inverse agonists on basal AC activities. Equilibration of columns before sample application is not required, and columns are disposed of after

use, the amount of liquid [^{32}P] waste and the possibility of contamination of both personnel and equipment thereby being reduced. Because of its simple design, the AC assay can be readily used for extensive studies. We routinely process 60 tubes in one AC assay.

Constitutive activity of G_s-coupled GPCRs in the AC assay can be assessed in three different ways. Firstly, the stimulatory effect of GTP on basal AC activity in the presence of the constitutively active GPCR can be monitored (Figure 8.5A) [22, 23, 65]. In the absence of a constitutively active GPCR, GTP, in contrast to its hydrolysis-resistant analogues GTPγS or guanosine 5'-[β,γ-imido]triphosphate (GppNHp) [70, 122, 123],

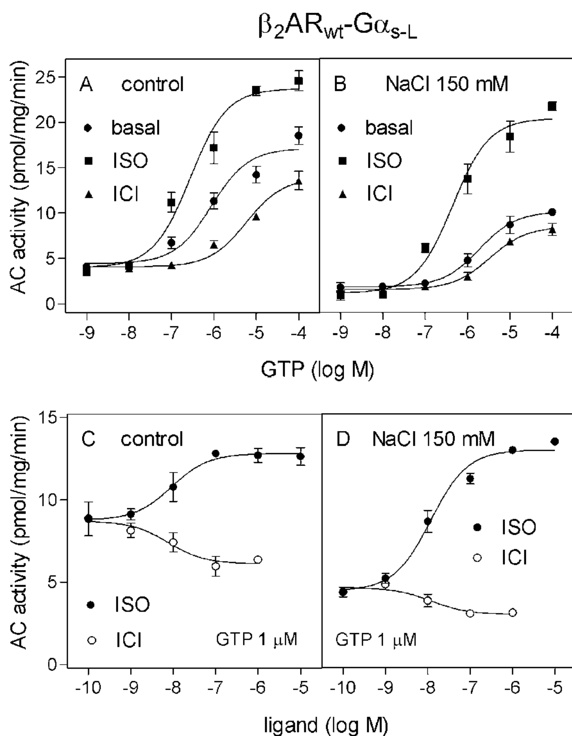

Fig. 8.5 Effects of isoproterenol and ICI 118 551 on AC activity expressing β_2AR_{wt}-$G\alpha_{s\text{-}L}$ in the absence and in the presence of NaCl. AC activity was determined as described in Section 8.2.5. Reaction mixtures contained Sf9 membranes (20 μg of protein per tube) expressing β_2AR_{wt}-$G\alpha_{s\text{-}L}$ (2.3-2.6 pmol mg^{-1}) and various additions. (A) and (B) concentration/response curves for GTP in the presence of distilled water (basal), isoproterenol (ISO) (10 μM), and ICI 118 551 (ICI) (1 μM). (C) and (D) concentration/response curves for ISO and ICI in the presence of GTP (1 μM). The experiments shown in (A) and (C) were conducted in the absence of NaCl; the experiments shown in (B) and (D) were conducted in the presence of NaCl (150 mM). Data shown are the means ± SDs of three independent experiments and were analyzed by non-linear regression.

does not exhibit a stimulatory effect on AC activity [32, 65]. Secondly, the inhibitory effects of inverse agonists on AC activity supported variously by GTP, GTPγS, or GppNHp can be examined (Figures 8.5A and 5C) [31, 32, 54, 58, 67, 118]. It should be noted that the relative inhibitory effects of ICI 118 551 on basal AC activity in Sf9 membranes expressing β_2AR_{wt}-$G\alpha_{s\text{-}L}$ are larger with submaximally stimulatory GTP concentrations (100 nM–1 µM) than with maximally stimulatory GTP concentrations (10–100 µM). Thus, for a detailed analysis of the effects of inverse agonists, GTP should be used at a submaximally stimulatory concentration. Thirdly, AC activity in the presence of GTP can be assessed in membranes expressing constitutively active GPCRs at different levels. Such studies revealed a correlation between GPCR expression level and GTP-dependent AC activity and that β_2AR_{CAM} exhibits higher constitutive activity than β_2AR_{wt} [31, 66]. Because of intrinsic differences in the AC activities of various membrane preparations, numerous membrane preparations have to be studied, so this approach is quite labor-intensive.

As has been discussed in Sections 8.2.3. and 8.2.4., constitutive activity of the β_2AR_{wt} can be assessed in the GTPase and [^{35}S]GTPγS binding assay when β_2AR_{wt}-$G\alpha_{s\text{-}L}$ fusion proteins are examined [32, 54]. The β_2AR_{wt}-$G\alpha_{s\text{-}L}$ fusion protein is also very efficient at activating AC. Intriguingly, β_2AR_{wt} exhibits a considerably higher constitutive activity in the AC assay than in the GTPase and [^{35}S]GTPγS binding assay, as assessed by the inhibitory effects of ICI 118 551 [32]. This striking discrepancy is explained by a model in which the available AC molecules in Sf9 membranes are rate-limiting for signal transduction (i.e., most or even all available AC molecules in the membrane interact with, and are efficiently activated by, β_2AR_{wt}-$G\alpha_{s\text{-}L}$ molecules, whereas the majority of the expressed β_2AR_{wt}-$G\alpha_{s\text{-}L}$ molecules have no AC partner to interact with) [32]. Indeed, it is well established that the concentration of $G\alpha_s$ molecules in cell membranes is much higher than the concentration of AC molecules [92, 124]. The problem of AC availability can be partially overcome by avoiding AC studies with membranes expressing β_2AR_{wt}-$G\alpha_{s\text{-}L}$ at high levels (>5 pmol mg^{-1}). Rather, membranes expressing β_2AR_{wt}-$G\alpha_{s\text{-}L}$ at low levels (2–3 pmol mg^{-1}) should be used for AC studies [22, 23, 32]. Even when such low-expressing membranes are used, the effects of inverse agonists in the AC assay are still larger than in the GTPase- and [^{35}S]GTPγS-binding assays [22, 23, 32, 40]. In contrast to G_s-coupled receptors, coupling of G_i-coupled receptors to AC appears to be loose [125]. These findings highlight the importance of assessing constitutive activity at multiple steps of the G protein cycle if at all possible. In addition, these data illustrate that it cannot be taken for granted that signal transfer from the G protein to the effector is linear. Rather, substantial bias may be introduced into data analysis because of nonlinear signal transfer from one signaling protein to another. Although this issue has been studied in at least some detail only for the AC-linked GPCRs, it is likely that similar nonlinearities of signal transfer are also of relevance for PLC-linked GPCRs. Thus, the assessment of different read-outs for one given GPCR may explain why inverse agonist efficacies are different among different studies and research groups [1].

As has been discussed in Section 8.2.4., Na^+ stabilizes the R state of G_i/G_o-coupled GPCRs, thereby reducing basal GTPase- and [^{35}S]GTPγS-binding signals generated by constitutively active GPCRs. Comparison of the effects of various salts on basal GTP

hydrolysis and [^{35}S]GTPγS binding in systems expressing G_i/G_o-coupled receptors clearly showed that it is the cation Na^+, and not the anion Cl^-, that accounts for the inhibitory effects of NaCl [17, 21, 73]. NaCl also reduces basal, GTP-dependent AC activity in Sf9 membranes expressing β_2AR-G$\alpha_{s\text{-}L}$ and thereby diminishes the inhibitory effects of the inverse agonist ICI 118551 (compare Figure 8.5A with 8.5B and Figure 8.5C with 8.5D) [23]. At first glance, the effects of NaCl on constitutive activity of G_i-coupled GPCRs and G_s-coupled GPCRs are similar (compare Figures 8.4 and 8.5). However, upon closer examination it turned out that in the case of the β_2AR_{wt}, it is actually the Cl^- anion, and not the Na^+ cation, that accounts for the inhibitory effect of NaCl. The order of efficacy of anions in reducing the constitutive activity of β_2AR_{wt}-G$\alpha_{s\text{-}L}$ is $I^- > Br^- > Cl^-$ [23]. In contrast, the cations Li^+, Na^+, K^+, Rb^+, and Cs^+ exhibit similar effects on constitutive β_2AR_{wt} activity when added to membranes as Cl^- salts [23]. The inhibitory effects of monovalent anions on constitutive β_2AR_{wt} activity are explained by a model in which Cl^- increases the GDP-affinity of G$\alpha_{s\text{-}L}$ and consequently reduces the efficacy of the agonist-free β_2AR_{wt} at promoting GDP/GTP exchange [23] (see also Chapter 9). From the experimental perspective for the assessment of constitutive activity of G_s-coupled GPCRs, future studies will have to control and define Cl^- concentration precisely. In addition, previous literature data will have to be reexamined for the extent to which the presence of Cl^- had an impact on constitutive GPCR activity [1]. At this time, it is unclear whether the impact of Cl^- on constitutive GPCR activity is only of technical importance for experiments with membranes or whether changes in intracellular Cl^- concentration could regulate constitutive GPCR activity *in vivo*.

In principle, the AC assay can also be used to assess constitutive activity of G_i-coupled GPCRs that mediate AC inhibition [10, 11]. Usually, the inhibitory effects of G_i-coupled GPCRs on AC activity is assessed in the presence of the direct AC activator, forskolin, to provide a sufficiently large basal signal [126, 127]. However, in comparison to AC activation by G_s-coupled GPCRs, which can be severalfold [66, 128–130], AC inhibition by G_i-coupled GPCRs is relatively small (at best 50%) [126, 127], providing only a small signal to noise ratio. This is probably the reason why constitutive activity of G_i-coupled GPCRs has not been studied in detail by use of AC activity in membranes as readout. In fact, much larger GPCR signals can be obtained by use of the steady-state GTPase assay (see Section 8.2.3) and the [^{35}S]GTPγS binding assay (see Section 8.2.4). In any case, stabilization of an R state of a G_i-coupled GPCR by an inverse agonist should result in an increase in forskolin-supported AC activity in cell membranes. Such regulation has been demonstrated for the cannabinoid 1-receptor (CB_1R), but the increase in AC activity induced by CB_1R inverse agonists is rather small (15–20%) [46]. We failed to detect an increase in forskolin-supported AC activity in Sf9 membranes expressing the constitutively active FPR1 and G$\alpha_{i2}\beta_1\gamma_2$ with CsH as inverse agonist (unpublished results), whereas inverse agonism of CsH in the GTPase and [^{35}S]GTPγS binding assays is profound [21, 29].

A procedure for the AC assay is described below; it has been successfully applied to various G_s-coupled GPCRs coexpressed with, or fused to, mammalian Gα_s in Sf9 cells [32, 41, 65, 87]. This AC procedure has also been adapted to Sf9 membranes expressing GPCRs without mammalian Gα_s [2, 32, 41, 65]. In this case, GPCRs couple to the

$G\alpha_s$-like G proteins of the insect cells, and the AC activities are considerably lower than in the presence of mammalian $G\alpha_s$. However, thanks to the extremely low blank values and high sensitivity of the method used, detection of low AC activities is not problematic. We have also applied this procedure to AC activation in S49 wild-type lymphoma cell membranes [131] and to AC inhibition in $G\alpha_s$-deficient S49 *cyc*⁻ lymphoma cell membranes [132].

Before experiments, membranes are sedimented by a 15 min centrifugation at 4 °C and 15 000 × *g* and carefully resuspended in assay buffer (12.5 mM $MgCl_2$ and 1 mM EDTA in 75 mM Tris/HCl, pH 7.4). The washing procedure is important, to remove any remaining endogenous GPCR agonists and traces of GTP that could induce an increase in AC activity. Firstly, GTP or distilled water (5 µl each) is added to tubes, followed by the addition of agonist or inverse agonist (5 µl). Then, 20 µl of Sf9 membranes (15–20 µg of protein per tube) suspended in assay buffer are added. If AC activities are very low, membrane protein can be increased up to 100–150 µg per tube without adverse effects on blank values. Tubes are kept on ice until the preincubation at 37 °C is initiated. The assay buffer added with the membranes supplies the required divalent cations and buffer for the AC assay (final concentrations: 5 mM $MgCl_2$, 0.4 mM EDTA, and 30 mM Tris/HCl, pH 7.4). Assay tubes (containing a volume of 30 µl) are placed in a heat block set at 37 °C and preincubated for 3 min before the addition of reaction mixture (20 µl) containing (final) 40 µM [α-^{32}P]ATP (3000 Ci $mmol^{-1}$; Perkin–Elmer; 1.0–2.0 µCi per tube), 0.1 mM cAMP (Sigma) to prevent degradation of the newly formed [^{32}P]cAMP, and a NTP-regenerating system consisting of 2.7 mM mono(-cyclohexyl)ammonium phosphoenolpyruvate (Sigma), 0.125 IU of pyruvate kinase (Sigma), and 1 IU of myokinase (Sigma) [133]. A mixture of mono(cyclohexyl)ammonium phosphoenolpyruvate, pyruvate kinase, myokinase, and cAMP, pH 7.4, is kept as a 3.3-fold concentrated stock solution in 375 µl aliquots at –80 °C. One aliquot of this mixture provides the components for 20 assay tubes. Blank values are obtained by processing tubes without membranes. Reactions are conducted for 20 min and are terminated by addition of 20 µl of 2.2 N HCl. Denatured membrane protein is sedimented by a 3 min centrifugation at room temperature and 15 000 × *g*. Sixty-five µl of the supernatant fluid are applied onto disposable chromatography columns (E & K Scientific, Campbell, CA, USA) filled with 1.3 g of neutral alumina (super 1, type WN-6, neutral; Sigma) each. cAMP is eluted into 20 mL scintillation counting tubes by addition of 4 mL of 0.1 M ammonium acetate, pH 7.0, into the column reservoir. Elution is completed within 20 min. The 0.1 M ammonium acetate solution is prepared from a 9 M stock solution and should be kept at 4 °C and protected from light because of its sensitivity to contamination with fungus. In preliminary experiments, and in agreement with Alvarez and Daniels [45], we found the recovery of [^{32}P]cAMP as determined by the elution efficincy of [^{3}H]cAMP to be reproducibly ∼80%. We do not routinely assess recovery of [^{32}P]cAMP any longer. [^{32}P]cAMP is determined by liquid scintillation counting after the addition of 15 mL of Ecolume cocktail. Alternatively, samples can be counted in water (Čerenkov radiation), but counting efficiency is >50% lower. Ecolume counting is recommended when assessing low AC activities (e.g., with membranes expressing GPCRs without mammalian $G\alpha_s$ or with membranes expressing GPCRs at low levels). Samples are carefully mixed and counted for 1 min. Blank values

are routinely <0.01% of total amount of [α-^{32}P]ATP added, and generally less than 0.5–1.0% of the total amount of [α-^{32}P]ATP are consumed. We keep the original [α-^{32}P]ATP stock in 20 µl aliquots at –20 °C. Under these conditions, [α-^{32}P]ATP is stable for 4 weeks (longer periods of storage time are not relevant because of the short $t_{1/2}$ of [^{32}P]) without increase in blank values.

AC activity (pmoles of cAMP formed per mg of membrane protein per min) is calculated as follows:

$$\text{pmol mg}^{-1}\ \text{min}^{-1} = \frac{(\text{cpm total} - \text{cpm blank}) \times \text{pmol ATP unlabeled} \times 1.35}{\text{cpm added} \times \text{min incubation} \times \text{mg protein}}$$

Explanations:

cpm total: Radioactivity ([^{32}P]cAMP) counted in the 65 μl aliquot analyzed by column chromatography from all tubes except blank tubes.

cpm blank: Radioactivity ([^{32}P]cAMP) counted in the 65 μl aliquot analyzed by column chromatography from tubes containing no membranes.

pmol ATP unlabeled: Absolute amount of substrate present in the tube (i.e., with 40 μM ATP, 2.000 pmoles of ATP are present in a 50 μl reaction volume). Since the specific activity of [α-^{32}P]ATP is very high (3000 Ci mmol^{-1}), the radiolabeled compound does not significantly contribute to the total amount of substrate present.

1.35: Factor correcting for the fact that only 65 μl out of 70 μl in the tube are counted and that [^{32}P]cAMP recovery is 80%.

cpm total added: The radioactivity of the [α-^{32}P]ATP added to each tube (no processing through alumina column).

min incubation: Routinely, assays are conducted for 20 min.

mg protein: Absolute amount of membrane protein added per tube. Each tube contains 15–20 μg of membrane protein, but the protein amount can be easily increased to 100 μg per tube if higher amounts of [^{32}P]cAMP should be recovered and if sufficient amounts of membrane protein are available.

8.3 Measurement of Constitutive Activity of GPCRs in Intact Cells

For a variety of GPCRs the measurement of second messengers, such as the increase or decrease in the cyclic AMP (cAMP) level or the increase in inositol phosphate (IP) formation in intact cells, is a widely used method to investigate constitutive activity (for a review see [1]) . Most of these studies were performed by expression of recombinant receptors in host cells (e.g., HEK-293, COS-7, or CHO cells, see Chapters 9–15). A major advantage of such experimental settings is based on the application of specific inhibitors of second messenger degradation (e.g., phosphodiesterase inhibitors for cAMP assays, LiCl for IP production). Therefore, the receptor-generated signal (R

to R* isomerization) can be greatly by amplified due to the continuous second messenger accumulation. The activation of PLC can additionally be monitored by the subsequent increase in intracellular Ca^{2+} concentration through interaction with fluorescent dyes or Ca^{2+} binding proteins [134] (see below).

Another approach now widely used to assess constitutive activity in living cells is the use of reporter gene assays. These take advantage of the modulation of the activity of transcription factors by many GPCR-induced signals. These factors bind directly or indirectly to regulatory elements in the promoter regions of their target genes to enhance or repress transcription. Reporter gene assays for monitoring of the activation of a variety of transcription factors and their binding to regulatory elements have been developed [135]. This includes the cAMP-response element binding protein/cAMP response element (CREB/CRE), activator protein 1/tetradecanoyl phorbol acetate-response element (AP-1/TRE), serum response factor/serum response element (SRF/SRE), or nuclear factor κ B/(NF-κB)/ NF-κB *cis*-enhancer element (NF-κB/κB) (see Figure 8.6). The influence of GPCR signaling on the regulation of transcription in these systems is subsequently monitored by constructs carrying the regulatory element in a given promoter, resulting in expression of, for instance, luciferase and β-galactosidase, for which the enzyme activity can be assayed in cell lysate (see below). A scheme showing the signaling pathways monitored by the respective reporter gene assay is given in Figure 8.6. The principal advantage of these assays is their high sensitivity, reliability, convenience, and adaptability to high-throughput measurements [136, 137]. Reporter-gene assays have been used to assist the deorphanisation of various orphan GPCRs, such as the histamine H_4-receptor (H_4R) [52, 138]. They are also helpful in characterization of virally pirated chemokine receptors, with so far unknown ligands (see Chapter 15).

Nevertheless, experiments measuring constitutive activity in living cells should be interpreted with caution for several reasons. Firstly, the presence of endogenous agonists secreted by, or diffusing from, the cultured cells may cause an increase in "basal" activity. To avoid contamination with endogenous ligands, several strategies can be implemented. If possible, repeated washings of the cells with serum-free medium or physiological buffer before the assay should be a standard procedure. If available, ligand-degrading enzymes can also be added to the culture medium. Another strategy is to exclude the presence of endogenous ligands in the medium by suitable analytical methods, such as high pressure liquid chromatography. Secondly, receptor regulatory events such as upregulation of expression or desensitization may contribute to or prevent the detection of constitutive activity. Therefore, especially in whole cells, to claim a constitutive activity of a given GPCR, researchers should show the three hallmarks of constitutive activity: 1) increased "basal" signaling, 2) inhibition of the basal activity by an inverse agonist, and 3) sensitivity of this inhibitory effect to an excess of a neutral antagonist (see Chapters 2–7). If these tools are not available for a given GPCR, the construction of mutants without constitutive activity and the coexpression of G proteins or allosteric inhibitors of GPCR signals, such as Regulator of G protein Signaling (RGS) proteins [139, 140], may be helpful for further exploration of constitutive activity. It has to be stressed, however, that the expression of the individual components (e.g., similar expression levels of the wild-type receptors and of their consti-

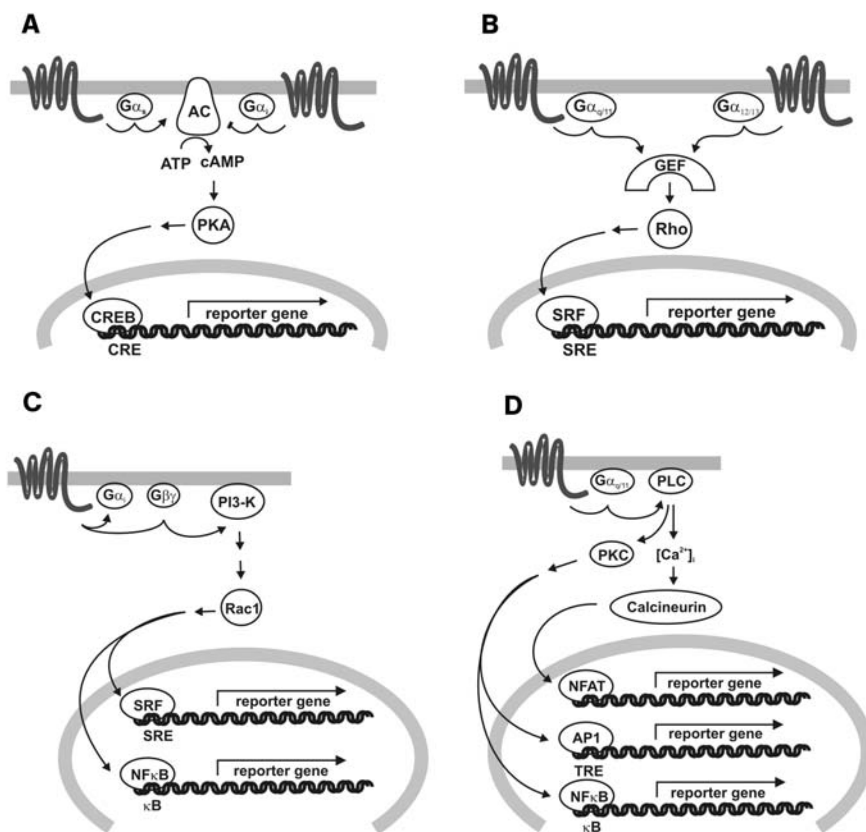

Fig. 8.6 Reporter gene assay usable to determine the activity of GPCRs. (A) Schematic view of a CREB-based reporter gene assay to determine the activity of either G_s- or G_i-coupled GPCRs. AC: adenylyl cyclase. PKA: protein kinase A. CREB: cAMP-response element binding protein. CRE: cAMP response element. (B) Schematic view of a SRE-based reporter gene assay to determine the activity of either $G_{q/11}$- or $G_{12/13}$-coupled GPCRs. GEF: guanine nucleotide exchange factor. SRF: serum response factor. SRE: serum response element. (C) Schematic view of reporter gene assays to determine the activity of GPCRs activating the PI3K-dependent signaling. PI3K: phosphoinositide-3-kinase. NF-κB: nuclear factor κB. κB: NF-κB *cis*-enhancer element. (D) Schematic view of reporter gene assays to determine the activity of GPCRs activating the PLC–PKC pathway. PLC: phospholipase C. PKC: protein kinase C. AP1: activator protein 1. TRE: tetradecanoyl phorbol acetate-response element.

tutively active or inactive mutants) have to be well controlled in the use of such coexpression systems.

We provide comprehensive procedures to determine cAMP accumulation and IP formation in living cells below. As an example for a reporter gene assay we describe the use of SRE-controlled luciferase production as a sensitive read-out for the activation of a variety of GPCR-induced signaling pathways. Finally, the use of aequorin-based assays to determine changes in intracellular Ca^{2+} concentrations is discussed.

8.3.1
Quantitative Determination of cAMP Concentrations in Cell Culture Lysates

cAMP is one of the most important second messengers, mediating a variety of physiological responses. cAMP levels in cells are tightly regulated by two enzyme families: the ACs and phosphodiesterases (PDEs) (for review see [141]). AC activity is regulated by two distinct heterotrimeric G proteins, G_s and G_i, which stimulate and inhibit the enzyme activity, respectively. Some GPCRs activating these G proteins possess constitutive activity: the G_s-coupled βARs and H_2R (see Figure 8.7A and Chapters 9, 10, and 13), for example, possess constitutive activity. The determination of cAMP accumulation in the presence of a PDE inhibitor has thus been used to characterize constitutive activity [7, 84]. To unmask the constitutive activity of a G_i-coupled receptor, such as the histamine H_3-receptor (H_3R; see Figure 8.6B) [49], however, the cellular cAMP formation has to be increased. Most often, the diterpene forskolin, a direct stimulator of AC, is used for this purpose at concentrations of 10–20 μM [142]. Constitutive activity of a G_i-coupled GPCR *reduces* forskolin-supported cAMP accumulation, whereas an inverse agonist actually *increases* depressed cAMP accumulation (see Figure 8.7B).

Different methodological approaches to the determination of cAMP concentrations in cells have been developed. Most of them are based on binding of cytosolic cAMP to a specific antibody. Various detection systems for the cAMP–antibody complex are commercially available, including systems based on radioactivity, luminescence, fluores-

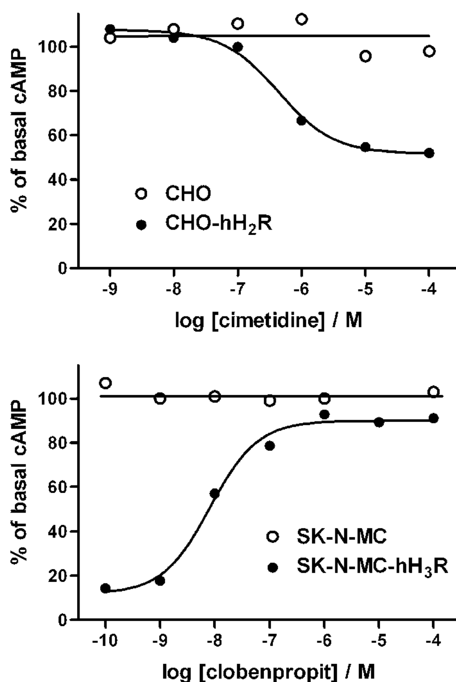

Fig. 8.7 Constitutive activity of the human H_2 and H_3 receptors determined by cAMP production in living cells. (A) Basal cAMP production was determined in CHO cells that either do not (CHO) or do stably express the human H_2R (CHO-hH_2R) when exposed to increasing concentrations of the inverse H_2R agonist cimetidine. Data are adapted from [84]. (B) Forskolin-induced (10 μM) cAMP production was determined in SK-N-MC cells that either do not (SK-N-MC) or do express the human H_3R (SK-N-MC-hH_3R) when exposed to increasing concentrations of the H_3R inverse agonists clobenpropit. Data are adapted from [49]. Cells were incubated for 10 min at 37 °C in the presence of 300 μM of the phosphodiesterase, inhibitor isobutylmethylxanthine.

cence, and colorimetry. Nearly all assay systems are competitive: cellularly formed cAMP competes with [^{3}H]cAMP and externally added cAMP for a limited amount of antibody. The most recent systems use fluorescence energy resonance transfer (FRET) technology (Bridge-it®, Mediomics, St. Louis, MO, U.S.A.) or proximity-dependent chemical energy transfer technology (AlphaScreen®, Perkin–Elmer). These systems are highly sensitive (80 to 100 fmol cAMP per well) with a "few handling steps procedure" and therefore very useful for high-throughput screens in formats up to 384-well [143]. Nevertheless, these "easy to handle kits" are rather expensive.

A simple and reliable method for determining cAMP concentrations in small scale usage is the cAMP enzyme immunoassay (cAMP-EIA, R&D). This assay is based on the competitive binding of cytosolic cAMP, extracted from cells with 0.1 M HCl, to a specific rabbit polyclonal antibody in the presence of alkaline phosphatase-labeled cAMP. The cAMP–antibody complex is captured by binding to a microplate coated with a goat anti-rabbit antibody. The amount of bound cAMP is determined by addition of the colorless alkaline phosphate substrate *p*-nitrophenyl phosphate, which is converted into *p*-nitrophenol and phosphate. The yellow *p*-nitrophenol is then detected in a microplate spectrophotometer at 405 nm. To quantify the cAMP concentrations, a standard curve is measured in parallel. Under standard conditions, a cAMP concentration between 0.8 pM and 200 pM can be detected. If lower concentrations are expected, the samples and standards can be acetylated with acetic anhydride and triethylamine, which increases the sensitivity of the assay tenfold [144].

We provide a short transfection and assay procedure for COS-7 cells below:

The day before transfection, COS-7 cells are seeded at a density of 0.2×10^6 cells per well in a 12-well plate. For transfection, 1 μg of receptor plasmid DNA plus 10 μL Polyfect® (Qiagen) per well are used according to the manufacturer's procedure. Forty-eight hours after transfection, the medium is removed and the cells are incubated in 400 μL of an assay buffer containing DMEM, 20 mM HEPES/NaOH, pH 7.4, and 100 μM of the PDE inhibitor isobutylmethylxanthine (IBMX) for 30 min at 37 °C. The incubation is stopped by removal of the assay buffer and the immediate addition of 400 μL ice-cold 0.1 N HCl. The cells are scraped off and transferred into a 1.5 mL microcentrifuge tube. After centrifugation at $20\,000 \times g$ for 15 min at 4 °C, 100 μL of the supernatant are used for the enzyme immunoassay, the manufacture's recommendations being strictly followed (cAMP-EIA, R&D).

8.3.2
Determination of Inositol Phosphate Formation in Living Cells

The most commonly used method to determine GPCR-induced phospholipase C (PLC) activity in living cells is the labeling of the cellular phosphatidylinositol 4,5-bisphosphate (PIP_2) pool by incorporation of *myo*-[^{3}H]inositol. Upon stimulation of PLC, [^{3}H]inositol-1,4,5-trisphosphate ([^{3}H]IP_3) is generated, and is subsequently degraded to [^{3}H]IP_2 and [^{3}H]IP. In the presence of 10 mM Li^+ (added as LiCl), however, the degradation of [^{3}H]IP to [^{3}H]inositol is blocked [145] and so the accumulation of [^{3}H]IP_x can be used to quantify PLC activity (see Figure 8.8).

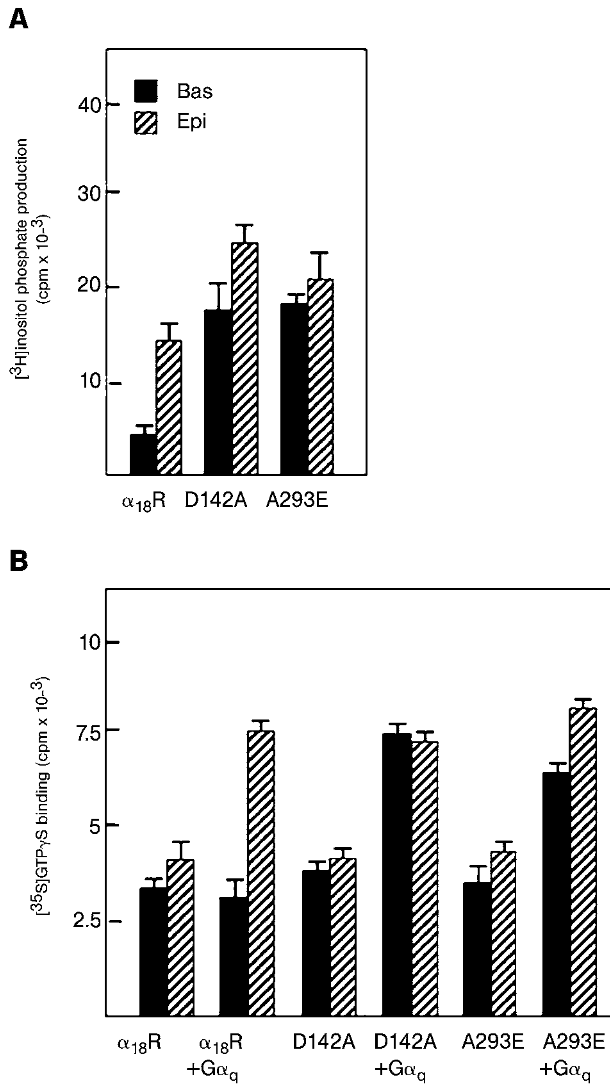

Fig. 8.8 Constitutive activity of the α_{1B} adrenoceptor and its mutants determined in living cells and membrane preparations. (A) Total inositol phosphate (IP) production was measured in COS-7 cells expressing the wild-type α_{1B}AR and its constitutively active mutants $Asp^{142}Ala$ and $Ala^{293}Glu$ either in the absence (Bas) or in the presence of 10^{-4} M epinephrine (Epi) as described in [178]. The receptor densities measured in membrane preparations were about 280, 110, and 210 fmol per 10^6 cells for the α_{1B}AR, α_{1B}AR $Asp^{142}Ala$ (D142A), and α_{1B}AR $Ala^{293}Glu$ (A293E), respectively. One million cells yielded about 200–300 μg of protein. (B) [^{35}S]GTPγS binding was measured in membranes from cells transfected with cDNAs encoding the receptors alone or in combination with $G\alpha_q$. Membranes were incubated either in the absence (Bas) or in the presence of 10^{-4} M epinephrine (Epi) for 15 min and the radiolabeled $G\alpha_q$ was immunoprecipitated with antibodies specific for its C-tail as described [30]. The data presented in this figure were kindly provided by S. Cotecchia, Lausanne, Switzerland.

The IP formation assay can be adapted to numerous cultured cells, including primary cells of various origins. Nevertheless, with regard to constitutive activity, the assay is often used in systems in which the GPCR under investigation (see, for example, Chapters 11 and 13) is stably or transiently expressed, in COS-7 cells, for example. Note that the method used for transfection in transiently expressing systems can be of considerable influence for the subsequent IP formation. For example, the use of some transfection reagents such as FuGENE 6®, Lipofetamine Plus®, or Lipofectamine 2000® can disturb the outcome of the IP formation assay, while others such as Lipofectamine® or Polyfect® are tolerated [146]. The use of electroporation also could not be recommended in this report, due to the appearance of an unacceptably high background [146]. We provide a procedure for the IP formation assay evaluated for COS-7 cells below:

Cells are cultured in 12-well plates in Dulbecco's modified Eagle medium (DMEM) containing 10% (vol/vol) fetal calf serum, 100 U mL^{-1} penicillin, and 100 μg mL^{-1} streptomycin. If the cells have to be transfected before the assay, they are allowed to grow to a density of about 50–60% confluence (see above). For transfection, up to 1 μg of receptor plasmid DNA and 10 μL Polyfect® (Quiagen) per well are used according to the manufacturer's recommendations 48 h prior to the assay. Sixteen to twenty hours before the assay, the medium is replaced with inositol-free cell culture medium containing 2 μCi mL^{-1} *myo*-[^{3}H]inositol (Amersham Biosciences). Note that regular cell culture medium contains considerable concentrations (40 μM) of *myo*-inositol, which will result in poor labeling in many cell types due to isotope dilution. The *myo*-[^{3}H]inositol-containing medium is then replaced by 500 μL Hank's buffered saline solution (HBSS) containing 10 mM LiCl to block inositol phosphatase. Receptor agonist, antagonist, or inverse agonist are also added at the desired concentration at this step, and cells are incubated for 30 min. The medium is then replaced by 250 μL ice-cold methanol. Cells are scraped off and transferred to a 2 mL microcentrifuge tube. The dish is washed with an additional 250 μL ice-cold methanol, which is also transferred to the same microcentrifuge tube. Then, 500 μL of chloroform and 250 μL of distilled H_2O are added, and the mixture is vigorously mixed. For phase separation the tubes are centrifuged for 5 min at 2000 × g in a precooled centrifuge.

In the meantime, ion-exchange columns are prepared by loading each column with 2 mL of a 1:1 (mass/mass) slurry of AG 1-X8 resin (mesh 200–400, BioRad) in H_2O. Columns are washed three times with 5 mL of distilled H_20 before use. From the aqueous (upper) phase obtained by centrifugation, 600 μL are applied to each column, which is subsequently washed with 6 mL H_20 and 5 mL of 0.1 M formic acid, 50 mM ammonium formate. Inositol phosphates are eluted with 3 mL of 0.1 M formic acid, 500 mM ammonium formate. After addition of 10 mL scintillation cocktail, such as AquaSafe® (Zinser), the production of radioactive inositol phosphates is determined by liquid scintillation counting. Normalization of the counted [^{3}H]IP radioactivity either to the protein content of the sample or to the total labeling (obtained by counting a part – e.g., 1 mL – of the wash fraction) to account for variability in cell number or labeling efficiency is recommended.

Although not frequently used to detect constitutive activity of PLC-β-stimulating GPCRs, two alternative assays to determine PLC activity should be mentioned. The

first is the determination of IP_3 production by a mass assay [147]. The advantage of this assay is that it monitors the production of IP_3 directly and that the cells under investigation do not have to be labeled with *myo*-[^{3}H]inositol. Since an excellent review and procedure for this assay is available [148], we explain the principle of the assay only briefly. Cells or tissues are denatured by treatment with trichloroacetic acid (TCA). After extraction of TCA with H_2O-saturated diethyl ether and neutralization of the samples, the aqueous phase is used for a displacement assay in a crude membrane preparation of bovine adrenal cortex. This membrane contains a sufficiently high concentration of the IP_3 receptor protein, which exhibits an equilibrium dissociation constant (K_d) of <5 nM for IP_3 [148]. The binding of a trace amount of [^{3}H]IP_3 is used in a displacement experiment with increasing concentrations of unlabeled IP_3 to obtain a standard curve. The IP_3 content of the cell or tissue extract is then quantified by the displacement of [^{3}H]IP_3 within the range of the standard curve.

The second assay can be used to follow GPCR-induced PLC-β activation by a fluorescence-based technique [149]. It uses a fusion protein consisting of enhanced green fluorescent protein and the pleckstrin homology domain of the PLCδ1 (EGFP-$PH_{PLC\delta}$) [150]. The pleckstrin homology domain of PLCδ1 ($PH_{PLC\delta}$) binds with high affinity and selectivity to PIP_2 [151]. Thus, recombinantly expressed EGFP-$PH_{PLC\delta}$ enriches over the plasma membrane through this association. Nevertheless, $PH_{PLC\delta}$ was found to exhibit an approximately 20 times greater affinity for the soluble IP_3 than for the membrane-bound PIP_2 [152]. Therefore, upon stimulation of a GPCR inducing PLC-β activation and thus IP_3 production, EGFP-$PH_{PLC\delta}$ translocates from the membrane to the cytosol. The translocation can be monitored by fluorescence microscopy and can thus be used to follow GPCR-induced IP_3 production in real time on the single-cell level [149].

8.3.3
Determination of G Protein Activation by SRF-mediated Gene Transcription

Activation of G proteins of the G_q, G_{12}, and G_i subfamilies induces the activation of RhoGTPases such as RhoA, Rac1, and Cdc42 [153, 154]. Besides many other cellular functions, the RhoGTPases stimulate SRF (serum response factor), which subsequently induces gene transcription through binding to *c-fos* SRE (see Figure 8.6) [155, 156]. Therefore, a firefly luciferase expression vector (pSRE.L) controlled by SRE.L, a derivative of the *c-fos* SRE that contains an intact high-affinity binding site for SRF but cannot bind ternary complex factor, can be used to monitor the activity of GPCRs and G proteins in transfected cells [157]. For example, GPCRs giving rise to the stimulation of G_q and G_{12} subfamily members induce RhoA activation [157, 158], whereas $G_{i/o}$ proteins induce Rac1 [159–161] (see Figure 8.6) and possibly RhoA activation through free Gβγ dimers [162]. To allow for the assay variability, a co-transfection of a second reporter gene, such as *renilla* luciferase, under control of a constitutively active promoter, is often used for normalization [163, 164]. As an example, this method has been used to characterize the constitutive activity of the virally pirated chemokine receptor US28 (see Figure 8.9 and Chapter 15). Below we give a short procedure for a dual luciferase assay used in COS-7 cells cultured on 48-well plates:

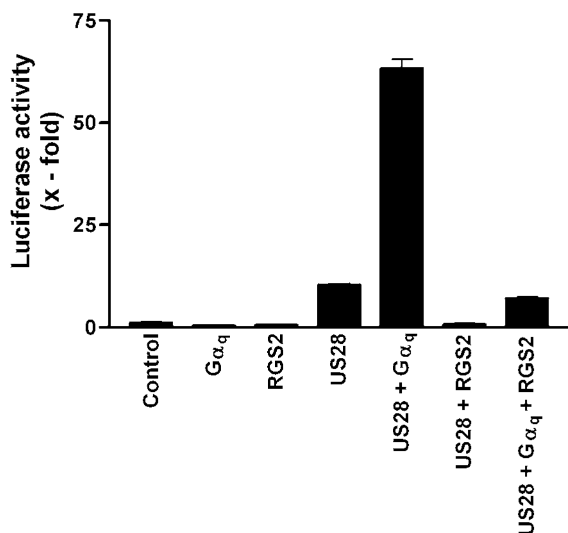

Fig. 8.9 Constitutive activity of the virally pirated chemokine receptor US28 determined by serum response factor mediated gene transcription. Luciferase production was measured in COS-7 cells transfected with control vectors alone and with the vectors US28, $G\alpha_q$, or RGS2 and their indicated combinations. The firefly and *renilla* luciferases encoding vectors, pSRE.L and pRL-TK, were present in each transfection. The induction of the firefly expression (given as the ratio of firefly:*renilla* luciferase activities (*x*-fold)) was determined 48 h after transfection by use of the dual luciferase assay kit (Promega). The data shown were kindly provided by B. Möpps, Ulm, Germany.

Cells are allowed to grow to a density of about 50–60 % confluency. For transfection, up to 250 ng of receptor plasmid DNA including 43 ng of pSRE.L plus 7 ng of the renilla luciferase-expressing plasmid pRL-TK (Promega) and 1.5 μL Polyfect® (Quiagen) per well are used according to the manufacturer's procedure. Forty-eight hours after transfection, cells are lyzed by treatment with 50 μL Passive Lysis Buffer® (Promega). Twenty-five μL of the lysate are subjected to the dual luciferase assay with 50 μL of Luciferase Assay Reagent II® and 50 μL of Stop and Glo Reagent® (Promega) for detection of firefly luciferase and *renilla* luciferase activities, respectively, according to the manufacturer's procedure. The ratio of the activity of firefly luciferase to the activity of the *renilla* luciferase in cells transfected only with pSRE.L and pRL-TK is set to 1. Any condition resulting in the induction of the firefly luciferase will therefore be expressed as x times this ratio.

By use of other reporter gene constructs, such as pNFκB-TA-Luc®, pNFAT-Luc®, pCRE-Luc®, or pAP1(PMA)-TA-Luc® (BD Biosciences), this assay can be adapted to monitor the activation of various other transcription factors (see Figure 8.6) [165].

8.3.4 Deorphanization and Constitutive Activity of GPCRs by Aequorin-based Ca^{2+} Determinations

Ca^{2+} ions are implicated in the regulation of a great variety of intracellular processes. For example, activation of GPCRs can induce rises in intracellular Ca^{2+} subsequent to the activation of PLC-β through IP_3 production [166]. Several techniques are commonly used for intracellular Ca^{2+} monitoring. On one hand, patch-clamp and Ca^{2+} selective microelectrodes give cumulative measurements of Ca^{2+} fluxes in a restricted number of cells. On the other, intracellular Ca^{2+} concentration dynamics in large populations of cells can be visualized with fluorescent dyes such as fura-2, indo-1, and fluo-3 [134]. This method is difficult to use for detection of constitutive GPCR activity, however, possibly due to continuous loss of Ca^{2+} from internal reserves. However, techniques to monitor rises in intracellular Ca^{2+} concentrations have recently been advanced by the expression of recombinant Ca^{2+}-sensitive molecular probes.

One category of assay uses the bioluminescent protein aequorin [167]. The active protein is formed in the presence of molecular oxygen from apo-aequorin and its luciferin, coelenterazine [168]. The binding of Ca^{2+} to aequorin, which contains three so-called "EF-hand structures" characteristic of Ca^{2+} binding sites, induces a conformational change resulting in the oxidation of coelenterazine. The reaction product coelenteramide is in an excited state, and blue light (λ_{max}, 470 nm) is emitted when it returns to its ground state [169]. Moreover, apo-aequorin can easily be targeted to specific subcellular compartments to enhance the efficiency [170]. As the release of Ca^{2+} from internal reserves is induced by the PLC reaction product IP_3, the aequorin bioluminescence can be used to monitor the activity of GPCRs coupling to the G_q or G_I families of G proteins [170]. The system can, however, be made usable for other GPCRs by coexpression of the promiscuous G protein α subunit $G\alpha_{16}$, which activates PLC in response to large variety of GPCRs [170, 171]. Recently, CHO cells stably expressing aequorin (CHO-aeq) have been used to screen for constitutive active mutants (CAMs) of the angiotensin II subtype 1A receptor ($AT_{1A}R$) [172]. The mutants have been transiently expressed in the CHO-aeq cells, which were screened for activity of the AT_2R receptor agonist nicotinoyl-Tyr-Lys(Z-Arg)-His-Pro-Ile-OH (CGP42112A) to induce Ca^{2+} release. As CGP42112A is very weak partial agonist at the wild-type $AT_{1A}R$, those mutants at which CGP42112A behaved as full agonist were defined as CAMs and further characterized.

The sensitivity and reliability of the system has recently been enhanced by the construction of a fusion protein of apo-aequorin with green fluorescent protein (GFP) [173], combining the Ca^{2+} sensitivity and fluorescence properties of aequorin and GFP, respectively. The new molecule exhibited an efficient intramolecular chemiluminescence resonance energy transfer, which resulted in a higher quantum yield of aequorin. Therefore, the molecule offers the possibility that calcium activities can be effectively monitored by classical epifluorescence. Both methods, co-transfection of apo-aequorin with $G\alpha_{16}$ or the GFP-apo-aequorin fusion protein, have been used to monitor GPCR/agonist interaction, thereby allowing identification of ligands for orphan receptors [174–177]. The GFP-apo-aequorin fusion protein system can efficiently

monitor changes in intracellular Ca^{2+} concentration in cells cultured on 96-well plates [175] or most probably on even smaller plate formats, even at single-cell levels [173]. Thus, it can be envisioned that the system might be used to screen for constitutive activity of, and inverse agonists for, GPCRs in a manner similar to that described above for CHO-aeq cells [172]. We therefore give below a procedure that has been used to study the $S1P_5$ receptor expressed in CHO-G5A cells, which stably express the GFP-apo-aequorin fusion protein [175]:

CHO-G5A cells are maintained in Ham's F12 medium supplemented with 10% (vol/vol) FCS, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 500 μg mL^{-1} geneticin. For calcium measurements, cells are grown on clear-bottomed, 96-well plates (Falcon) and transfected with 50 ng receptor cDNA per well by treatment with FuGENE 6® (Roche) according to the supplier's manual. Two days after transfection, cells are loaded with 5 μM coelenterazine in calcium-free HBSS for 3 h at 37 °C. At least 45 min prior to the experiments, the solution is replaced by HBSS containing 1.8 mM $CaCl_2$. Measurements are performed with a luminometer plate reader. Light generation by the calcium-sensitive probe is simultaneously recorded after ligand application for 60 to 120 min.

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II
Constitutive Activity of Selected GPCR Systems

9
Constitutive Activity of β-Adrenoceptors: Analysis in Membrane Systems

Roland Seifert

9.1
Introduction

β-Adrenoceptors (βARs) are divided into three subtypes: β_1AR, β_2AR, and β_3AR [1–3]. βARs predominantly couple to the G protein G_s, which activates the effector adenylyl cyclase (AC) [4, 5]. AC catalyzes the formation of the second messenger cAMP from ATP [6], and cAMP then modulates cell functions through activation either of cAMP-dependent protein kinase or of cyclic nucleotide-gated (CNG) cation channels [7]. The β_2AR can also couple to G_i proteins, which are involved in the regulation of several pathways including AC inhibition, and to G_q proteins, which mediate activation of phospholipase C-β isoenzymes [8–14].

Constitutive activity is the ability of a G protein-coupled receptor (GPCR) to undergo spontaneous – that is, agonist-independent – isomerization from an inactive (R) state to an active (R*) state [15–17]. In the R* state, GPCRs activate G proteins and induce effector activation (or inhibition). Agonists stabilize the R* state and increase basal G protein and effector activity, whereas inverse agonists stabilize the R state and decrease basal G protein activity (see Chapters 2 and 3). Thus, constitutive GPCR activity can be detected through an increase in basal G protein and/or effector activity relative to an appropriate control such as mock-transfected cells or through the ability of an inverse agonist to reduce the elevated basal G protein and effector activity.

Among βARs, the best studied GPCR subtype in terms of constitutive activity is the β_2AR, followed by the β_1AR [17]. Until now, only little attention has been paid to constitutive activity of the β_3AR [18, 19]. The focus on the β_2AR is explained by the fact that this GPCR has historically served as the model for GPCRs in general [1, 20]. However, given the abundance of full agonists, partial agonists, and antagonists (which are potential inverse agonists) for these GPCRs [2, 3, 19, 21], analysis of constitutive activity of the β_1AR and β_3AR is technically just as feasible as that of the β_2AR.

This chapter focuses on the analysis of constitutive activity of βARs in membrane systems. Analysis of constitutive activity of βARs in membrane systems has been very informative for analysis of models of GPCR activation and βAR coupling to various G proteins because experimental conditions can be very precisely controlled and multi-

ple steps of the G protein cycle can be analyzed under comparable experimental conditions. Most importantly, experiments with carefully washed membranes ensure the absence of contaminating endogenous agonists that may mimic apparent constitutive activity in intact cell studies (see Chapter 1) [22–24]. Specifically, an endogenous agonist present in trace amounts may induce a moderate extent of G protein activation that is blocked by the addition of an antagonist, conferring apparent inverse agonist properties to the antagonist. The advantage of ensuring the absence of endogenous agonists in experiments with membranes has been recognized for a long time [25, 26].

In addition, studies with membranes allow for control of GTP concentration, an important variable in analysis of the constitutive activity of βARs [27–29]. The stimulatory effect of GTP on basal AC activity is an accurate measure of the constitutive activity of a βAR, since it does not rely on the use of an inverse agonist [28, 30, 31]. When inverse βAR agonists are used in the AC assay, it should be kept in mind that the apparent efficacy of inverse agonists depends on the GTP concentration in the assay. Specifically, at submaximally stimulatory GTP concentrations (100 nM–1 μM), inverse agonists are more efficacious than at maximally stimulatory GTP concentrations (10 μM–1 mM). In other words, at high GTP concentrations, inverse βAR agonists tend to act only as partial inverse agonists [28].

9.2 Analysis of βAR/G_s Protein Coupling in Membranes

Figure 9.1 illustrates coupling of βARs to the G protein G_s. Like all heterotrimeric G proteins, G_s consists of an α subunit ($G\alpha_s$) and a Gβγ complex [4, 5]. In the basal state, $G\alpha_s$ tightly binds GDP. A βAR in the R* state, be it an agonist-free or an agonist-bound βAR, promotes the dissociation of GDP from $G\alpha_s$ (*step 1*). It is generally assumed that GDP dissociation from Gα constitutes the rate-limiting step of the G protein cycle [4, 5]. However, at least in the case of the retinal G protein transducin, the hydrolysis of GTP (*step 5*) can become the rate-limiting step of the G protein cycle under certain conditions [32]. If the βAR in the R* state is agonist-bound, a ternary complex consisting of agonist, βAR, and nucleotide-free heterotrimeric G_s forms (*step 2*). This ternary complex is characterized by high agonist affinity [33–35]. Both the agonist-free and the agonist-bound βAR then promote the binding of GTP to $G\alpha_s$ (*step 3*). GPCRs in the R* state thus act as guanine nucleotide exchange factors (GEFs) on G proteins. The binding of GTP to $G\alpha_s$ induces a conformational change in this subunit, resulting in dissociation of the heterotrimer into $G\alpha_{s\text{-}GTP}$ and the Gβγ complex. Moreover, the βAR uncouples from G_s. In the case of an agonist-occupied βAR, this results in a decrease in agonist affinity [33–35]. $G\alpha_{s\text{-}GTP}$ then activates the effector AC (*step 4*). Termination of G protein activation is accomplished by the GTPase activity of $G\alpha_s$ (*step 5*). Finally, $G\alpha_{s\text{-}GDP}$ and the Gβγ complex reassociate.

Historically, analysis of βARs in membranes has largely relied on measurements of AC activity (*step 4*) since cAMP formation can be monitored with high sensitivity by use of [α-^{32}P]ATP as substrate [1, 5, 6]. While agonist competition studies using radiolabeled antagonist ([^{3}H]dihydroalprenolol or [^{125}I]iodocyanopindolol) can be con-

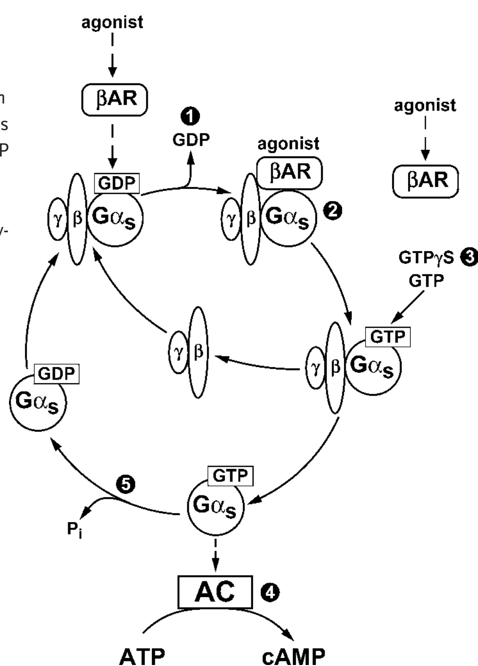

Fig. 9.1 $\beta AR/G_s$ protein coupling: G protein activation/deactivation cycle. A βAR in the R* state promotes GDP dissociation form $G\alpha_s$, which is the rate-limiting step of the G protein cycle. The circled numbers indicate the methods available for studying the G protein cycle. **1**) GDP dissociation assay. **2**) High-affinity agonist-binding assay (ternary complex formation). **3**) GTPγS binding assay. **4**) AC assay. **5**) Steady-state GTPase assay.

ducted in numerous systems expressing βARs in the fmol–subpicomol mg^{-1} membrane protein range, the detection of ternary complex formation (see Chapter 2) requires an excess of G protein relative to βAR and/or high expression levels of both βAR and G_s [31, 33, 34, 37, 38]. βAR-catalyzed GDP dissociation can be detected directly only in avian erythrocyte membrane systems [25, 39]. Experimentally, βAR-catalyzed GTP binding is difficult to monitor because the affinity of $G\alpha_s$ for GTP (~100–200 nM range) is not sufficiently high to be exploited in radioligand binding studies [28]. In addition, GTP is subject to hydrolysis, and the affinity of GDP towards G proteins is lower than the affinity of GTP [28]. In contrast, the hydrolysis-resistant GTP analogue guanosine 5'-[γ-thio]triphosphate (GTPγS) binds with very high affinity (1–5 nM range) to $G\alpha_s$ [14, 40]. Accordingly, radiolabeled GTPγS ([^{35}S]GTPγS), which is commercially available at sufficiently high specific activity (1,000–1,500 Ci $mmol^{-1}$), can be used to monitor βAR-catalyzed GDP/GTP exchange in binding studies [14, 40, 41]. In addition, the βAR-promoted steady-state hydrolysis of [γ-^{32}P]GTP to GDP and [^{32}P]P_i can be monitored to assess $\beta AR/G_s$ coupling directly at the G protein level [14, 40, 41].

It should be emphasized, however, that the GTPγS binding and steady-state GTPase assays are applicable only to selected systems: namely turkey erythrocyte membranes (GTPase) [42] and *Spodoptera frugiperda* (Sf9) insect cell membranes expressing βAR-$G\alpha_s$ fusion proteins (GTPase and GTPγS binding) [14, 40, 41]. In membranes expressing β_2AR-$G\alpha_s$ fusion proteins, βAR-catalyzed GDP dissociation can also be monitored indirectly by studying *inhibitory* effects of agonist on AC activity in the absence of GTP, reflecting formation of the GDP-free form of $G\alpha_s$ [27, 40]. GDP-free $G\alpha_s$ is less effi-

cient than the GDP-bound $G\alpha_s$ at activating AC. However, the signal-to-noise ratio of this indirect GDP dissociation assay is too small to be exploited for extensive mechanistic studies.

In βAR-$G\alpha_s$ fusion proteins, the βAR C terminus is tethered to the $G\alpha_s$ N terminus (Figure 9.2). As a result of the tether, there is a close proximity and a defined 1:1 stoichiometry between the two coupling partners [43]. In addition, the tether prevents $G\alpha_s$ from dissociating into the cytosol [44], so βAR/G_s coupling is much more efficient in fusion proteins than in conventional coexpression systems. In fact, βAR-stimulated GTPγS binding and GTP hydrolysis are barely detectable in coexpression systems, even if the signaling proteins are expressed at very high levels [31, 38]. These data indicate that, in coexpression systems, only a small portion of the expressed $G\alpha_s$ molecules actually participates in the coupling process. To this end, the fusion protein technique has been applied to the β_1AR [30, 45] and β_2AR [38], but there is no reason to assume that β_3AR-$G\alpha_s$ fusion proteins are not sensitive systems for the analysis of constitutive activity. Although Gβγ complexes are not required for fusion protein function [46], it is clear that fusion proteins can interact with Gβγ complexes [38]. In fact, the coexpression of β_2AR-$G\alpha_s$ fusion proteins with mammalian $G\beta_1\gamma_2$ complex in Sf9 insect cells enhances the apparent constitutive activity of the β_2AR, indicative of improved coupling of the agonist-free β_2AR to $G\alpha_s$ by Gβγ complexes [38].

Undoubtedly, GPCR-Gα fusion proteins provide highly sensitive model systems with which to detect ternary complex formation, GTPγS binding, effector activation, and steady-state GTP hydrolysis, and they greatly facilitate analysis of the molecular mechanisms of GPCR activation (see also Chapter 3) [35, 41, 47, 48]. However, one has to be very careful in extrapolating experimental data obtained with such fusion proteins to the *in vivo* situation. Specifically, the splice variants with β_2AR coupled to $G\alpha_s$ behave differently in terms of constitutive activity when analyzed in a fusion protein

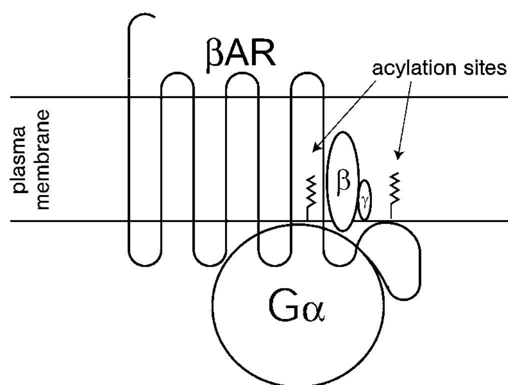

Fig. 9.2 Structure of βAR-Gα fusion proteins. The βAR N terminus faces towards the extracellular space, the βAR C terminus towards the intracellular space. The GPCR C terminus is linked to the Gα N terminus. βARs are palmitoylated at the C terminus, while Gα subunits are myristoylated and/or palmitoylated at the N terminus. Acylation is important for membrane attachment of proteins. In addition, the covalent link between GPCR and Gα prevents dissociation of Gα into the cytosol after G_α activation, which is particularly relevant for $G\alpha_s$ proteins [116, 117]. Several modifications that allow for immunological detection have been incorporated into βAR-Gα fusion proteins, specifically an N-terminal FLAG epitope and a hexahistidine tag between βAR C terminus and Gα N terminus.

system than in a coexpression system [27, 31, 38]. Additionally, it is unclear why the β_1AR and β_2AR on one hand and the structurally related histamine H_2-receptor (H_2R) on the other exhibit different patterns of constitutive activity when fused to $G\alpha_s$ splice variants [27, 30, 45, 49]. Finally, it cannot be taken for granted that only intramolecular coupling takes place in GPCR-Gα fusion proteins [14, 27]. Rather, cross-talk between various GPCR-Gα fusion protein molecules occurs [50], which may complicate data interpretation.

9.3
Development of the Concept that βARs Are Constitutively Active

The first evidence for constitutive activity of βARs was provided in a 1984 collaborative paper by the Lefkowitz and Birnbaumer groups [51]. In phospholipid vesicles containing purified G_s (at that time still referred to as N_s, N being the acronym for nucleotide-binding protein), addition of β_2AR purified from guinea pig lung resulted in a significant increase in basal steady-state GTPase activity. These data were correctly interpreted as evidence for coupling of the agonist-free β_2AR to G_s. Five years later, Murray and Keenan [25] demonstrated that propranolol, a classic β-adrenoceptor antagonist, reduced AC activity stimulated by the hydrolysis-resistant GTP analogue guanosine 5'-[β,γ-imido]diphosphate (GppNHp) in chicken erythrocyte membranes expressing the avian βAR. The stereoselectivity of the effect of propranolol and the lack of inhibitory effect of the membrane-stabilizing compound lidocaine on AC activity indicated that the effect of propranolol was mediated through the agonist-free βAR. Thus, in this pioneering study, propranolol, until then classified as a neutral antagonist, actually exhibited inverse agonistic activity. This concept was corroborated and extended by Jakobs' group in an elegant study with turkey erythrocyte membranes [26].

In 1993, Lefkowitz and co-workers generated a β_2AR mutant in which four amino acids in the third intracellular loop were replaced by the corresponding amino acids of the α_{1B}-adrenoceptor (α_{1B}AR) [52]. The rationale for those mutations was that mutations at homologous positions in the α_{1B}AR sequence induced constitutive activity (see Chapter 11) [53, 54]. When expressed in Chinese hamster ovary (CHO) cells, this β_2AR mutant caused a much higher basal (GTP-dependent) AC activity than the wild-type β_2AR (β_2AR_{wt}) expressed at comparable levels [52]. Accordingly, this β_2AR mutant is referred to as a constitutively active β_2AR mutant (β_2AR_{CAM}) [35, 55, 56]. This term, however, should not be interpreted in the sense that β_2AR_{wt} is not constitutively active; β_2AR_{CAM} just possesses higher constitutive activity than β_2AR_{wt} [35, 52, 56]. In fact, when expressed at very high levels ensuring high abundance of R*, the constitutive activity of β_2AR_{wt} is massive (see Chapter 10) [57]. The βAR inverse agonists *erythro*-DL-1-(7-methylindan-4-yloxy)-3-isopropylaminobutan-2-ol (ICI 118 551) and betaxolol were found to reduce basal (GTP-dependent) AC activity in CHO cell membranes expressing β_2AR_{CAM} [52]. In addition, various other βAR inverse agonists including timolol, labetalol, and pindolol were shown to reduce basal (GTP-dependent) AC activity in Sf9 insect cell membranes and Chinese hamster fibroblast cell membranes expres-

sing β_2AR_{wt} [58]. All these findings established the concept of constitutive activity of β_2AR_{wt} and β_2AR_{CAM}.

Early electrophysiological studies with isolated human and guinea pig cardiomyocytes and the βAR antagonists atenolol and propranolol suggested that the β_1AR is constitutively active as well (see Chapter 10) [22]. Subsequently, point mutations in the third intracellular loop of the β_1AR were shown to induce constitutive activity in this GPCR [59]. More recently, the wild-type β_1AR (β_1AR_{wt}) was found to be constitutively active as assessed by stimulatory effects of increasing β_1AR_{wt} expression levels in COS-7 cells on intracellular cAMP concentrations [24]. This concept was corroborated by stimulatory effects of GTP on basal AC activity in Sf9 cell membranes expressing β_1AR-$G\alpha_s$ fusion proteins [30] and inhibitory effects of various βAR inverse agonists including alprenolol, pindolol, atenolol, carvedilol, and propranolol on basal (GTP-dependent) AC activity in CHO cell membranes expressing non-fused β_1AR [19].

Very recently, ICI 118 551 and (±)-2-hydroxy-5-[2-({2-hydroxy-3-[4-(1-methoxy-4-trifluoromethyl-1*H*-imidazol-2-yl)phenoxy]propyl}amino)ethoxy]-benzamide (CGP 20712) were documented as inverse agonists at the β_3AR as assessed by their inhibitory effects on basal (GTP-dependent) AC activity in CHO cell membranes expressing the β_3AR [19]. It is as yet unknown whether β_3AR polymorphisms exhibit different extents of constitutive activity (see Chapter 5) [60]. Collectively, these data show that all wild-type βAR subtypes exhibit constitutive activity, that several antagonists at these GPCRs are actually inverse agonists, and that certain βAR mutants exhibit increased constitutive activity relative to their wild-type counterparts. The relative abundance of βAR inverse agonists among βAR antagonists is in accord with analogous data for other GPCRs [17, 21].

9.4
Probing Models of GPCR Activation with β_2AR_{wt} and β_2AR_{CAM} with Inverse Agonists

As is discussed in Chapters 1 and 2, the precise molecular mechanisms of constitutive activity and inverse agonist action are still unknown. This is at least in part due to the unavailability of high-resolution crystal structures of GPCRs bound to agonists and inverse agonists [61]. Studies with mammalian and insect cell membranes expressing β_2AR_{wt} and β_2AR_{CAM} have substantially advanced our knowledge of the mechanisms of actions of both agonists and inverse agonists [52, 62–64], but an important technical problem in those studies was the fact that β_2AR_{CAM} is structurally unstable and so expresses only at lower levels than β_2AR_{wt} [52, 56]. This problem was partially overcome by stabilizing β_2AR_{CAM} with a βAR ligand (agonist or inverse agonist) during the expression period [56]. As an alternative approach, fusion proteins of β_2AR_{wt} and β_2AR_{CAM} with $G\alpha_s$ were generated [35]. These fusion proteins ensured defined 1:1 stoichiometries of the coupling partners. Through analysis of steady-state GTPase activity and ternary complex formation, the properties of the two β_2AR variants could be analyzed in an expression level-independent manner. Thus, although the fusion pro-

tein system is, undoubtedly, artificial, it allows for the comparison of two receptor isoforms under clearly defined conditions.

In order to explain the phenomenon of constitutive activity, the classic *ternary complex model* was elaborated into the *extended ternary complex model* (see also Chapters 2 and 3) [15, 52, 62]. The extended ternary complex model assumes that agonists stabilize GPCRs in the R* state, whereas inverse agonists stabilize the R state. In $GPCR_{CAM}$, the basal equilibrium between R and R* is assumed to be shifted towards R* relative to $GPCR_{wt}$, with the R* states in both $GPCR_{CAM}$ and $GPCR_{wt}$ being essentially equivalent [15]. Therefore, according to the extended ternary complex model, basal G protein and effector activity should be higher with $GPCR_{CAM}$ than with $GPCR_{wt}$. Additionally, inverse agonists should exhibit higher efficacy at $GPCR_{CAM}$ than at $GPCR_{wt}$. Moreover, while GTP and GTP analogues decrease agonist affinity through disruption of the ternary complex and destabilization of R*, GTP and GTP analogues should increase inverse agonist affinity by stabilizing the R state (see Chapters 2 and 13). These predictions from the model have been confirmed experimentally. Firstly, mammalian and insect cell membranes expressing β_2AR_{CAM} exhibit higher basal (GTP-dependent) AC activity than membranes expressing β_2AR_{wt} at comparable levels [52, 56]. Secondly, the inverse agonist ICI 118 551 exhibits relatively larger inhibitory effects on basal GTPase activity in Sf9 membranes expressing β_2AR_{CAM}-$G\alpha_s$ than in membranes expressing β_2AR_{wt}-$G\alpha_s$ [35]. Thirdly, GTPγS increases the affinity of ICI 118 551 at β_2AR_{CAM}-$G\alpha_s$ [35]. Because of the lower constitutive activity of β_2AR_{wt} relative to β_2ARC_{AM}, GTPγS did not increase the affinity of β_2AR_{wt}-$G\alpha_s$ for ICI 118 551 [35].

However, the extended ternary complex model does not explain why timolol, pindolol, labetalol, and dichloroisoproterenol acted as inverse agonists to inhibit basal (GTP-dependent) AC activity in Sf9 membranes expressing β_2AR_{wt}, whereas all those ligands were partial agonists in intact Sf9 cells expressing β_2AR_{wt} [58]. A follow-up study revealed that dichloroisoproterenol actually acted stochastically as partial agonist or inverse agonist in Sf9 cells [65]. This stochastic behavior of dichloroisoproterenol was converted into inverse agonism after agonist treatment of Sf9 cells resulting in desensitization [65]. Additionally, agonist treatment of Sf9 cells increased the inverse agonistic efficacy of the weak inverse agonist labetalol but not the efficacy of the strong inverse agonist timolol [65]. With respect to GTP-dependent AC activity in Sf9 membranes expressing β_2AR_{wt}-$G\alpha_s$, dichloroisoproterenol and labetalol are partial agonists, whereas timolol is a strong inverse agonist [28]. Unlike Bouvier's group, who were working with non-fused β_2AR, we did not observe stochastic properties of dichloroisoproterenol when working with β_2AR-$G\alpha_s$ fusion proteins. In our hands, dichloroisoproterenol was always a partial agonist, never an inverse agonist [14, 27, 28, 35]. Kobilka's group found dichloroisoproterenol to be a partial agonist with respect to GTP-dependent AC activation in Sf9 membranes expressing β_2AR_{wt}, but with regard to fluorescence changes in the *N,N'*-dimethyl-*N*-(iodoacetyl)-*N'*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine-labeled purified β_2AR_{wt}, dichloroisoproterenol behaves like the inverse agonist ICI 118 551 [64]. Finally, the full agonist isoproterenol increases the apparent affinity of $G\alpha_s$ for GTPγS, whereas ICI 118 551 decreases the apparent affinity of $G\alpha_s$ for GTPγS, indicative of an *active inhibitory conformation* of the inverse agonist-bound β_2AR_{wt} with regard to the GTPγS affinity of $G\alpha_s$ [14]. Evi-

dence for an active inhibitory effect of inverse agonist-bound GPCR on G protein activation was also observed for the cannabinoid subtype 1 receptor (CB_1R) [66].

These complex effects of ligands at the β_2AR, behaving as inverse agonist in one setting and as a partial agonist in another setting, indicate that the extended ternary complex model, assuming stabilization of a single R state by an inverse agonist, is a simplification. Rather, those experimental findings are compatible with the existence of *multiple ligand-specific GPCR conformations*. During the past decade, this model has gained substantial support from numerous GPCRs from different GPCR families (see Table 1.1 in Chapter 1) [67–71]. Some of the strongest support for the existence of ligand-specific GPCR conformations comes from sophisticated fluorescence studies with purified β_2AR and with use of the antagonist alprenolol and the agonists isoproterenol, salbutamol, and dobutamine as probes [72]. These ligand-specific GPCR conformations apparently possess different capabilities with respect to G protein coupling, depending on the specific experimental conditions chosen. The precise structural basis of ligand-specific GPCR conformations is still unknown. The most rigorous strategy to approach this problem is the crystallization of β_2AR_{wt} bound to different ligands in complexation with $G\alpha_s$. Given that the crystal structures of the light receptor rhodopsin [73] and of $G\alpha_s$ [74] have already been resolved, the production of β_2AR_{wt} crystals should ultimately become possible.

9.5 Probing Models of GPCR Activation with β_2AR_{wt} and β_2AR_{CAM} and with Partial and Full Agonists

Both the *ternary complex model* and the *extended ternary complex model* predict a correlation between the efficacy of agonists in stabilizing the ternary complex and their efficacy in promoting multiple G protein activation/deactivation cycles as assessed by AC activity or steady-state GTPase activity (see also Chapters 2 and 3) [15, 33]. In fact, such correlations have been observed for the avian βAR and the human β_2AR_{wt} [34, 35]. The extended ternary complex model also predicts that the binding affinities and potencies of full and partial agonists should be greater at $GPCR_{CAM}$ than at $GPCR_{wt}$. Again, the experimental data obtained for comparisons of β_2AR_{wt} with β_2AR_{CAM} [52] and of β_2AR_{wt}-$G\alpha_s$ with β_2AR_{CAM}-$G\alpha_s$ [35] are in agreement with the predictions of the extended ternary complex model [15].

However, in contrast with the data obtained for the avian βAR and the human β_2AR_{wt} [34, 35], there is no correlation between the efficacy of partial agonists in stabilizing the ternary complex and that in promoting multiple G protein activation/deactivation cycles in Sf9 membranes expressing β_2AR_{CAM}-$G\alpha_s$ as assessed by steady-state GTPase activity [35]. Specifically, the partial agonists ephedrine, dichloroisoproterenol, and dobutamine are all more efficient than the full agonist (–)-isoproterenol with respect to ternary complex stabilization, but with regard to activation of GTP hydrolysis the opposite is true [35]. Additionally, the (+) and (–) stereoisomers of isoproterenol differ substantially from each other in terms of ternary complex stabilization at β_2AR_{CAM}-$G\alpha_s$, but in the steady-state GTPase assay the efficacies of the two agonists are

actually similar [35]. Dissociations in the effects of agonists on ternary complex formation relative to activation of multiple G protein activation/deactivation cycles are not restricted to β_2AR-Gα_s fusion proteins expressed in Sf9 insect cells. In particular, at the β_2AR_{wt} and β_2AR_{CAM} expressed in CHO cells, dobutamine surpasses salbutamol in terms of ternary complex formation but not in terms of AC activation [52]. These data indicate that certain agonists stabilize non-signaling or frozen ternary complexes, which are ternary complexes not participating in guanine nucleotide exchange. Non-signaling ternary complexes are not accommodated by the extended ternary complex model but are interpretable by the *cubic ternary complex model* and have been observed for several GPCRs other than the β_2AR (see Chapter 2) [21, 69, 70].

Another important prediction of the extended ternary complex is the assumption that the R* states of $GPCR_{wt}$ and $GPCR_{CAM}$ are essentially identical [15]. Thus, one would predict that, despite the differences in basal G protein and effector activity induced by $GPCR_{CAM}$ and $GPCR_{wt}$ [52], the maximum agonist-stimulated G protein and effector activities should be identical for $GPCR_{CAM}$ and $GPCR_{wt}$ [15]. However, in CHO cell membranes expressing β_2AR_{CAM}, the maximum isoproterenol-stimulated AC activities are, on average, higher than the corresponding isoproterenol-stimulated AC activities observed with membranes expressing β_2AR_{wt} at comparable levels [52]. Similar data have also been obtained for β_2AR_{CAM} and β_2AR_{wt} expressed in Sf9 membranes [63]. These data suggest that agonist-bound β_2AR_{CAM} exhibits higher biological activity than agonist-bound β_2AR_{wt}. In other words, agonist-bound β_2AR_{CAM} appears to be *superactive*. This was corroborated in fluorescence studies with *N,N'*-dimethyl-*N*-(iodoacetyl)-*N'*-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)ethylenediamine-labeled purified β_2AR_{CAM}. In particular, isoproterenol induces larger decreases in fluorescence in β_2AR_{CAM} than in β_2AR_{wt} [63]. The greater fluorescence changes in β_2AR_{CAM} relative to β_2AR_{wt} presumably reflect the loss of conformational constraints in the mutant GPCR, resulting not only in stronger biological activity (superactivity) but also in greater structural instability [63]. Together with the data obtained with β_2AR_{wt}-Gα_s and β_2AR_{CAM}-Gα_s fusion proteins [35], these data indicate that the R* state stabilized by a given agonist in β_2AR_{CAM} is qualitatively different from the corresponding R* state stabilized in β_2AR_{wt}.

9.6 Probing Models of GPCR Activation with β_2AR_{wt} and Purine Nucleotides

G proteins are thus named because they bind GTP [4, 5, 75], but they also bind other purine nucleotides. The order of affinity of purine nucleotides for G proteins is GTP > ITP > XTP [28, 76–79]. The starting point for the use of GTP, ITP, and XTP as tools for probing of mechanisms of GPCR activation lay in early data showing that various GPCRs coupled to the same G protein exhibited different responses in the presence of GTP, ITP, and XTP [76–78]. It should be emphasized that the intracellular concentrations of ITP and XTP under physiological conditions are probably too low to be of relevance for G protein signaling [75, 80, 81], and that XTP and ITP are thus mainly pharmacological tools to analyze G protein and receptor function.

In frog and turkey erythrocyte membranes, there is a linear correlation between the efficacies of βAR agonists in stimulating AC in the presence of GTP and steady-state GTPase [42]. In agreement with those data, a linear correlation between the efficacies of a series of partial and full agonists in stimulating AC activity in the presence of GTP and GTPase exists for β_2AR-Gα_s expressed in Sf9 membranes [28]. These data are consistent with the generally accepted G protein cycle (Figure 9.1), indicating that GTP hydrolysis is an obligatory event in G protein deactivation [4, 5]. Thus, similar correlations were expected for AC activation in the presence of ITP and ITPase activity, as well as for AC activation in the presence of XTP and XTPase. However, the correlation of ligand efficacies for AC activation with ITP and ITPase activity was considerably weaker ($r^2 = 0.644$) than the corresponding correlation for AC activation with GTP and GTPase ($r^2 = 0.990$) [28]. In the presence of ITP, for example, (–)-isoproterenol activates AC more efficiently than salbutamol, but the opposite is actually the case for ITP hydrolysis. In addition, G_s does not exhibit measurable XTPase activity although agonists efficiently activate AC in the presence of XTP [28]. These findings suggest that in the case of XTP, despite its being a non-physiological NTP, NTP dissociation rather than NTP hydrolysis must be the predominant mechanism of G protein deactivation.

A prediction of the extended ternary complex model is that the efficacies of agonists and inverse agonists in modulating G_s protein function should be independent of the NTP present [15]. This is not true, however. Most notably, ICI 118 551 in the presence of XTP acts as a weak partial agonist with respect to AC regulation in Sf9 membranes expressing β_2AR-Gα_s, whereas in the presence of GTP, ICI 118 551 exhibits the expected strong inverse agonistic activity [28]. With regard to agonists, dobutamine exhibits the predicted strong partial agonism for AC activation in the presence of GTP and ITP, whereas in the presence of XTP, dobutamine is, most unexpectedly, only a weak partial agonist [28].

The discrepancies in the effects of agonists and inverse agonists on AC activity in the presence of various NTPs and NTPase activity can be explained by assuming the existence of ligand-specific β_2AR conformations [67–71]. According to this model, ligand-specific conformational changes are passed on to G_s, resulting in ligand-specific nucleotide binding, hydrolysis, and dissociation. Obviously, this model implies that the β_2AR must be in some physical contact with the G protein during the entire G protein cycle, because steps downstream from nucleotide binding to the G protein could not otherwise be directly regulated by a GPCR. Additional evidence for continuous contact between the β_2AR and Gα_s throughout the G protein cycle was provided by the finding that ternary complex formation is not necessarily abolished even at saturating purine nucleotide concentrations [28]. It should be emphasized that evidence for continuous physical interaction between GPCR and G protein is not restricted to β_2AR-Gα_s fusion proteins, but is also observed in several non-fused systems [82]. Ultimately, ligand-specific β_2AR activation results in ligand-specific AC activation. Thus, the β_2AR conformation stabilized by ICI 118 551 is very efficient in preventing GTP binding to Gα_s, and somewhat efficient in promoting XTP binding to Gα_s. Additionally, the β_2AR conformation stabilized by dobutamine may be more efficient in promoting the binding of GTP and ITP to Gα_s than the binding of XTP. Furthermore, the β_2AR bound to (–)-isoproterenol and salbutamol may be similarly

efficient in stimulating ITP binding to Gα_s, but salbutamol-bound β_2AR may be more efficient than (–)-isoproterenol-bound β_2AR in stimulating ITP hydrolysis, resulting in a lower efficacy of salbutamol in activation of AC in the presence of ITP.

9.7 Constitutive Activity of the β_2AR Coupled to Various Gα_s Proteins

The β_2AR is a prototypical G$_s$-coupled receptor [1, 4, 5]. The G protein Gα_s exists in three isoforms: the short- and long-splice variants of Gα_s – G$\alpha_{s\text{-}S}$ and G$\alpha_{s\text{-}L}$, respectively – and the olfactory G protein Gα_{olf} [37, 83–86]. The β_2AR can couple to all three Gα_s proteins [37, 87]. In addition, insect cells express a Gα_s-like G protein to which the β_2AR can also couple [38, 58, 64]. All Gα_s isoforms activate AC. Finally, an extra large Gα_s splice variant has been described; this protein activates AC but does not couple to the β_2AR [88].

The nucleotide-binding pocket of a Gα subunit is embedded in the cleft between the *ras*-like domain and the α-helical domain [89]. The two domains are connected to one another through a linker. Intriguingly, G$\alpha_{s\text{-}S}$ possesses a shorter linker than G$\alpha_{s\text{-}L}$ between the α-helical domain and the *ras*-like domain [27, 74]. Because of the lower mobility of the domains and/or the size of the nucleotide-binding pocket, G$\alpha_{s\text{-}S}$ exhibits a higher GDP affinity than G$\alpha_{s\text{-}L}$ [27, 84]. G$\alpha_{s\text{-}S}$ also possesses a higher GDP affinity than Gα_{olf} [40]. Previous studies using coexpression systems and various Gα_s isoforms did not find differences in the coupling of the β_2AR to Gα_s isoforms [37, 87].

The discovery of the constitutive activity of the β_2AR and the development of the fusion protein technique in the early 1990s (see also Chapter 6) [52, 90] provided a novel approach for searching for differences in the coupling of the β_2AR to Gα_s isoforms. In fact, the β_2AR fused to G$\alpha_{s\text{-}L}$ exhibits a higher constitutive activity than the β_2AR fused to G$\alpha_{s\text{-}S}$ as assessed by the efficacy of the inverse agonist ICI 118 551 in the AC assay in the presence of GTP, the steady-state GTPase assay, and the GTPγS binding assay [14, 27, 29]. Moreover, GTP exhibits a much stronger stimulatory effect on AC activity in membranes expressing β_2AR-G$\alpha_{s\text{-}L}$ than in membranes expressing β_2AR-G$\alpha_{s\text{-}S}$, reflecting the enhanced prevalence of R* in the former system, resulting in increased GTP binding to G$\alpha_{s\text{-}L}$ [27, 29]. Additionally, partial agonists exhibit stronger potencies and efficacies in the AC, GTPase, and GTPγS binding assays in Sf9 membranes expressing β_2AR-G$\alpha_{s\text{-}L}$ than in membranes expressing β_2AR-G$\alpha_{s\text{-}S}$ [14, 27]. Differences in inverse agonist and partial agonist effects similar to those seen between β_2AR-G$\alpha_{s\text{-}S}$ and β_2AR-G$\alpha_{s\text{-}L}$ have also been reported between β_2AR-G$\alpha_{s\text{-}S}$ and β_2AR-Gα_{olf} [40]. These differences are explained in terms of a model in which the agonist-free β_2AR and β_2AR bound to partial agonists are more efficient in promoting GDP dissociation from the Gα_s proteins exhibiting lower GDP affinity (G$\alpha_{s\text{-}L}$ and Gα_{olf}) than from the Gα_s protein exhibiting higher GDP affinity (G$\alpha_{s\text{-}S}$) [27, 40]. As a result of these differences in GDP affinity, at any given time, a larger fraction of the β_2AR molecules fused to G$\alpha_{s\text{-}L}$ or Gα_{olf} is in the R* state, promoting G$_s$ protein activation, than is the case for the β_2AR molecules fused to G$\alpha_{s\text{-}S}$. Thus, since G$\alpha_{s\text{-}L}$ and Gα_{olf} confer the hallmarks of constitutive activity to the β_2AR without a structural (muta-

tional) change in the receptor itself, the β_2AR fused to $G\alpha_{s-L}$ or $G\alpha_{olf}$ possesses a higher *apparent* constitutive activity than the β_2AR fused to $G\alpha_{s-S}$. Other examples of the impact of G proteins on constitutive GPCR activity are discussed in Chapter 6.

The hallmarks of high constitutive activity in β_2AR-$G\alpha_{s-L}$ in Sf9 membranes are abrogated by monovalent anions in the order of efficiency $I^- > Br^- > Cl^-$ [29]. The effects of anions were observed at concentrations between 50–150 mM. While the effects of Cl^- on constitutive activity could be of physiological relevance, I^- and Br^- are certainly only of significance as pharmacological tools. Presumably, monovalent anions increase the GDP affinity of $G\alpha_{s-L}$ more than the GDP affinity of $G\alpha_{s-S}$ [29, 91] As a result of the increase in the GDP affinity of $G\alpha_{s-L}$, the agonist-free β_2AR is now less efficient in promoting GDP/GTP exchange at $G\alpha_{s-L}$, while the efficiency of the agonist-bound β_2AR in promoting nucleotide exchange is increased. Regardless of the physiological relevance of the effects of anions of β_2AR-$G\alpha_{s-L}$, those studies indicate that detection of constitutive activity of the β_2AR in membrane systems is strongly dependent on the ionic composition of the incubation medium. Finally, it must be emphasized that the effects of *monovalent anions* on constitutive activity of the *G_s-coupled β_2AR* are distinct from the well established effects of *monovalent cations* on *G_i/G_o-coupled GPCRs* (see also Chapter 8) [17].

The apparent constitutive activity of β_2AR-$G\alpha_{s-L}$ is also sensitive to the pH value of the incubation medium. When assessed in the GTPase assay, the inhibitory effects of ICI 118 551 at pH 6.5 are much larger than at pH 8.0 [92]. Thus, R to R* isomerization is facilitated by protonation. One of the target amino acids for protonation is Asp^{130}, which is homologous to Asp^{142} in the α_{1B}AR. Protonation of Asp^{142} had previously been linked to GPCR activation (see also Chapter 11) [93].

The differences in apparent constitutive activity of the β_2AR fused to $G\alpha_s$ isoforms [14, 27, 29, 40] initiated a follow-up study in which the β_2AR was co-expressed with $G\alpha_{s-S}$ and $G\alpha_{s-L}$ in Sf9 cells [31]. Surprisingly, the GPCR exhibited high constitutive activity regardless of the $G\alpha_s$ splice variant with which the β_2AR was co-expressed. In other words, the β_2AR co-expressed with $G\alpha_{s-S}$ or $G\alpha_{s-L}$ exhibited properties similar to β_2AR-$G\alpha_{s-L}$ [27, 31]. One explanation for this striking discrepancy between co-expression systems and fusion protein systems could be that fusion constrains the flexibility of $G\alpha_{s-S}$ to a greater extent than that of $G\alpha_{s-L}$, so that small biochemical differences between the two $G\alpha_s$ isoforms observed in the non-fused form become exaggerated upon fusion [41, 84]. Thus, the physiological relevance of the increased apparent constitutive activity of the β_2AR fused to $G\alpha_{olf}$ and $G\alpha_{s-L}$ relative to the β_2AR fused to $G\alpha_{s-S}$ is elusive.

9.8
Probing Models of GPCR Activation with β_2AR Coupled to Various Classes of G proteins

The β_2AR does not couple only to G_s proteins, but also to G proteins of the G_i and G_q families [8–14]. These data prompted us to examine the constitutive activity of the β_2AR-Gα_{i2}, β_2AR-Gα_{i3}, β_2AR-Gα_q, and β_2AR-Gα_{16} fusion proteins expressed in Sf9 membranes with use of the GTPγS binding assay as read-out [14]. Overall, the inhibitory effects of ICI 118 551 on GTPγS binding to these fusion proteins were much smaller than for β_2AR-G$\alpha_{s\text{-}L}$ [14]. Additionally, the potencies and efficacies of the full agonist isoproterenol and of the partial agonists salbutamol, dobutamine, ephedrine, and dichloroisoproterenol at the β_2AR-Gα_{i2}, β_2AR-Gα_{i3}, β_2AR-Gα_q, and β_2AR-Gα_{16} fusion proteins were generally lower than at β_2AR-G$\alpha_{s\text{-}L}$ [14]. Thus, there is no evidence for constitutive activity of the β_2AR fused to Gα_i and Gα_q proteins. In agreement with the data on fusion proteins, there is no evidence for constitutive activity of the β_2AR coupled to G_i proteins in cardiac myocytes. This conclusion was reached by studying the effect of pertussis toxin on basal signaling [94]. Pertussis toxin ADP-ribosylates Gα_i proteins and thereby blocks the coupling of agonist-free GPCRs to G_i proteins, resulting in a decrease in basal G protein activity [95].

While the analysis of partial agonists did not provide evidence for constitutive activity of β_2AR-Gα_i and β_2AR-Gα_q fusion proteins globally, a more detailed analysis of the effects of partial agonists on β_2AR-Gα_i and β_2AR-Gα_q fusion proteins provided valuable insights into the mechanisms of action of those ligands. The order of efficacy of ligands in activating GTPγS binding to β_2AR-Gα_{i2} was isoproterenol $>>$ salbutamol $>$ dobutamine $>$ ephedrine $\sim$ dichloroisoproterenol, while the rank order of efficacy at β_2AR-Gα_{i3} was isoproterenol $>>$ salbutamol $\sim$ dobutamine$>$ ephedrine $>$ dichloroisoproterenol [14]. The corresponding order of efficacy at β_2AR-Gα_{16} was isoproterenol $>$ salbutamol $>>$ dobutamine $\sim$ ephedrine $>>$ dichloroisoproterenol, and at β_2AR-Gα_q the order of efficacy was isoproterenol $\sim$ salbutamol $>>$ dobutamine $\sim$ ephedrine $>>$ dichloroisoproterenol. There are also large differences in the orders of potency of agonists at the various β_2AR-Gα fusion proteins. These data are compatible with the existence of ligand-specific receptor conformations. The various ligand-specific β_2AR conformations differ from each other in their ability to activate various G proteins, a process known as agonist-induced stimulus trafficking [71]. During the past ten years, ligand-specific activation of G proteins was also reported for various other receptors in non-fused systems [71], indicating that the data obtained with the β_2AR-Gα fusion proteins are not an artifact of the fusion.

9.9
Comparison of the Constitutive Activities of the β_1AR and the β_2AR

There is longstanding interest in defining the functional similarities and differences between the β_1AR and the β_2AR [96–103], an issue complicated by the fact that that the β_1AR exists in two polymorphisms that may differ from each other in their biological activities. Specifically, the originally cloned Gly^{389}-β_1AR possesses an allele frequency of $\sim$25 %, whereas the Arg^{389}-β_1AR possesses an allele frequency of $\sim$75 % [104, 105]. It

has been suggested that Arg^{389}-β_1AR may be much more efficient than Gly^{389}-β_1AR in stabilizing the ternary complex and activating AC [105]. Since Arg^{389}-β_1AR was identified more than a decade later than Gly^{389}-β_1AR, most studies have actually been performed with Gly^{389}-β_1AR.

Studies with isolated human and guinea pig cardiomyocytes suggested that the β_1AR exhibits constitutive activity (see also Chapter 10) [22]. However, when expressed in human embryonic kidney (HEK-293) cells, the human β_1AR exhibited little if any constitutive activity [59]. Upon separate expression in cardiomyocytes from β_1/β_2AR double knock-out mice, the β_2AR exhibits constitutive activity, but the β_1AR does not [102]. At comparable expression levels in COS-7 cells, the β_2AR is more efficient than the β_1AR in inducing an increase in basal cAMP accumulation [24], indicating that the β_2AR has higher constitutive activity than the β_1AR. Evidence for constitutive activity of the β_1AR was also obtained in cardiac tissue from mice overexpressing the β_1AR, but in comparison with cardiac tissue from mice overexpressing the β_2AR, constitutive activity of the β_1AR appears to be small [24, 57]. Constitutive activity of the β_1AR and the β_2AR was also assessed by comparing the effects of a series of βAR antagonists (and, therefore, potential inverse agonists) on basal (GTP-dependent) AC activity in CHO cells expressing the two GPCRs at comparable levels. The basal AC activities were similar for the β_1AR and the β_2AR [19]. Additionally, atenolol, carvedilol, metoprolol, bisoprolol, propranolol, CGP-20712, and ICI 118 551 exhibited similar inverse agonistic efficacy at both βARs [19], indicative of similar constitutive activity of the β_1AR and β_2AR. When compared as fusion proteins with $G\alpha_{s-L}$, Gly^{389}-β_1AR and β_2AR exhibit similar constitutive activity as assessed by the stimulatory effect of GTP on basal AC activity [28, 30]. Moreover, there is no difference in constitutive activity of Gly^{389}-β_1AR and Arg^{389}-β_1AR fused to $G\alpha_{s-L}$ [30, 45].

Collectively, these data show substantial differences in the constitutive activity of the β_1AR in comparison to the constitutive activity of the β_2AR in various experimental settings. Overall, constitutive activity of the β_1AR does not manifest itself as consistently as constitutive activity of the β_2AR. Future studies will have to determine which factors account for the differences in constitutive activity of the two βAR subtypes under various experimental conditions. Possible factors could be differential distribution of βAR subtypes and G_s proteins in membrane compartments and differential interaction of βAR subtypes with G protein βγ complexes [106–109].

9.10 Conclusions

Studies with membranes expressing β_2AR_{wt} and β_2AR_{CAM} have provided valuable insights into the molecular basis of constitutive GPCR activity, the mechanism of action of inverse agonists, and mechanisms of GPCR activation in general. Collectively, the data discussed in this chapter suggest that a multi-state model of GPCR activation, assuming ligand-specific GPCR conformations, is currently the most appropriate model to conceptualize the mechanisms of GPCR activation [71]. In order to corroborate this model further, crystal structures of GPCRs bound to various ligands in the complex with G proteins are urgently needed [61].

It is well documented that the constitutive activity of GPCRs is altered by structural changes in the receptor protein, such as those brought about by artificial and natural mutations, receptor polymorphisms, receptor editing, and receptor splicing [17, 110–112]. Many of those structural changes are discussed in this book (see Chapters 4, 5, 10–15). This chapter shows that, in addition to structural changes in GPCRs, the type of G protein, the ionic composition, the pH, the type of purine nucleotide, the concentration of purine nucleotide, and the fusion of a GPCR to Gα can all have profound effects on the apparent constitutive activity of GPCRs. The data discussed here document that constitutive activity of GPCRs is not a static receptor property but a process regulated by many exogenous factors. Future studies will have to address the role of regulators of G protein signaling (RGS) proteins and effector systems such as AC and PLC on constitutive GPCR activity. Researchers should carefully control the variables discussed in this chapter when designing experiments on constitutive GPCR activity. Some of the variables discussed here may explain discrepancies in the literature regarding the extent of constitutive activity of a given GPCR in various studies and/or laboratories (for a review see [17]). Finally, it will be important to examine whether any of the variables shown to regulate constitutive GPCR activity *in vitro* are of physiological relevance (see Chapter 7).

The data discussed here may also have therapeutic implications. Numerous of the inverse agonists for the β_1AR and β_2AR, including metoprolol, bisoprolol, carvedilol, and atenolol, are clinically used for the treatment of cardiovascular disorders, including chronic heart failure [113–115]. Evidence is beginning to accumulate that inverse agonism at the β_1AR is of clinical relevance for the treatment of chronic heart failure (see also Chapter 7) [24, 115]. Future clinical studies will have to compare carefully the effects of inverse agonists, neutral antagonists, and weak partial agonists at βARs in cardiovascular disorders.

It is also clear that partial β_2AR agonists activate various G proteins in a ligand-specific manner. The differences in G protein specificity of the partial agonists examined so far are certainly too small to be exploited therapeutically. However, systematic drug development programs, combining classic structure/activity assessments of compounds, molecular modeling, and high-throughput screening encompassing known and novel classes of compounds may ultimately yield (partial) GPCR agonists that selectively activate a single G protein pathway.

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10
Constitutive Activity of β-Adrenoceptors: Analysis by Physiological Methods

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10.1
Introduction

The βARs are transmembrane, G protein-coupled receptors: all three subtypes (β_1AR, β_2AR, and β_3AR) may exist in cardiomyocytes, although only the β_1- and β_2ARs couple to adenylyl cyclase (AC) to increase contraction [1]. The β_1AR is expressed at higher levels than the β_2AR, often more than fourfold, within the ventricular membrane [2]. Both βARs couple to G_s, but β_2ARs can also couple to G_i in tissues including rat ventricle [3]. The primary pathway mediating βAR-G_s effects is AC/cAMP, the final effect of which is largely mediated by cAMP-dependent protein kinase A (PKA). PKA produces increases in contractile force and rate of contraction and relaxation of the heart by phosphorylation of protein targets including phospholamban, troponin I, the L-type Ca^{2+} channel, and the sarcoplasmatic reticulum Ca^{2+} release channel. However, it should be remembered that non-cAMP-dependent effects of βAR stimulation have been periodically reported [4–6]. The βARs and ACs co-localize in microdomains within cells, such as caveolae or other lipid rafts. This form of compartmentation of the components of the signal transduction pathway within the membrane increases the efficiency of communication between different proteins [7, 8]. In resting cells, β_1ARs have been located both within caveolae [9] and in non-caveolar plasmalemmal membranes and internal membranes, whereas β_2ARs are confined to caveolae [10]. Once occupied by agonists, β_2ARs translocate out of caveolae into clathrin-coated pits for internalization [10, 11]. Less efficient coupling of β_2ARs than of β_1ARs to AC in rat neonatal cardiomyocytes is attributed to the sequestration of β_2ARs from their effectors [12]. In addition to providing close assembly of the reaction partners to facilitate coupling, caveolae also co-localize regulatory molecules. In particular, caveolin appears to exert negative regulation on cAMP formation, because disruption of caveolae formation by removal of cholesterol from the membrane actually enhances agonist-stimulated cAMP formation [10].

10.2 Constitutive Activity and Inverse Agonism: Definition and Detection

Constitutive activity and inverse agonism are interrelated concepts, each of which is needed to define the other. Both can be explained in terms of the *extended ternary complex model* of ligand/receptor interaction (see Chapter 2), since this proposes an equilibrium between an inactive conformation of the receptor (R) and an active form (R*). Only the active form can couple to the appropriate G protein (R*G), and thus transduce an effect. However, ligands can stabilize, and therefore effectively increase, the concentrations of either R or R*. Conventional agonists stabilize R*, thus increasing the concentration of the active form and activating (or, less frequently, inhibiting) downstream pathways. Constitutive activity arises when receptor number is high relative to the sensitivity of the transduction system. Since R and R* are in equilibrium, a large amount of total receptor will imply a large amount of R*, and this may reach levels sufficient to produce a physiological effect even before the increase due to agonist binding. Clearly, the crucial factors are the sensitivity of the transduction system and how it is measured.

To take the β_1AR in the cardiomyocyte as an example, detection of constitutive activity through an increase in cAMP may or may not have a different threshold level from measurement of contraction. In the ventricular cardiomyocyte the thresholds differ, since basal contraction is not affected by cAMP antagonists [13, 14] and blockade of cAMP breakdown is required to raise levels sufficiently to stimulate contractility. Cardiomyocytes from the sino-atrial node, however, do show tonic support of basal contraction by cAMP [15], so contraction would be at least as sensitive a readout as cAMP accumulation. Contraction could even be more sensitive, since effects of cAMP will be multiplied by a number of cascades to produce the final effect, but this ultimately depends on the system of measurement. Gene transcription by the cAMP response element (CRE) requires considerably lower (but more sustained) cAMP turnover and could again be a more sensitive indicator of constitutive activity than cAMP levels themselves (see also Chapter 8) [16]. Additionally, GPCRs can signal by a number of pathways, each of which may have a different capacity to display the effects of constitutive activity. Membrane-delimited pathways of ion channel activation are observed with the βARs [17], and different susceptibilities to constitutive activation may underlie the discrepancy between L-type Ca^{2+} channel activity and contraction described below. Physiological consequences of constitutive activity of βARs are, therefore, difficult to predict.

Inverse agonism occurs when ligands bind with higher affinity to the R state than to the R* state and shift the equilibrium between R and R* towards the inactive form. Therefore, in the presence of inverse agonists, constitutive activity of receptors is reduced and G protein activation decreased. The detection of inverse agonism depends even more critically on the measurement conditions, since there must be sufficient difference between the basal and constitutively active state to allow the detection of the decrease by the inverse agonist. Additionally, not every inverse agonist will completely abolish all constitutive activity; their "efficacy" will depend on the relative affinity for R and R*. An inverse agonist may be partial, with an efficacy between that of a full in-

verse agonist and a neutral antagonist (which binds equally to R and R*). Absence of an observable decrease in activity cannot be taken as evidence that a compound does not possess inverse agonism [18]. This must be shown under conditions where a known inverse agonist does have an effect. In turn, the action of the inverse agonist must be blocked by a known neutral antagonist at the same receptor under the same conditions, to exclude alternate effects (as are observed for the β_2AR with some antagonists; see below). Both thermodynamically and intuitively, it would be predicted that neutral antagonists, in which binding to R and R* are equal, should be outnumbered by either agonists or inverse agonists. In a survey of the literature, it was shown that 322 out of 380 antagonists (at 73 GPCRs) are actually inverse agonists and only 58 satisfied the criteria for a neutral antagonist [18].

Having described the theoretical properties relating to constitutive activity and inverse agonism, we will now consider how this translates into the physiological effects of β_1- and β_2ARs in the heart. For both β_1- and β_2ARs the situation is complicated by the presence of agonist binding at a site separate from the principal catecholamine site that may cause allosteric activation [19] and, in the case of the β_2AR, by coupling through two different G protein pathways. Finally, interactions between the β_1- and β_2ARs themselves can produce new pharmacological profiles.

10.3 β_1-Adrenoceptors

10.3.1 Constitutive Activity of Overexpressed β_1ARs

The phenotypes of transgenic animals overexpressing the cardiac β_1AR demonstrate an early and severe cardiomyopathy [20]. *In vivo* effects can always be attributed to an increased response to endogenous transmitters, rather than to constitutive activity of the receptor itself. Constitutive activity of the β_1AR was more convincingly demonstrated by the increased basal rates of contraction of atria isolated from these animals, retained even after *in vivo* treatment with reserpine to deplete catecholamines. Inverse agonism and neutral antagonism was clearly demonstrated on the atrial strips through the use of a range of βAR antagonists. However, expression of the β_1AR in COS-7 cells resulted in minimal increases in cAMP levels, and the reduction in activity by the inverse agonist (±)-2-hydroxy-5-[2-({2-hydroxy-3-[4-(1-methyl-4-trifluoromethyl-1*H*-imidazol-2-yl)phenoxy]propyl}amino)ethoxy]benzamide monomethanesulfonate (CGP 20712A) was also minor [20]. Similarly, *in vitro* overexpression of the human β_1AR in cardiomyocytes from transgenic mice lacking both β_1- and β_2ARs did not produce any increases in basal contractility or cAMP levels, and CGP 20712A was without effect on these parameters [21]. This may be a system-dependent effect, since we have been able to observe clear constitutive activation of contraction after transfection of adult rat ventricular cardiomyocytes with the same β_1AR adenovirus [22]. The magnitude of the increase in basal contraction was dependent both on the concentration of virus used and on the time after exposure, and correlated well with the increase in

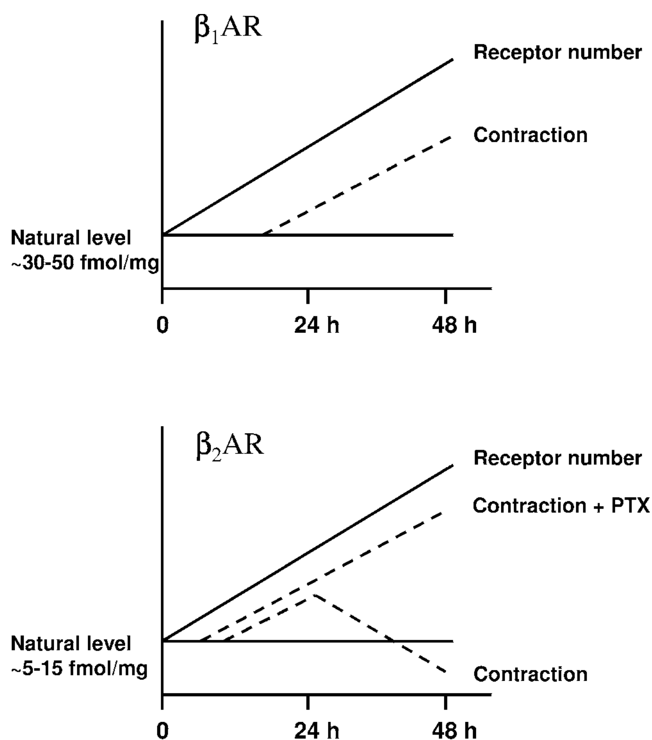

Fig. 10.1 Effects of adenoviral overexpression of β_1- and β_2ARs in adult rat ventricular cardiomyocytes. In each case, receptor number increases linearly with time after adenoviral transfection (abscissae). For β_1ARs, there are times and/or concentrations where receptor number is increased but contraction is not affected. This is presumably because cAMP (or L-type Ca^{2+} channel activation) has not yet reached the threshold for stimulation of contraction. For β_2ARs, there is an initial increase in contraction at 24 h, but by 48 h the basal amplitude is below that of control cardiomyocytes (cultured but untransfected, or transfected with control GFP virus). Treatment of cardiomyocytes with pertussis toxin (PTX), to prevent coupling of the β_2AR to G_i proteins, restores the constitutive activation of contraction. This implies that the overexpressed β_2ARs are producing both constitutive activation of contraction through G_s and constitutive depression of contraction through G_i. Time-dependent effects may indicate either differences in threshold for the two effects or a cAMP-dependent switch of the β_2AR from G_s to G_i coupling.

β_1AR expression level measured by radioligand binding (Figure 10.1). One complicating factor is the increase in cardiomyocyte death during culture after exposure to the β_1AR (but not control) adenovirus. The propensity of the β_1AR to trigger apoptosis (and necrosis) has been widely reported [6, 23], and probably underlies not only the effects on isolated cardiomyocytes but also the cardiomyopathy in the transgenic mouse. We suggest that this may explain the negative effects in the *in vitro* transfection of the β_1/β_2AR double KO mouse, with the most strongly expressing cardiomyocytes being selectively lost. Alternatively, the development of these mice without β_1- and β_2ARs might have disrupted the development of the subcellular compartments in which they would be expected to localize.

10.3.2
Is there any Evidence for a Physiological Effect of Constitutively Active Receptors in Normal Cardiomyocytes?

In most mammalian species, β_1AR stimulation increases activation of cardiac voltage-dependent L-type Ca^{2+} channels through PKA-mediated phosphorylation [24–26]. Early work in isolated guinea pig and human cardiomyocytes [27] suggested that the β_1AR-selective antagonist atenolol does indeed behave as an inverse agonist by several criteria (Figure 10.2): peak L-type Ca^{2+} current (I_{Ca}) was reversibly reduced by atenolol and by the active stereoisomer (–)-propranolol, while (+)-propranolol was ineffective. The effect of atenolol on I_{Ca} was concentration-dependent; the half-maximum effect was in good agreement with the affinity of atenolol for βARs. However, it was not examined whether the action of atenolol on I_{Ca} was blocked by a neutral antagonist, as would be required for the complete definition of an inverse agonist (see above). Furthermore, constitutive activity of the "empty" (agonist-free) receptors had to be amplified by stimulation with the direct AC activator forskolin in order to give a clearly detectable read-out. In the absence of forskolin, significant reduction of I_{Ca} required much higher atenolol concentrations (10 μM), at which nonselective effects cannot be excluded. Interestingly, in dog ventricular cardiomyocytes without prior amplification of signal transduction pathway, atenolol (1 μM) failed to reduce I_{Ca} whereas the selective β_1AR antagonist CGP 20712A significantly and reversibly reduced I_{Ca} by about one third of control [28]. A small inverse agonist action with CGP 20712A was also detected in rat ventricular cardiomyocytes [29].

Although constitute activity of cardiac β_1ARs and inverse agonist action of several β_1AR-selective antagonists were readily detectable when L-type Ca^{2+} current was used as a read-out of the signal transduction pathway, we failed to observe similar effects on cell shortening of guinea pig ventricular cardiomyocytes stimulated with forskolin [30]. Five minutes of exposure to atenolol (10 μM) in forskolin-treated cardiomyocytes reduced electrical field stimulated cell shortening in some cells, but on average the mean reduction to $79.9 \pm 3.8\%$ ($n = 4$) of pre-drug control did not reach the level of statistical significance when tested against run-down within the 5 min period in time-matched controls exposed to forskolin only ($89.9 \pm 4.1\%$, $n = 9$). In the absence of forskolin, atenolol was ineffective ($105.9 \pm 6.9\%$ of control, $n = 5$). For comparison, the muscarinic acetylcholine receptor agonist carbachol (10 μM) completely reversed forskolin-induced augmentation of contractility, in that it decreased cell shortening to $55.2 \pm 7.4\%$ of control ($n = 11$) in the presence of forskolin, as compared to no effect ($104.8 \pm 4.2\%$, $n = 11$, $P < 0.001$) in its absence (unpublished results).

We also failed to detect inverse agonism of atenolol in forskolin-stimulated papillary muscle or in left atria from reserpine-treated guinea pigs. In contrast, Böhm's group classified metoprolol, nebivolol, and bisoprolol as inverse agonists with force of contraction as read-out parameter in human atrial and ventricular trabeculae [31]. These effects were detected both in the presence and in the absence of forskolin to facilitate coupling of β_1AR to AC and were reversed by bucindolol (a less effective inverse agonist), to rule out involvement of endogenous catecholamines. In cardiac tissue from reserpinized rats, almost all βAR antagonists tested (with the exception of pindolol)

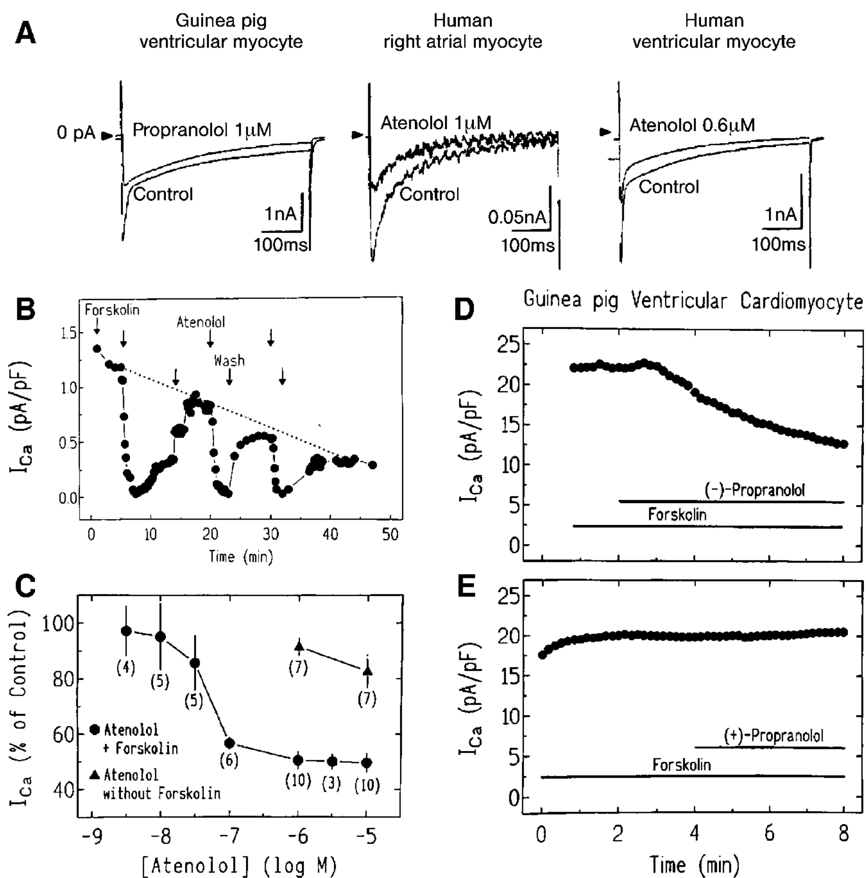

Fig. 10.2 Evidence for inverse agonism of (±)-atenolol and (–)-propranolol at β_1ARs with sarcolemmal Ca^{2+} channels as effector system in guinea pig and human cardiomyocytes. A) L-type Ca^{2+} current (I_{Ca}) traces in response to clamp steps from –40 mV holding potential to 0 mV in the presence of forskolin (0.5 μM) before (control) and after 5 min exposure to the antagonists indicated. B) Time course of peak I_{Ca} during repeated exposure and wash out of (±)-atenolol (1 μM) in a forskolin-stimulated human ventricular cardiomyocyte. The dotted line is the extrapolated current "run-down". C) Concentration dependence of the inverse agonism of atenolol on I_{Ca} in guinea pig ventricular cardiomyocytes pretreated without (triangles) and with (closed circles) forskolin. D, E) Time course of the effects of (–)-propranolol and (+)-propranolol (1 μM each) on forskolin-stimulated I_{Ca} in a guinea pig ventricular cardiomyocyte. Data adapted from Mewes et al., 1993 [13], with permission of the publisher.

exhibited inverse agonism, which was most prominent in right atrial and virtually absent in left ventricular tissue [32]. Therefore, detection of inverse agonism clearly depends not only on the drug involved but also on the conditions and the cardiac tissue investigated.

10.3.3 Substates of the β_1AR: the Putative β_4AR

An atypical βAR effect was first observed with certain βAR ligands with partial agonist activity (e.g., pindolol and CGP 12177A [(±)-4-(3-*tert*-butylamino-2-hydroxypropoxy)-benzimidazol-2-one]), which block the effects of catecholamines in human heart tissue with high affinity at β_1- and β_2ARs, but produce cardiostimulant effects (positive inotropy, lusitropy, and chronotropy) at concentrations ~100 times higher [33, 34]. The cardiostimulant effects of these "unconventional" partial agonists are relatively resistant to blockade by classical β_1AR antagonists, including propranolol, but are blocked with moderate affinity by bupranolol and carvedilol [33, 35, 36]. Initially, a pharmacologically distinct G_s-coupled receptor, the 'putative' β_4AR, was proposed to mediate these effects [37, 38]. In common with the activation of 'classical' β_1ARs, β_4AR agonists produce positive inotropy, chronotropy, and lusitropy and effect signal transduction in human and rat heart by a G_s/AC pathway increasing cAMP and activation of PKA [37, 39]. [^{3}H]-CGP 12177A labels a site thought to mediate the cardiostimulant effects of CGP 12177A in human [40] and rat [41, 42] atria and ventricles. Catecholamines compete stereoselectively at the receptor in radioligand binding studies with low affinity [41]. Agonist-specific differences in antagonist affinity showed two different affinity states of the recombinant β_1ARs in cell lines [43] and in ferret heart preparations [35].

Various lines of evidence have now shown that the β_4AR is in fact an alternate state or conformation of the β_1AR, in addition to the 'classical' catecholamine activation site. CGP 12177A stimulates AC in cell lines expressing recombinant rat and human β_1ARs [44–47], and its cardiostimulant effects show the characteristic resistance to βAR antagonism of the β_4AR [48]. In a more relevant primary cell line, the adult rat cardiomyocyte, adenovirally overexpressed β_1ARs demonstrated similar increases in the cardiostimulant potencies of both isoproterenol and CGP 12177A [49]. An obligatory role for β_1ARs in the cardiostimulant effect of CGP 12177A was demonstrated in β_1/β_2AR double KO mice [50]. CGP 12177A increased sinoatrial rate and left atrial contractile force in hearts from wild-type and β_2AR KO mice, but its effects were absent in β_1/β_2AR double KO mouse heart. There was parallel desensitization and resensitization of β_1- and β_4ARs in a rat model of heart failure [51]. A similar desensitization in cardiostimulant responses (maximal effect and potency of CGP 12177A) through both states of the β_1AR was seen in the right atrium of failing human heart [40]. At the same time, there was a decrease in both the high-affinity (catecholamine site) and the low-affinity (β_4AR site) [^{3}H]-CGP 12177A binding sites in right atrium from failing human myocardium, in relation to the non-failing tissue. The most compelling evidence for two separate ligand binding sites on the β_1AR comes from recent experiments in which mutagenesis was able to disrupt the classic catecholamine responses of the overexpressed β_1AR selectively while retaining the CGP 12177A effects through the propranolol-resistant low-affinity site [52].

Effects mediated through the low-affinity site of the β_1AR may be of more than academic interest. Pindolol, in the presence of β_1- and β_2AR blockade by propranolol, induces a significant 10% increase in heart rate in healthy volunteers [53], which can be prevented by predosing with carvedilol [54]. In part, the continuing interest

in the pharmacology of the β_4AR is due to its potential involvement in arrhythmogenesis. CGP 12177A produces ventricular extrasystoles in whole ferret ventricle [55], arrhythmic Ca^{2+} transients in atrial and ventricular rat cardiomyocytes [42], and arrhythmic Ca^{2+} transients in mouse ventricular cardiomyocytes with a potency 40 times greater than that of isoproterenol through the 'classical' β_1AR [56]. The mechanism for this effect is not fully elucidated. CGP 12177A is more potent than norepinephrine in shortening the ventricular monophasic action potential but does not alter the refractory period, suggesting differential coupling to individual ion channels [55]. CGP 12177A also increases both intracellular Ca^{2+} transient and current through the L-type Ca^{2+} channel [56].

10.4 β_2-Adrenoceptors

10.4.1 Constitutive Activity of Overexpressed β_2ARs

There is little doubt that the β_2AR has the capacity for constitutive activation of contraction. Expression of the β_2AR in CHO cells produced a much greater increase in cAMP concentration than observed with the β_1AR at similar expression levels [20]. The same is true for the heart: *in vitro* overexpression of human β_2AR in cardiomyocytes from transgenic mice lacking both β_1- and β_2ARs increased both basal contractility and cyclic AMP levels [21]. In transgenic mice overexpressing the β_2AR (TGβ2), basal contractility of isolated atria was shown to be near the maximum isoproterenol-stimulated level *in vivo* in the first study [57]. However, different results were seen when other groups came to study these animals. While the beating frequencies of isolated right atria continued to be elevated in several studies [58, 59], which was linked to activation of the hyperpolarization-activated current I_f [60], a loss of the constitutive activity of contraction was observed. The basal force of contraction of left atria [61] or contraction amplitudes of isolated left ventricular cardiomyocytes [58] from TGβ2 mice were either the same as littermates or even lower under some conditions. Other groups confirmed increased basal contraction in cardiomyocytes [62] or isolated hearts [59], but at levels well below that which would be expected after maximum isoproterenol stimulation. This was not due to loss of the β_2AR, which remained detectable by radioligand binding. Often, isoproterenol gave a biphasic effect in myocardium from these mice, with an initial positive response (although lower than in littermates) and a second negatively inotropic phase [61, 63]. Several groups were able to restore the original phenotype by treatment of mice or cardiomyocytes with pertussis toxin [64, 65], which also abolished the negative inotropic response to isoproterenol [63, 66]. Pertussis toxin ADP-ribosylates G_i protein α subunits and thereby blocks coupling of G_i-linked GPCRs to their cognate G protein [67].

It was therefore hypothesized that G_i-dependent coupling had been increased in some substrains of these mice [68]. This could be due either to an increase in G_i expression (or a selection of animals with higher G_i expression) [65] or to an increased

probability of the β_2AR coupling with G_i. A switch of the β_2AR from G_s- to G_i-coupled pathways has been shown in HEK293 cells, brought about by phosphorylation of the β_2AR itself by PKA [69]. Mutation of consensus PKA sites yielded a β_2AR that stimulated cAMP accumulation through G_s but did not activate G_i-dependent pathways. Manipulation of conditions in a manner that would be expected to increase PKA activity decreased the positive phase of the response to isoproterenol in atria from the later TGβ2 strains and increased the negative component [66]. This was interpreted to mean that PKA-dependent phosphorylation of the overexpressed β_2AR had decreased G_s coupling and increased G_i coupling. Reduction of cAMP thorough G_i activation can be by sequestration of $G\alpha_s$ through release of $G\beta\gamma$ subunits, or by a direct inhibitory effect of $G\alpha_i$ on ACV and ACVI isoforms [70]. Additionally, a number of pathways can be activated through either $G\alpha_i$ or $G\beta\gamma$ in the cardiac myocytes; these include phosphatidylinositol-3-kinase (PI3 kinase)–Akt (also referred to as protein kinase B (PKB)) [68], p38-mitogen activated kinase (p38 MAPK) [71], the Na^+/Ca^{2+}-exchanger [72], the muscarinic acetylcholine receptor-gated atrial potassium channel (I_{KACh}) [73], and nitric oxide synthase type 3 (eNOS) [74]. Each of these pathways has the potential to produce a negative inotropic effect, depending on their presence and activation state in the ventricular cardiomyocyte.

β_2AR-dependent early activation of contraction, followed by later inhibition, has been recapitulated by overexpression of the human β_2AR in adult rat ventricular cardiomyocytes by use of adenoviral transfection (S. E. Harding, unpublished results). β_2AR levels detected by radioligand binding rose with time after transfection, and at 24 h an increase in basal contractility, indicative of constitutive activation, was detected (Figure 10.1). After 48 h the receptor number continued to rise, but contraction amplitude was now lower than in control (untransfected or green fluorescent protein (GFP) transfected) cardiomyocytes. Pertussis toxin treatment increased contraction at 48 h to levels above those in control cells. We suggest that a switch from G_s to G_i coupling occurred within the cardiomyocyte as the β_2AR and cAMP levels rose and that, by 48 h, G_i effects were not only sufficient to suppress G_s but also to have a constitutive negative inotropic effect. Importantly, this was not due simply to a decrease in cAMP levels, because basal contractility in the rat cardiomyocyte is not tonically supported by cAMP [14]. Na^+/Ca^{2+}-exchanger (NCX) activation may underlie the tonic negative inotropic effect. We have shown that overexpression of NCX decreases the contraction amplitudes of rat ventricular cardiomyocytes, but that overexpression at levels insufficient to depress contraction allows β_2AR-G_i activation to suppress both β_2AR-G_s and β_1AR-G_s stimulation by isoproterenol [75]. This would imply that overexpression of the β_2AR in rat cardiomyocytes is having both constitutive positive inotropic effects through G_s/AC and constitutive negative inotropic effects through G_i/NCX, with the balance having shifted towards G_i after 48 h. Blocking with pertussis toxin does not allow distinction between $G\alpha_i$- or $G\beta\gamma$-mediated effects.

10.4.2
Inverse Agonism at the β_2AR

Decreases in contraction were seen in the original, hypercontractile, TGβ2 mice with inverse agonists, in line with the hypothesis that binding to an inactive form of the receptor (R) was reducing constitutive activity. Convincing blockade by a neutral antagonist (alprenolol) of the effects of the inverse agonist ICI 118551 ((±)-1-[(7-methyl-2,3-dihydro-1*H*-inden-4-yl)oxy]-3-[(1-methylethyl)amino]-2-butanol) was observed. In fact, results obtained with this mouse were instrumental in the development of theories of inverse agonism and constitutive activity for the β_2AR (see also Chapters 1–3 and 9) [76, 77].

However, some of this apparent inverse agonism was still observed in the substrains in which basal contractility was no longer raised. Even more surprisingly, despite their reduced βAR number, low cAMP levels, and depressed contractility [13], this effect was observed in cardiomyocytes from the failing human heart. The EC_{50} value of ICI 118551 for this effect was 13 nM for failing human heart and 100 nM for the TGβ2 mouse (Figure 10.3); these values are considerably higher than the K_B for inhibition at β_2AR-G_s, which has been reported as 0.5 nM [78]. The site of action was still the β_2AR, since blockade of β_1ARs did not prevent the negative inotropic effect and β_3AR agonists did not mimic it [13]. Overexpression of the β_2AR by use of adenoviral vectors in rat or rabbit cardiomyocytes also enhanced the effect or induced it de novo [13]. For this to be an inverse agonist effect at β_2AR-G_s, it would require basal contraction to be dependent for support on cAMP, and for this support to be withdrawn in the presence of ICI 118551. However, antagonism of cAMP with concentrations of the PKA inhibitor (*Rp*)-adenosine-3':5'-cyclic monophosphothioate triethylamine ((*Rp*)-cAMPS) sufficient to decrease the effects of isoproterenol substantially did not decrease basal contraction in the cardiomyocytes either from the TGβ2 animals (Figure 10.4A) or from failing human heart [13]. It is therefore unlikely that cAMP is constitutively supporting basal contraction, so the effects of inverse agonists cannot be due to a decrease in the abundance of R*, the activated G_s-coupled form of the β_2AR.

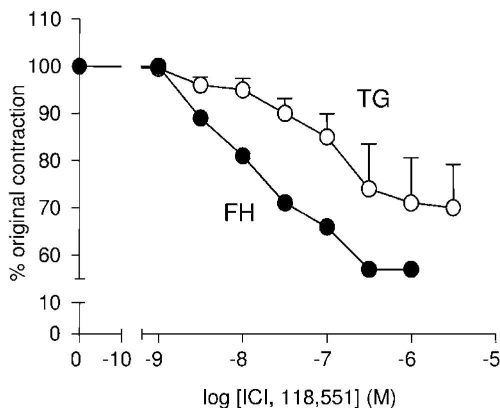

Fig. 10.3 Concentration-response curve for ICI 118551 in TGβ_2 cardiomyocytes (TG, open symbols, $n = 6$ myocytes/4 hearts) and a failing human ventricular cardiomyocyte ((FH, closed symbols). Human myocytes were studied in 8 mM Ca^{2+} and mouse in 4 mM Ca^{2+}. Reprinted from Gong et al., 2002 [13], with permission.

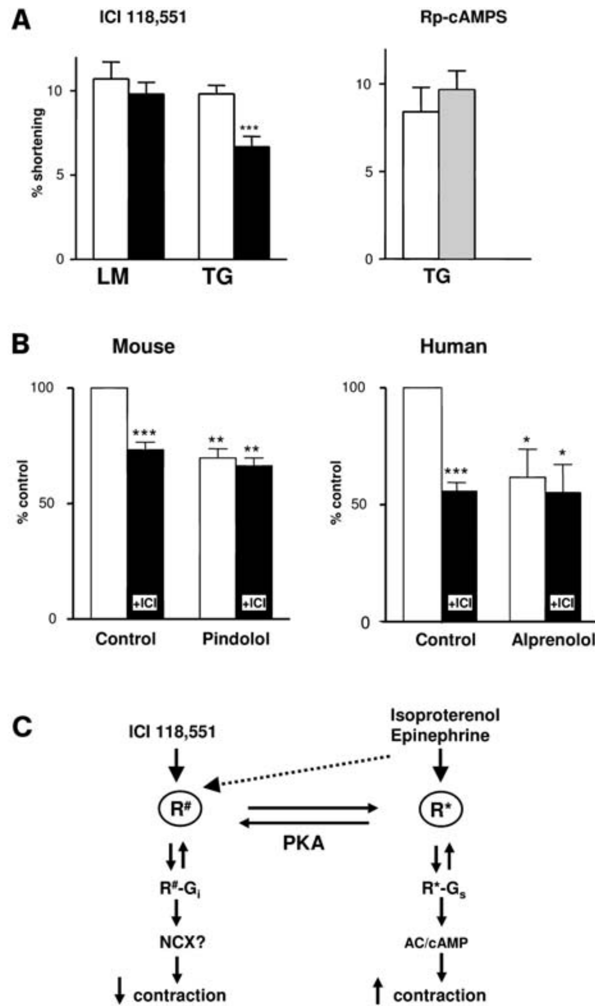

Fig. 10.4 Depression of contraction by ICI 118 551 (1–3 µM) does not fulfil the criteria for inverse agonism of β_2AR-G_s coupling in ventricular cardiomyocytes from transgenic mouse overexpressing the β_2AR (TGβ2) or failing human heart.
A) Basal contraction is not increased in TGβ2 (TG) cardiomyocytes relative to littermate (LM) controls, nor depressed by a cAMP antagonist (Rp-cAMPS, 100 µM). ICI 118 551 (1 µM) depresses contraction in TG but not LM myocytes. A similar pattern is seen in failing human cardiomyocytes (Gong et. al., 2002 [13]). Therefore, β_2AR-G_s (R*) is not constitutively active to a sufficient extent to increase contraction.
B) A partial agonist (pindolol) or a neutral antagonist (alprenolol) would be predicted to block the effects of an inverse agonist. However, both have similar effects to ICI 118551 and the combined effects of pindolol (10 µM) or alprenolol (10 µM) are non-additive with those of ICI 118 551 (1–3 µM), suggesting a common pathway.
C) An alternative scheme suggests dual coupling of the β_2AR to G_s or G_i. Theories of stimulus trafficking predict that agonist/antagonist orders of potency differ when a GPCR couples to different G proteins. We suggest that ICI 118551 is an agonist at the β_2AR-G_i-coupled form, and is inducing a negative inotropic effect through a pathway unrelated to cAMP. Isoproterenol and epinephrine are agonists at both β_2AR-G_s and β_2AR-G_i. See text for more details.

Further, neither a neutral antagonist (alprenolol) nor a partial β_2AR-G_s agonist (pindolol) was able to prevent the negative inotropic effect of ICI 118 551; each of these in fact had significant negative effects that were non-additive with ICI 118 551 in failing human /TGβ2 cardiomyocytes (Figure 10.4B). Thus, the criteria for inverse agonism are no longer fulfilled for ICI 118 551 in these tissues (Figure 10.4).

10.4.3
βAR Antagonists: Inverse Agonists at β_2AR-G_s or Full Agonists at β_2AR-G_i?

The mechanism that we have proposed [13] to explain these anomalous effects of βAR antagonists is that of stimulus trafficking [79]. β_2ARs, like other GPCRs, have been shown to possess a number of active states that differ when coupling to different G proteins. By use of recombinant systems, for example, the β_2AR was found to couple to G_s, G_i, or G_q, as evidenced by ligand-regulated [^{35}S]guanosine 5'-[γ-thio]triphosphate) binding to β_2AR-G protein fusion proteins (see also Chapters 8 and 9) [80]. The order of potency of agonists and antagonists at these two states is not necessarily the same: this is a hallmark of stimulus trafficking [79]. In the experiments with fusion proteins, clear differences were observed for agonist efficacy between the G_s-, G_i-, or G_q-coupled forms of the β_2AR [80]. G_i-dependent coupling clearly has negative inotropic potential in cardiac muscle, through the various mechanisms described in Section 10.4.1. If the active states for G_s and G_i are R^* and $R^\#$, respectively, then we suggest that ICI 118 551 and some other β_2AR antagonists that are antagonists at R^*-G_s can also be agonists at $R^\#$-G_i (see Figure 10.4). Preferential binding to $R^\#$-G_i could therefore have a direct negative inotropic effect. Activation of G_i-dependent coupling by ICI 118 551 is supported by the ability of pertussis toxin to inhibit the negative inotropic effect of this compound in β_2AR-overexpressing cardiomyocytes from rabbit, rat, or mouse heart or from failing human ventricle, as well as by the induction of a negative inotropic effect after G_i-overexpression in rat [13]. However, other studies do not support this concept, with ICI 118 551 being unable to regulate GTPγS binding to the β_2AR-$G\alpha_i$ fusion protein [80].

Isoproterenol is a full agonist at R^*-G_s, and there is also evidence for agonism both at G_i- and at G_q-coupled forms of the β_2AR [80]. This would account for the reported ability of high concentrations of isoproterenol to activate β_2AR/G_i-dependent pathways in rat cardiomyocytes [81], and for the biphasic effect of isoproterenol in atria from TGβ2 mice [63, 66]. Epinephrine behaves in the same way as isoproterenol in TGβ2 atria, but norepinephrine does not [82]. Data from the β_2AR-$G\alpha_i$ fusion protein experiments suggest that epinephrine has a lower efficacy than isoproterenol in stimulation of GTPγS binding [80].

The scheme in Figure 10.4 could account not only for the negative inotropic effect of ICI 118 551 in the absence of constitutive activation of β_2AR-G_s, but also for the ability of increased G_i to decrease responses through G_s. This has been observed in a number of situations including adenoviral up-regulation of G_i in rat cardiomyocytes [83], transgenic mice overexpressing the β_2AR [65, 64], and failing human heart [84, 85]. In each case G_i expression was increased, βAR responses were decreased (interestingly, both

through β_1- and through β_2AR), and these responses were restored by pertussis toxin. The fact that prolonged stimulation by catecholamines through the R*-G_s pathway produces desensitization of that pathway is well known, and loss of βARs certainly contributes to the effect [86].

However, G_i levels are increased in rats exposed to isoproterenol [87], and βAR desensitization is reversed by pertussis toxin in cardiomyocytes from norepinephrine-treated guinea pigs and failing human heart [85]. Up-regulation of G_i secondary to chronic stimulation of R*-G_s therefore appears to contribute to catecholamine-induced desensitization.

The mechanism of the reduction of G_s-mediated activation by G_i is not entirely clear. If R^*-G_s and $R^\#$-G_i are in equilibrium, then binding to $R^\#$-G_i could also reduce the amount of R^*-G_s: however, if $R^\#$ represents a phosphorylated form of the β_2AR then equilibrium conditions might not be established between R^* and $R^\#$. Activation of a separate negative inotropic β_2AR-G_i pathway could also explain the responses seen, especially the ability of β_2AR-G_i activation to suppress not only the β_2AR-G_s- but also the β_1AR-G_s-dependent positive responses [65, 75].

10.4.4
Involvement of the β_2AR in the "Putative β_4AR" Effect

Although there is strong evidence, detailed above, that the primary mediator of β_4AR-type effects is a low-affinity propranolol-resistant state of the β_1AR, there are data that also suggest a role of the β_2AR. Overexpression of β_2ARs in rat cardiomyocytes enhances the positive inotropic effect of CGP 12177A in a manner similar to that seen after β_1AR overexpression, although the magnitude of the effect was lower [88]. Once again, antagonists at the high-affinity site did not prevent the effect. CGP 12177A also has a weak cardiostimulant effect via β_2ARs (efficacy only 10–20% of that at expressed β_1ARs) on cAMP accumulation in cell lines overexpressing recombinant β_2ARs [44, 45]. CGP 12177A may be activating a novel low-affinity site on the β_2AR, similar to the novel state of the β_1AR. The concept that the β_2AR can exist in more than one active conformation has been suggested previously (see also Chapter 9) [89, 90]. The effects of CGP 12177A seen after β_2AR expression exhibit other characteristics of the β_2AR, in that the effect is time-dependent, with an early (24 h) enhancement of the positive effect lost by 48 h. The attenuation of the effect with time can be restored by pretreatment with pertussis toxin, suggesting that the CGP 12177A cardiostimulant site, like the β_2AR, can couple through G_i as well as G_s [13]. The possibility that the cardiostimulant effects of CGP 12177A are mediated by an interaction with β_1- and β_2AR homo- or heterodimers has also been suggested [48, 91].

10.5
Homo- and Heterodimerization of β_1- and β_2ARs

Increasing numbers of GPCRs are now known to exist as dimeric or oligomeric complexes, with receptor–receptor interaction producing subtle (or sometimes substantial) alterations in signaling properties [92]. Both GPCR homodimers and heterodimers between various GPCRs have been shown [92]. Homodimerization of both β_1ARs [93] and β_2ARs [94, 95] has been observed *in vitro* and *in vivo*, and intermolecular interactions between receptors may have both functional and structural implications for G protein-mediated signaling. For example, the correct targeting of the β_2AR to the plasma membrane has been prevented by disruption of the sixth transmembrane domain, which appears to be required for homodimerization [95]. A peptide derived from this domain inhibits both dimerization and βAR G_s/AC coupling [94]. Agonist stimulation was also found to stabilize the homodimer, whereas inverse agonists bound with greater affinity to the monomeric state, providing further evidence that conversion between monomer and dimer forms may play a physiological role [94].

β_1- and β_2ARs coexist within the same cardiomyocyte [96], giving the potential for heterodimerization between these two receptors to modify both constitutive activity and the response to inverse agonists. Heterodimerization of β_1- and β_2ARs has been observed in cell lines [97] and in mouse cardiomyocytes [98] co-overexpressing β_1- and β_2ARs. β_1- and β_2AR co-localization on the cell membrane produced novel functional properties, with a leftward shift in the concentration-response curve to isoproterenol of cardiomyocyte contraction when β_1- and β_2ARs were co-expressed in cardiomyocytes from double β_1-/β_2AR KO mice [98]. The K_d values for CGP 20712A and ICI 118551 were also reduced. This suggests that β_1- and β_2ARs can form high-affinity heterodimers with a novel binding site and altered ligand binding properties. In contrast, this same group (Lakatta and co-workers) demonstrated similar efficacy and potency of isoproterenol-stimulated cAMP production when β_1- and β_2ARs were expressed alone compared with co-expression, despite formation of heterodimers [97]. However, a unique functional property of β_1-/β_2AR heterodimers was observed, with complete loss of the isoproterenol stimulation of extracellular signal-related kinase (ERK)1/2 activation, which is usually seen when β_2ARs (but not β_1ARs) are expressed alone. Homo- and heterodimerization of β_1- and β_2ARs therefore have the potential to alter receptor trafficking, receptor/ligand interactions, and subsequent signaling pathways significantly. The question remains as to which of these are physiologically relevant effects, since there are as yet no reports of βAR dimerization in the native heart.

10.6
Conclusions

The relation between constitutive activity and physiological effects shows an interesting contrast between the β_1- and β_2ARs in the cardiomyocyte. β_1ARs are able to show constitutive activity in normal cardiomyocytes as well as when overexpressed, although

this crucially depends on the parameter being studied. Constitutive activity of the β_1ARs controls not only contractility but also cell viability, and this is seen both *in vivo* and *in vitro*. The β_2AR can also produce constitutive activity, but the coupling to (at least) two different G proteins (G_s and G_i) means that two separate pathways are activated. Effects interpreted as inverse agonism may result simply from activation of the alternate pathway. The potential for heterodimer formation between the β_1- and β_2ARs could produce new pharmacological traits, and it remains to be established whether other manifestations of constitutive activity will result from this phenomenon. It is increasingly clear that the spatial relation between the β_1- and β_2ARs themselves, as well as with their various signal transduction factors, is an essential factor in our understanding, and this should always be kept in mind when relating the effects of overexpression to the physiology of the native system.

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11
Constitutive Activity at the α_1-Adrenoceptors: Past and Future Implications

Susanna Cotecchia, Francesca Fanelli, Alexander Scheer, and Tommaso Costa

11.1
Introduction

11.1.1
The α1-Adrenoceptors: Main Structure–Functional Features

The mammalian GPCRs can be divided into three main classes according to sequence homology: class I or rhodopsin-like (the largest subfamily), class II or secretin-like, and class III or glutamate-metabotropic-like [1]. In class I, the adrenoceptors (ARs) mediate the functional effects of epinephrine and norepinephrine. The AR family includes nine different gene products: three β (β_1, β_2, β_3), three α_2 (α_{2A}, α_{2B}, α_{2C}), and three α_1 (α_{1A}, α_{1B}, α_{1D}) receptor subtypes.

Within the subfamily of the α_1AR subtypes [2], extensive mutational analysis performed by various investigators has helped to identify the structural determinants involved in each of the three main "classical" functional properties of GPCRs: 1) ligand-binding, 2) coupling to G protein-effector systems, and 3) desensitization.

The molecular interactions of the endogenous catecholamines – epinephrine and norepinephrine – with different AR subtypes have been explored in different studies. Epinephrine and norepinephrine each contain a protonated amino group separated from the aromatic catechol ring by a β-hydroxyethyl chain. Mutagenesis studies of the α_{1B}AR [3, 4] suggested that the catecholamine amino group enters into an electrostatic interaction with the carboxylate side chain of an aspartate on helix 3, $Asp^{125(3.32)}$, which is highly conserved in all GPCRs that bind biogenic amines (Figure 11.1) (the amino acid numbering in parentheses, used only for the amino acids in the helical bundle, is that proposed in [5]). Similar findings were obtained for mutation of the homologous aspartate in the α_{1A}AR subtype (S.C., unpublished results). For both the α_{1A}AR and α_{1B}AR, the catechol *meta*- and *para*-hydroxy groups of epinephrine and norepinephrine make weak hydrogen bonding interactions with serine residues in helix 5, which are conserved in all catecholamine-binding GPCRs (Figure 11.1) [3, 4].

The roles of a cluster of aromatic amino acids ($Phe^{F303(6.44)}$, $Phe^{310(6.51)}$, and $Phe^{F311(6.52)}$ in helix 6 and $Tyr^{348(7.53)}$ in helix 7) in ligand binding of the α_{1B}AR

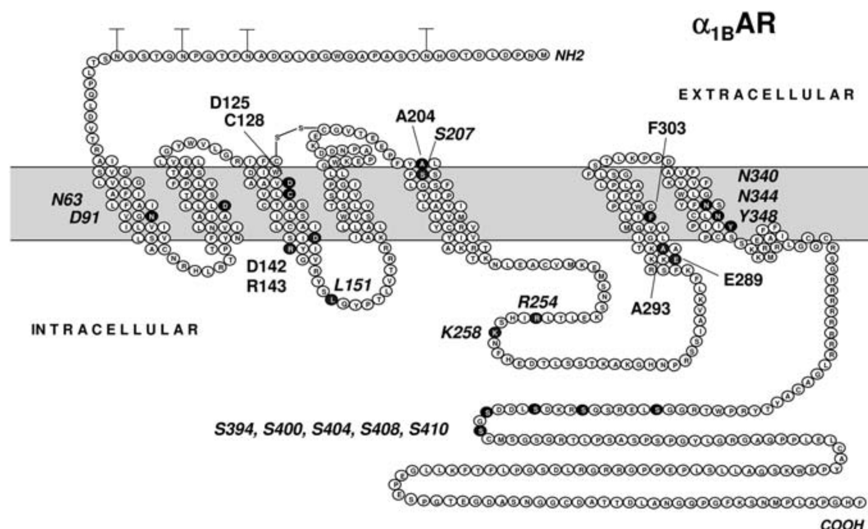


Fig. 11.1 Topographical model of the α_{1B}AR. The sequence of the hamster α_{1B}AR is topographically arranged according to its alignment with the crystal structure of bovine rhodopsin. The four N-linked glycosylation sites of the receptor are indicated with crosses. The black circles indicate most of the amino acids mentioned in the text, which can be divided into three groups: i) in bold, main sites at which constitutively activating mutations have been described (Asp$^{125(3.32)}$, Cys$^{128(3.35)}$, Asp$^{142(3.49)}$, Arg$^{143(3.50)}$, Ala$^{204(5.39)}$, Glu$^{289(6.30)}$, Ala$^{293(6.34)}$, and Phe$^{303(6.44)}$), ii) in italic, key amino acids playing a role in catecholamine binding (Asp$^{125(3.32)}$, Ser$^{207(5.42)}$), coupling to G_q (Leu^{151}, Arg^{254}, Lys^{258}), and phosphorylation (Ser^{394}, Ser^{400}, Ser^{404}, Ser^{408} and Ser^{410}), and iii) also in italic, highly conserved polar residues within the helical bundle (Asn$^{63(1.50)}$, Asp$^{91(2.50)}$, Asn$^{340(7.45)}$, Asn$^{344(7.49)}$, Tyr$^{348(7.53)}$).

were also investigated [6]. The results indicated that, among these aromatic residues, Phe$^{310(6.51)}$ is the only one involved in binding epinephrine, probably interacting with the catechol ring of the ligand. In fact, the substituted cysteine accessibility method revealed that the side chain of Phe$^{310(6.51)}$, but not that of Phe$^{311(6.52)}$, is both solvent-accessible and directed towards the agonist-binding pocket.

In contrast, very little is known so far either about the amino acids of the α_{1B}AR that interact with different antagonists, or about the structural basis underlying receptor selectivity for different ligands. Mutation of Asp$^{125(3.32)}$ (Figure 11.1) to alanine profoundly impaired the ability of the hamster α_{1B}AR to bind antagonists [3]. Experimental mutagenesis studies suggested that the conserved Tyr$^{338(7.43)}$ in the extracellular half of helix 7 should interact with prazosin. Indeed, replacement of this tyrosine with alanine induced a reduction of about 70-fold in the binding affinity of prazosin for the hamster α_{1B}AR [3].

In almost all tissues in which this effect has been examined, activation of the α_1AR subtypes causes polyphosphoinositide hydrolysis catalyzed by phospholipase C via pertussis toxin-insensitive G proteins of the $G_{q/11}$ family. Polyphosphoinositide hydrolysis results in an increase in intracellular inositol phosphate production. Several lines of evidence indicate that the main structural determinants involved in α_{1B}AR coupling to G proteins of the $G_{q/11}$ family are contained in the i3 loop. A detailed analysis of the

molecular basis of the receptor–G_q coupling was carried out by combining computational modeling and experimental mutagenesis of α_{1B}AR [7]. The functional analysis of a large number of receptor mutants in conjunction with the predictions of molecular modeling support the hypothesis that Arg^{254} and Lys^{258} in the i3 loop, as well as Leu^{151} in the i2 loop (Figure 11.1), are directly involved in receptor–G protein interaction and/or receptor-mediated activation of the G protein [8]. It is important to highlight the fact that mutations of the homologous leucine or hydrophobic residue in the i2 loop resulted in receptor–G protein uncoupling for other GPCRs as well [1].

The α_{1B}AR expressed in various cell types can undergo phosphorylation and desensitization upon exposure to agonists and also to the protein kinase C (PKC) activator phorbol-myristate-acetate [9, 10]. In particular, we demonstrated that a stretch of serines in the C terminus of the receptor represents the main phosphorylation sites (Figure 11.1) [11]. Three of them (Ser^{404}, Ser^{408}, and Ser^{410}) are involved in agonist-induced phosphorylation, whereas two others (Ser^{394} and Ser^{400}) represent the sites for PKC-mediated phosphorylation of the α_{1B}AR. Agonist-induced regulation of the receptor seems to be mainly mediated by members of the G protein-coupled receptor kinase (GRK) family. In fact, GRK2-mediated phosphorylation of Ser^{404}, Ser^{408}, and Ser^{410} is crucially involved in the desensitization of the α_{1B}AR.

11.1.2 The Discovery of Constitutively Activating Mutations and its Implications

The α_{1B}AR was the first GPCR in which point mutations were shown to trigger receptor activation [12]. A conservative substitution (Ala^{293}Leu) in the cytosolic extension of helix 6 (Figure 11.1) of the α_{1B}AR resulted in its constitutive (agonist-independent) activity. In the absence of agonist, cells expressing the mutated receptor exhibited higher basal levels of inositol phosphates than cells expressing the wild-type α_{1B}AR. To assess the role of this amino acid further, $Ala^{293(6.34)}$ was systematically mutated by substitution of each of the other 19 natural amino acids [13]. Remarkably, all possible amino acid substitutions of Ala^{293} in the α_{1B}AR induced variable levels of constitutive activity, highest for the Ala^{293}Glu mutant.

To extend the generality of this finding within the AR family, similar mutations were performed in the β_2AR and α_{2A}AR, which are coupled to G_s-mediated stimulation or G_i-mediated inhibition of adenylyl cyclase, respectively [14, 15]. Both β_2 and α_{2A}AR mutants exhibited increased constitutive activity, resulting in increased and decreased agonist-independent adenylyl cyclase activity, respectively.

The discovery of the constitutively active mutants (CAMs) in the AR family sparked the interest of a large number of groups towards the elucidation of two main aspects of GPCR function and drug action: a) the activation process of GPCRs, and b) constitutive activity of wild-type GPCRs and the identification of ligands with negative efficacy (i.e., inverse agonists). The use of CAM GPCRs in drug discovery has also attracted great interest and may result in interesting findings in the future [16]. In addition, the discovery of the CAM GPCRs encouraged the search for spontaneously occurring activating mutations of different receptors responsible for a number of human diseases [17].

In this article we review the main contribution provided by studies on constitutive activity of the α_1AR subtypes and highlight some of the questions on constitutive activity remaining to be answered.

11.2 Theoretical and Experimental Approaches for Study of Constitutive GPCR Activity

11.2.1 Theoretical Analysis of CAM GPCR Pharmacology

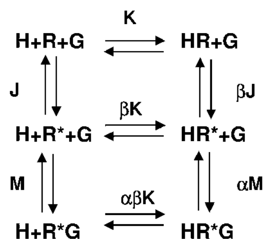
The concept that the activation of a receptor by an agonist involves conformational changes in the receptor molecule was originally developed in 1957 by del Castillo and Katz, who proposed that binding of acetylcholine to nicotinic acetylcholine receptor produces a conformational change resulting in channel opening [18]. This model invokes the existence of two receptor states: a closed (resting) and an open (active) one, the latter being stabilized by an agonist. However, the theoretical concept of how conformational changes in proteins may give rise to changes in functional properties, later called allosterism, was first developed for hemoglobin by Wyman in 1951 [19] (see also Chapter 1).

For GPCRs, the allosteric transition between different "states" of the receptor molecule had not been explicitly recognized by models describing receptor behavior. A detailed analysis of the properties of a β_2AR CAM was instrumental for the proposal of the *allosteric or extended ternary complex model* [15] (Figure 11.2; see also Chapter 9). This extended version of the ternary complex model introduced an explicit isomerization constant (J) regulating the equilibrium of GPCR between at least two interconvertible allosteric states: **R** (inactive or ground state) and **R*** (active). It is assumed that only **R*** can effectively interact with the G protein. In the absence of the agonist, **R** predominates, whereas agonists shift the equilibrium toward **R***, thus favoring its stabilization. Constitutively activating mutations would also appear to increase the isomerization constant J. From this simple formulation, more elaborate and complex models were also developed [20] (see also Chapters 2 and 3). This analysis gave rise to the hypothesis that constitutively activating mutations mimic, at least to some extent, the conformational change triggered by agonist binding to a wild-type GPCR.

The allosteric ternary complex model could explain some important pharmacological features of the β_2AR CAM, in particular: a) the increased affinity of agonists and partial agonists for the CAM in proportion to the ligand's efficacy even in the absence of G proteins, and b) the increased efficacy of partial agonists at the CAM.

The allosteric ternary complex model [15] has been widely cited for interpretation of the behavior of several constitutively active GPCR mutants. However, it should be emphasized that the pharmacological features of the constitutively active AR mutants mentioned above have not been systematically tested for other CAMs. At those GPCRs for which a limited number of ligands is available, for example, correlation between increased affinity of agonists and partial agonists in proportion to their efficacy cannot

Fig. 11.2 The allosteric ternary complex model. The model is described in Ref. 15. H = hormone or, more generally, receptor agonist, R = receptor, G = G protein. R undergoes an allosteric transition with a constant *J*, which leads to the formation of R*.



be rigorously tested. Extensive pharmacological characterization of receptor mutants is thus required to assess whether the allosteric ternary complex model can explain the behavior of most CAMs.

An important prediction of the model is that, if a mutation changes the intrinsic equilibrium between receptor conformations, there must be a linkage between the enhancement of constitutive activity and the increase in agonist affinity. However, when multiple activating mutations have been found at the same residue, correlation between the increase in affinity of the agonist and the degree of constitutive activity of the receptor mutants has been explored only in a few studies (see Chapter 9). Therefore, the question of whether the concept of allosteric transition can be generally used to interpret the behavior of CAM GPCRs remains open. This would also be important for elucidation of another unanswered question: are CAMs truly representative of the agonist-bound wild-type receptor?

11.2.2
Computational Modeling of the α_{1B}AR

It was suggested that, in the absence of agonist, structural "constraints" keep the wild-type receptor in its inactive state (**R**), whereas activating mutations release such constraints, triggering the conversion of the receptor into its active state (**R***), which couples to G proteins [15]. Once this hypothesis had been made, several studies addressed the following questions: What is the nature of such "constraints"? Are they similar for different GPCRs? How can mutations in apparently unrelated regions of a GPCR release these constraints?

To investigate the mechanisms of agonist-independent activation at a molecular level, we combined three-dimensional model building of the α_{1B}AR structure with computational simulation of receptor dynamics. The first α_{1B}AR model was constructed by use of an iterative ab initio procedure intended to incorporate the rapidly increasing amount of experimental data on rhodopsin and other homologous GPCRs [21]. The ab initio model of the α_{1B}AR was extremely useful for the development of a computational approach aimed at inferring the structural features differentiating the inactive from the active receptor states. Through comparison of the molecular dynamics (MD) trajectories of the wild-type receptor with those of the inactive or active mutants, this approach was successful in predicting the functionality of novel α_{1B}AR mutants [22, 23].

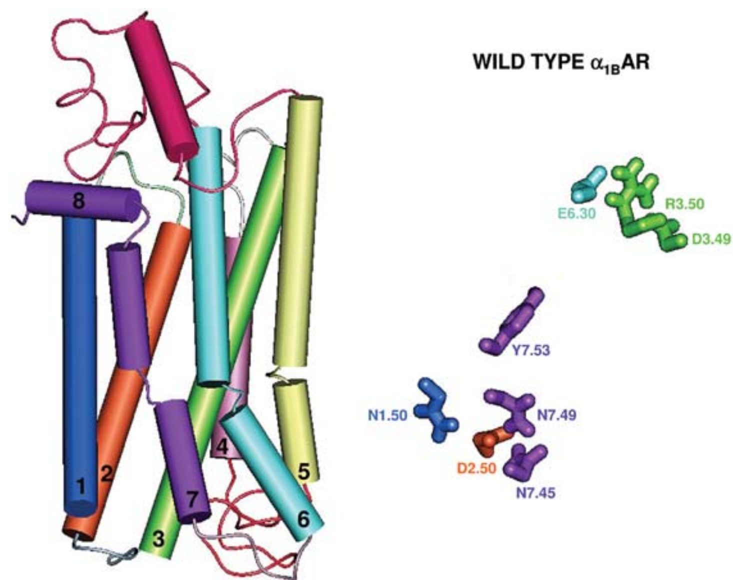


Fig. 11.3 Homology model of the wild-type α_{1B}AR. Comparative modeling and molecular dynamics simulations were performed as described in Ref. 25. *Left*: cylinder representations of the whole receptors are shown, viewed in a direction almost parallel to the membrane surface with the extracellular side on the bottom and the intracellular one on the top; the numbers on the cylinders indicate the numbers of the helices. *Right*: interactions involving highly conserved polar amino acids ($Asn^{1.50}$, $Asp^{2.50}$, $Asn^{7.45}$, $Asn^{7.49}$, $Tyr^{7.53}$), the $Asp^{3.49}$ and $Arg^{3.50}$ of the Glu/AspArgTyr motif, and $Glu^{6.30}$ in helix 6 are viewed in a direction almost perpendicular to the membrane surface from the intracellular side. The side chains of the amino acids are colored according to the helices to which they belong.

The interpretative and predictive abilities of the computational approach improved as soon as the first crystal structure of rhodopsin became available [24], allowing the use of comparative modeling to derive the initial structure of the α_{1B}AR (Figure 11.3). Comparative MD simulations of a wide number of engineered mutants of the α_{1B}AR allowed us to gain insight into the structural hallmarks of the inactive and active states, as well as to distinguish the amino acids and the receptor domains important for maintaining the ground state or promoting the active states from those potentially involved in G protein recognition [8, 25]. Despite the structural differences between the ab initio and the comparative models, both suggested that the highly conserved Glu/AspArgTyr motif at the cytosolic end of helix 3 in the α_{1B}AR (Figures 11.1 and 11.3) is particularly prone to undergo structural modification in response to activating mutations. Thus, the data indicate that this region "holds the switches" of receptor activation.

11.2.3
Measuring Constitutive Activity of the α_1AR Subtypes

In the majority of studies, constitutive activity of the α_1AR subtypes was assessed by measurement of agonist-independent accumulation of inositol phosphates in intact cells expressing the recombinant receptors (see also Chapter 8). Experiments measuring constitutive activity in intact cells have the main advantage of amplifying the receptor output signal in experiments measuring the accumulation of second messengers under conditions in which their degradation is inhibited by the inositol phosphatase inhibitor Li^+. However, intact cell results should be interpreted with caution for several reasons, including the presence of endogenous ligands activating the receptor or receptor regulatory events (e.g., desensitization or upregulation). To overcome these problems it may be advantageous to measure constitutive activity in membranes by direct monitoring of receptor-mediated G protein activation (see Chapters 8 and 9).

For GPCRs coupled to G_i, receptor-mediated binding of $[^{35}S]GTP\gamma S$ to $G\alpha_i$ in membrane preparations has been successfully used to measure constitutive activity. For GPCRs linked to the G_s or $G_{q/11}$ signaling pathway, however, receptor-mediated binding of $[^{35}S]GTP\gamma S$ gives smaller signals, probably because of the lower abundance of the G proteins in the cells.

A membrane assay measuring receptor-mediated production of inositol phosphates *in vitro* has previously been described [26]. The accumulation of inositol phosphates measured in this membrane assay, however, is much smaller than that observed in whole cells in which polyphosphoinositide turnover is preserved.

For the α_{1B}AR mutants, the increased constitutive activity of the $Ala^{293}Glu$, $Asp^{142}A$-la, and of a triple CAM has also been demonstrated in experiments measuring receptor-mediated nucleotide exchange in cells expressing fusion proteins between the receptor and the $G\alpha_{11}$ subunit [27] (see Chapters 6, 8, and 9). The agonist-independent binding of $[^{35}S]GTP\gamma S$ was substantially greater at fusion proteins expressing the CAMs than at those incorporating the wild-type receptor. Similar results were obtained in our laboratory in experiments measuring $[^{35}S]GTP\gamma S$ binding in membranes derived from cells co-expressing the wild-type α_{1B}AR or its constitutively active mutants with $G\alpha$ (see Chapter 8). These results are among the few demonstrating constitutive activity of G_q-coupled GPCRs at the level of receptor–G protein coupling in membranes, thus overcoming potential problems, such as receptor regulatory events, that occur in intact cells.

A crucial problem in experiments measuring constitutive activity of GPCRs in whole cells concerns the relationship between the receptor activity and the expression levels of different receptors. As predicted by the "allosteric ternary complex model", the constitutive activity measured in cells depends on the number of expressed receptor molecules in the active form [15]. Therefore, to compare the constitutive activity of different receptors these should be expressed at the same level, which is often difficult to achieve in transfected cells. Two main approaches have been used to overcome this problem.

In most studies on CAMs of the α_{1B}AR, different amounts of receptor DNA have been transfected to obtain similar expression levels [13, 22, 25]. In these studies the

constitutive activity of different CAMs could be directly compared between different receptors in the same experiment. In other studies, the constitutive activity of different receptors was normalized to their receptor number after assessment of the existence of a linear relationship between constitutive activity and receptor number for each receptor [28]. Both methods rely on the careful measurement of receptor expression. In the vast majority of studies on CAMs, receptor expression has been measured through ligand binding. In these cases, if the expression level of a receptor is underestimated, its constitutive activity normalized to receptor binding might be overestimated. This could happen for receptor mutants expressed at levels too low to obtain reliable binding data or displaying changes in the affinity for the ligand. Therefore, if highly specific antibodies for the GPCR concerned are available, it might be useful to monitor the expression of the protein by immunocytochemistry or Western blotting to confirm the ligand binding data.

Problems linked to the ligand binding measurement might, for example, explain the discrepancy between the results reported by two different groups on the α_{1B}AR mutants carrying mutations of Asp$^{125(3.32)}$ (the putative counterion for the protonated group of the ligands) on helix 3. Whereas Perez's group reported that mutations of Asp$^{125(3.32)}$ could increase the constitutive activity of the α_{1B}AR [28], we were not able to confirm these findings. In Perez's group's work, constitutive activity of the Asp^{125}Ala mutant was normalized to its expression measured by ligand binding, which was much lower than that of the wild-type receptor. In contrast, we found that the Asp^{125}Ala mutant was highly expressed at the protein level despite the fact that only very little binding could be measured ([3] and S.C., unpublished results). Therefore, the example of the Asp^{125}Ala mutant might represent a case in which it is problematic to normalize the constitutive activity of a receptor mutant to its expression measured by ligand binding.

11.3 Constitutively Activating Mutations of the α_1AR Subtypes

11.3.1 Where the Mutations have been Found

Among the three α_1AR subtypes, the largest number of activating mutations have been described in the α_{1B}AR (Figure 11.1), a few in the α_{1A}AR, and none in the α_{1D}AR subtype. For all the CAMs their constitutive activity was mainly assessed by measurement of inositol phosphate accumulation in whole cells, whereas for a few of them other biochemical pathways such as phospholipase A_2 or D have also been explored.

As mentioned above, all possible amino acid substitutions of Ala$^{293(6.34)}$ in the cytosolic extension of helix 6 in the α_{1B}AR induced variable levels of constitutive activity [13]. No quantitative relationship was found between the physicochemical properties of the substituting amino acids and the levels of agonist-independent activity of the CAMs. The greatest increase in constitutive activity was observed for the mutation of Ala$^{293(6.34)}$ into glutamate (see Chapter 8). All CAMs displayed increased affinity for the

full agonist epinephrine, which was correlated at least to some extent with their degree of constitutive activity.

Studies from our laboratory combining site-directed mutagenesis of the α_{1B}AR and MD simulations on computational models of the receptor highlighted the potential role played in receptor activation by the Glu/AspArgTyr motif at the cytosolic end of helix 3 (Figure 11.1), which is highly conserved in GPCRs of the rhodopsin-like class. Similarly to the case of Ala$^{293(6.34)}$ replacements, all the 19 possible natural amino acid substitutions of the aspartate (Asp$^{142(3.49)}$) of the Glu/AspArgTyr motif resulted in variable levels of agonist-independent activity [22] (see Chapter 8). The greatest constitutive activity was observed for the mutation of Asp$^{142(3.49)}$ into Thr. All CAMs carrying mutations of Asp$^{142(3.49)}$ displayed increased affinity for the full agonist epinephrine, which was correlated, at least to some extent, with their degree of constitutive activity. Increased constitutive activity was also found after mutation of the acidic residue of the Glu/AspArgTyr motif in other receptors including rhodopsin [29], the β_2AR [30], the histamine H_2 [31], the vasopressin V_2 [32] and muscarinic M_1 [33] receptors.

Unlike in the case of the mutants at Ala$^{293(6.34)}$, a linear relationship was found between the hydrophobic character of the amino acid replacing Asp$^{142(3.49)}$ and the constitutive activity of the receptor mutants (i.e., the stronger the hydrophobic character of the amino acid at position 142(3.49), the greater the extent of constitutive activity) [22]. According to the meaning of hydrophobicity as an empirical descriptor (obtained as the mean value of the free energy transfer from a polar to a nonpolar environment), the results on mutations of Asp$^{142(3.49)}$ in the α_{1B}AR generated the following hypothesis: a) the hydrophobic/hydrophilic character of Asp$^{142(3.49)}$ is regulated by protonation/deprotonation of this residue as demonstrated in rhodopsin for the homologous glutamate [34], and b) the increased hydrophobicity of protonated Asp$^{142(3.49)}$ promotes the translocation of its side chain towards a less polar environment, inducing changes in the interaction pattern of Asp$^{142(3.49)}$ itself, and, consequently, helix motions [23].

An interesting pattern of functional responses was also obtained by mutation of the arginine of the Glu/AspArgTyr sequence of the hamster α_{1B}AR into Lys, His, Asp, Glu, Ala, Iso, and Asn [35]. The charge-conserving mutation of Arg$^{143(3.50)}$ into lysine and histidine conferred high and low degrees, respectively, of constitutive activity to the receptor. Whereas the Arg$^{143(3.50)}$ mutant also showed a small but significant 40% increase in basal IP accumulation, none of the other replacements of Arg$^{143(3.50)}$ was constitutively active and all were dramatically impaired in their ability to mediate agonist-induced inositol phosphate response.

Altogether, these findings on the Glu/AspArgTyr sequence engendered the hypothesis that the aspartate might constitute a fundamental switch of the α_{1B}AR activation through the protonation/deprotonation of its side chain, whereas the main role of the conserved arginine is to mediate receptor activation.

Activating mutations of the α_{1B}AR were also found in the extracellular half of the seven-helix bundle. In particular, mutation of Cys$^{128(3.35)}$ in helix 3 into Ala, Phe, Leu, Met, Try, Ser, Tyr, and Thr gave constitutively active forms of the receptor, with large, bulky, or hydrophobic residues inducing greater constitutive activity [36]. The increase in constitutive activity correlated with increased affinity of the receptor mutants for

epinephrine. Increased constitutive activity was interpreted as the result of enhanced translational motion of helix 3 induced by the mutations.

It has been reported that constitutive activation of the α_{1B}AR could result from mutations of the $Asp^{125(3.32)}$ (i.e., the helix 3 aspartate of the putative ligand binding site) into alanine or lysine and those of $Lys^{331(7.36)}$ in helix 7 into Ala, Glu, Asp, His, Gln, Met, Leu, and Try [36]. The $Asp^{125(3.32)}Lys/Lys^{331(7.36)}Asp$ switch mutant displayed basal signaling activity similar to that of the wild-type receptor. These results, together with those from pH-dependent binding studies, suggested a potential mechanism of α_{1B}AR activation. The hypothesis was that, when the α_{1B}AR ligand binding pocket is empty, a salt bridge between $Lys^{331(7.36)}$ (helix 7) and $Asp^{125(3.32)}$ (helix 3) constrains the receptor in its ground state. Upon docking, the protonated amine of epinephrine competes with the protonated amine of $Lys^{331(7.36)}$ for the $Asp^{125(3.32)}$ counterion's negative charge. Destruction of the $Asp^{125(3.32)}/Lys^{331(7.36)}$ salt bridge might release a structural constraint, thus triggering the α_{1B}AR activation [36]. However, our group was not able to confirm that mutations of $Asp^{125(3.32)}$ increased the constitutive activity of the α_{1B}AR [3]. The reasons for this discrepancy, discussed above, should be further investigated. However, the rhodopsin-based models of the α_{1B}AR do not support the hypothesis that a salt bridge between $Asp^{(3.32)}$ and $Lys^{(7.36)}$ would stabilize the inactive state of the αARs [25].

Constitutive activation could also be induced by mutation of $Ala^{204(5.39)}$ into valine in the hamster α_{1B}AR and of Met^{292} into leucine in the rat α_{1A}AR. It is interesting to note that Ala^{204} and Met^{292} are residues involved in selectivity for agonists in the α_{1B}AR and the α_{1A}AR, respectively [37].

Recently, constitutively activating mutations of the α_{1B}AR have been described at two residues, $Glu^{289(6.30)}$ and $Phe^{303(6.44)}$, in the cytosolic half of helix 6 [25]. Mutations of $Glu^{289(6.30)}$, at the cytosolic end of helix 6, into Ala, Asp, Phe, Lys, and Arg resulted in a marked increase in the constitutive activity of the receptor as well as in its affinity for epinephrine. It is worth noting that, in one of the three so far released crystal structures of dark rhodopsin, $Glu^{289(6.30)}$ is involved in a salt bridge with the arginine of the Glu/AspArgTyr motif [24]. MD simulations on the α_{1B}AR models based upon the rhodopsin structure suggest that the salt bridge between positions 3.50 and 6.30 might be a feature of the inactive state [25].

Replacement of the highly conserved $Phe^{303(6.44)}$ with leucine also resulted in increased constitutive activity of the α_{1B}AR [25]. In contrast, mutations of this residue into Ala, Gly, Asn, and Tyr impaired the receptor-mediated inositol phosphate response. It was suggested that the replacement of $Phe^{303(6.44)}$ with leucine perturbs the helix 3–helix 6 packing interactions, thus resulting in constitutive activation of the α_{1B}AR.

Finally, it was recently also found for the human α_{1A}AR that mutations of $Asp^{123(3.49)}$, the aspartate of the Glu/AspArgTyr sequence, into isoleucine and of $Ala^{271(6.34)}$ into glutamate or lysine increase the constitutive activity of the receptor, similarly to the situation with the α_{1B}AR [38].

Altogether these findings support the hypothesis that the process of agonist-independent activation for both the α_{1A} and α_{1B}AR subtypes involves the breaking of interhelical interactions between the cytosolic halves of helices 3 and 6. Evidence for critical

roles of transmembrane domains 3 and 6 in GPCR activation is also provided by fluorescence studies with the β_2AR and is discussed in Chapter 3.

An interesting question concerns the functional relationship between activating mutations located in apparently distant regions of the receptors and whether they result in similar conformational changes. One way to address this question is to combine different activating mutations, but very few studies of this type have been done on different GPCRs. Interestingly, in the α_{1B}AR, the activating mutations Cys$^{128(3.35)}$ to Phe and Ala$^{293(6.34)}$ to Glu were combined and their functional effects were found to be synergistic, thus suggesting that each mutation acts independently and synergistically on the mechanism of receptor activation [39].

11.3.2
Constitutive Activation of Multiple Signaling Pathways

Despite the fact that the activation of the α_1AR subtypes causes polyphosphoinositide hydrolysis in almost all tissues in which this effect has been examined, these receptors can also stimulate other signaling pathways including the activation of phospholipases A_2 [40] and D [41] as well as extracellular signal-regulated kinases (ERKs) [42].

An intriguing question addressed by a few studies is whether GPCRs can adopt multiple active states that can selectively activate different signaling pathways. These active states could be induced either by activating mutations or by stimulation with different agonists.

In one study it was reported that a CAM of the α_{1B}AR (Ala^{293}Glu) was, like the wild-type receptor, able to activate both phospholipases C and D [41]. In another study, the abilities of two CAMs of the α_{1B}AR to activate multiple signaling pathways were compared [40]. Whereas the Ala^{293}Glu mutant could constitutively activate both phospholipases C and A_2, the Cys^{128}Phe mutant was able to activate only phospholipase C. These findings, together with those on some pharmacological differences between the two CAMs, have been interpreted in support of the hypothesis that the mutations can induce multiple states selective for different G proteins/effector pathways (see also Chapter 9). However, the biochemical mechanisms underlying the receptor-mediated activation of phospholipases C or A_2 (e.g., which G protein subunit is involved) have not been explored in detail in these studies and additional work should be performed to support this hypothesis.

11.4
A Putative Model of Receptor Activation for the α_{1B}AR

The results of computational and experimental mutagenesis of the α_{1B}AR suggest an important role of the conserved Glu/AspArgTyr motif in receptor activation. In particular, the results of molecular modeling emphasize that interactions between the arginine of the Glu/AspArgTyr motif and some amino acids forming a highly con-

served "polar pocket" ($Asn^{63(1.50)}$, $Asp^{91(2.50)}$, $Asn^{340(7.45)}$, $Asn^{344(7.49)}$, $Tyr^{348(7.53)}$) within the helical bundle contribute to stabilization of the ground state of the α_{1B}AR [21] (Figures 11.1 and 11.3).

In the latest computational model of the α_{1B}AR, shown in Figure 11.3 and based upon the first crystal structure of rhodopsin [24], the double salt bridge between the arginine of the Glu/AspArgTyr motif and both the adjacent aspartate and $Glu^{289(6.30)}$, as inherited from the rhodopsin structure, constitutes a feature of the ground state. At the experimental level, mutations of the $Glu^{289(6.30)}$ markedly increased the constitutive activity of the α_{1B}AR, similarly to the effect induced by mutation of the aspartate of the Glu/AspArgTyr motif [25].

In contrast, the structures of the active mutants, despite the different locations of the mutations, tend to share the release or weakening of one or both of the charge-reinforced H-bonding interactions involving $Arg^{143(3.50)}$ in the ground state. However, the lower degree of conservation of the anionic amino acid at 6.30 in relation to that at 3.49 supports the hypothesis that the latter plays a more important role in stabilizing the ground state of GPCRs. On similar lines, in the latest structures of rhodopsin $Arg^{3.50}$ retains its interaction with the adjacent glutamate while losing the charge-reinforced H-bond with $Glu^{6.30}$ [43, 44].

Altogether, these findings for the α_{1B}AR support the hypothesis that receptor activation involves the weakening or breaking of the interhelical interactions between the cytosolic halves of helices 3 and 6. This putative model of receptor activation seems to be shared by other GPCRs belonging to the rhodopsin-like class (reviewed in [45]). However, other potentially constraining interactions in GPCRs, beyond those involving helices 3 and 6, have also been described [46].

Interestingly, it has been suggested that a cluster of aromatic amino acids in helix 6 participates in the information transfer from the extracellular to the intracellular domains induced by structurally different agonists such as serotonin and the melanin concentrating hormone in their cognate receptors [47, 48]. It is suggested that the "most intracellular" member of this cluster, $Phe^{6.44}$ (Figure 11.1), may modulate the helix 3/helix 6 packing, consistently with the experimental results on the α_{1B}AR [25]. Another perturbation in the cytosolic domains shared by the constitutively active mutants and the agonist-bound forms is the breakage of the interaction found in the ground state between $Tyr^{7.53}$ of the AsnProXXTyr motif in helix 7 and a phenylalanine in helix 8. These observations are consistent with recent results on rhodopsin indicating that the AsnProXXTyr$(X)_{5,6}$Phe and Glu/AspArgTyr motifs provide, in concert, a dual control over the structural changes underlying activation in the photoreceptor [49].

In conclusion, the results of computational modeling suggest that the mutation- and agonist-induced active states of different GPCRs of the rhodopsin-like class, although different, share significant structural commonalties in the closeness of the Glu/AspArgTyr (helix 3) and AsnProXXTyr$(X)_{5,6}$Phe (helix 7) motifs (Figure 11.3). The transition between the inactive and active states should be thought of as a complex mechanism resulting from the interplay between loss and gain of interactions, involving highly conserved polar and aromatic amino acids.

A particularly important challenge for the future will be to explain how agonists can activate GPCRs, to what extent CAMs mimic the conformational changes induced by the agonists, and whether a common mechanism of activation is shared by GPCRs of different classes.

11.5 Constitutive Activity of Wild-type α_1ARs and Inverse Agonism

11.5.1 Constitutive Activity of Wild-type α_1AR Subtypes

The allosteric ternary complex model predicts that the constitutive activity measured in cells depends on the number of expressed receptor molecules in the active state **R*** (Figure 11.2). This prediction is clearly supported by findings demonstrating that increasing receptor density results in the progressive elevation of basal (agonist-independent) receptor-mediated production of second messengers [15].

Interestingly, the position of the equilibrium between **R** and **R*** (which depends on the magnitude of the equilibrium constant *J*) may vary for individual receptors and be responsible for the various level of constitutive activity of different wild-type GPCRs. It can also be speculated that different GPCRs might differ in their degrees of constitutive activity induced by activating mutations. This was clearly observed for the α_1AR subtypes, which differ both in the levels of constitutive activity displayed by the wild-type receptors and in those induced by mutations of homologous residues [38].

In HEK cells expressing the α_{1A} and α_{1B}Ars, as well as their respective CAMs carrying mutations of Ala^{271} or Ala^{293}, the following differences were observed: a) the agonist-independent activities both of the wild-type α_{1B}AR and of its CAMs were significantly higher than those of the wild-type α_{1A}AR or its CAMs, and b) both for the α_{1B}AR and for its CAMs, the epinephrine-induced inositol phosphate accumulations above basal were significantly higher than those of the α_{1B}AR or its CAMs expressed at comparable levels. This suggests that the agonist-occupied α_{1A}AR has greater efficacy than the α_{1B}AR in activating phospholipase C, whereas its spontaneous or mutation-induced isomerization towards the active states is lower [38]. These findings are in agreement with those from a previous study [50] describing the coupling efficiencies of different α_1AR subtypes expressed in HEK 293 or SK-N-MC neuroblastoma cells. In that study, the agonist-induced inositol phosphate response mediated by the α_{1A}AR was higher – whereas its agonist-independent activity was lower – than those of the α_{1B}AR expressed at similar level.

The properties of the α_{1D}AR subtype have been investigated very little, since both its expression and the agonist-induced inositol phosphate response mediated by this receptor have often been found to be much smaller than those of the other two subtypes [50]. Interestingly, the α_{1D}AR expressed in rat fibroblasts is constitutively active and internalized [42]. In fact, the basal activity of the α_{1D}AR was twice that of the α_{1B}AR and was increased after treatment with the inverse agonist prazosin, which caused its redistribution from the intracellular compartments to the plasma membrane. The con-

stitutive activity of the α_{1D}AR was also observed in physiological systems such as in aorta and mesenteric arteries, where it could inhibited by inverse agonists [51]. The constitutive activity of the α_{1A}AR or α_{1B}AR in physiological systems has not yet been investigated.

Taken together, these findings indicate that there are important differences in the constitutive activity of the α_1AR subtypes, which could have consequences in their signaling and regulatory properties *in vivo*. Such differences should be further explored and the elucidation of their physiological implications might represent an important area of investigation.

11.5.2
Inverse Agonism at the α_1ARs

The availability both of CAMs and of cell systems overexpressing wild-type GPCRs stimulated widespread interest in drugs with negative efficacy. Negative efficacy is commonly measured as the ability of receptor ligands to inhibit the agonist-independent activity of receptors. In most studies, ligands displaying negative efficacy have been denoted by the terms inverse agonists or negative antagonists. The terms do not make any specific reference to the mechanistic basis of their effect, which may, however, be more complex than commonly thought (see Chapter 2). In this chapter, receptor ligands displaying negative efficacy at the α_1AR subtypes are denoted by the term inverse agonists [52].

Both selective and nonselective antagonists for different AR subtypes are widely used in a variety of pathological conditions including hypertension, heart failure, and benign prostate hypertrophy as well as in mental diseases. Twenty-four αAR antagonists differing in their chemical structures were examined for their effect on the basal activity of both the wild-type α_{1A}AR and α_{1B}AR and on their CAMs [38]. Unexpectedly, the vast majority of αAR antagonists displayed inverse agonism. However, the various αAR antagonists differed in their negative efficacy and some of these differences depended on the α_1AR subtype. Indeed, a large number of structurally different αAR-antagonists including all the tested quinazolines were inverse agonists at both the α_{1A}AR and α_{1B}AR. In contrast, several *N*-arylpiperazines displayed different properties at the two α_1AR subtypes, being inverse agonists with profound negative efficacy at the α_{1B}AR, but not at the α_{1A}AR.

In the context of the allosteric ternary complex model, inverse agonists are assumed to have higher affinity for the inactive state **R**, decreasing the proportion of **R*** and thus resulting in the inhibition of receptor–G protein coupling [15]. However, the αAR inverse agonists did not display significantly different binding affinities for the constitutively active forms of the α_{1A} and α_{1B}AR subtypes. Thus, it was speculated that the mutation-induced increase in the isomerization constant J might not be sufficiently large to decrease the measurable affinity of the CAMs for the inverse agonists [38].

As mentioned above, inverse agonism has also been observed at the recombinant α_{1D}AR subtype as well as on the receptor expressed in arteries [50].

Several lines of evidence indicate that inverse agonists, beyond their ability to decrease constitutive activity, are also able to induce the "upregulation" of several CAMs. For the α_1AR subtypes, it has been reported that a variety of inverse agonists increase the receptor number measured by radioligand binding of a CAM, but not that of the wild-type α_{1B}AR [53]. The ability of inverse agonists to increase receptor number is linked to the instability of several CAMs. For example, CAMs of the histamine H_2 receptor (H_2R) and of the α_{1B}AR carrying mutations of the aspartate of the Glu/AspArgTyr motif displayed an increased instability compared to the wild-type receptors as demonstrated by time-dependent loss of binding activity at 37 °C, which could be prevented by incubation with inverse agonists [31]. Effects of inverse agonists on receptor "upregulation" have been reported for different GPCRs and have a variety of interpretations. It should be noted that, since the instability of CAMs is not accompanied by receptor degradation, the apparent "upregulation" induced by inverse agonists might not necessarily imply new receptor synthesis. In addition, as demonstrated for the H_2R, instability has been reported for both constitutively active and inactive receptor mutants [31]. Finally, the stabilization of receptor function is not a unique property of inverse agonists since it can be induced by various ligands acting as agonists or antagonists depending on the receptor system [31].

Altogether, an important question still to be answered is whether there are therapeutic differences and benefits in the clinical use of drugs displaying negative efficacy versus those that behave as neutral antagonists. In particular, inverse agonists with different degrees of negative efficacy might differ in their ability to induce upregulation of GPCRs upon chronic treatment. These problems remain an important challenge for future studies in the fields of both molecular and clinical pharmacology.

11.6 Receptor Regulation and Constitutive Activity of the α_1ARs

Because CAMs are thought to mimic, at least in part, the agonist-occupied forms of wild-type GPCRs it has been postulated that they should undergo constitutive phosphorylation and internalization. This hypothesis is supported by previous findings indicating that constitutively active β_2AR and α_{2A}AR with mutations in the distal portion of the i3 loop can be phosphorylated by G protein-coupled receptor kinases in the absence of the agonist [14, 54]. Thus, an interesting question, addressed by few studies, is whether CAMs carrying mutations in different receptor domains share similar regulatory properties.

The phosphorylation and internalization properties of two CAMs of the α_{1B}AR, the Ala^{293}Glu and Asp^{142}Ala mutants, were compared in one study [55]. Whereas the Ala^{293}Glu mutant displayed significantly higher agonist-independent phosphorylation than the wild-type α_{1B}AR, the Asp^{142}Ala mutant did not. In addition, whereas the Ala^{293}Glu mutant displayed increased constitutive internalization, the Asp^{142}Ala mutant was impaired in its ability to interact with β-arrestin and to undergo internalization. These findings demonstrated that constitutively activating mutations might have divergent effects on the regulatory properties of the receptor. Thus, the paradigm that

they result in constitutive phosphorylation and/or internalization cannot be generalized.

In addition, these results highlight the fact that the effects of different mutations on receptor regulation must be taken into account for interpretation of constitutive activity. For example, a mutation that decreases phosphorylation and/or internalization of a receptor might apparently increase its constitutive activity. Vice versa, mutations that increase its phosphorylation and/or internalization might be interpreted as inactivating the receptor.

The relationship between receptor phosphorylation and constitutive activity was further investigated at the α_{1B}AR [56]. To this end, the phosphorylation sites for G protein-coupled receptor kinases (Ser^{404}, Ser^{408}, and Ser^{410}) and protein kinase C (Ser^{394} and Ser^{400}) were mutated in the two CAMs Ala^{293}Glu and Asp^{142}Ala. Mutations of the phosphorylation sites increased the constitutive activities of the Ala^{293}Glu and Asp^{142}Ala mutants by about 40 and 200%, respectively.

Altogether, these findings provided evidence that receptor phosphorylation can induce a tonic attenuation of both the constitutive and the agonist-dependent response of the α_{1B}AR, which can be relieved through mutations in the phosphorylation domain. Therefore, mutations in domains involved in receptor regulation might also be a strategy to increase the constitutive activity of GPCRs.

11.7 Conclusions

Studies of CAMs of the α_1AR subtypes and of other GPCRs have had an important impact on our understanding of several basic aspects of GPCR function and drug action. Despite the large number of studies on CAMs, several important questions remain to be answered. Structural information at high resolution on GPCRs other than rhodopsin will be needed to test the predictions of molecular modeling and to improve our understanding of GPCR activation and drug action at a molecular level. Assessment of the relevance of constitutive activity of GPCRs *in vivo* will be important for understanding of the implications of drugs with negative efficacy, evaluation of their benefits, and improvement of future therapeutic strategies.

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12
Constitutive Activity of Muscarinic Acetylcholine Receptors: Implications for Receptor Activation and Physiological Relevance

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12.1
Introduction

The G protein coupled receptors (GPCRs), with seven transmembrane domains, comprise the largest gene family in humans, with approximately 1000 members. In 1989 it was shown that the δ opioid receptor (DOPR) exhibits agonist-independent activity, a phenomenon termed "constitutive activity" [1]. In 1993, the extended ternary complex model, incorporating the ternary complex model [2] and theory on allosteric transitions of enzymes [3], was proposed to account for agonist-independent activity of mutant α- and β-adrenoceptors (see Chapters 9–11) [4]. In this model, receptors spontaneously interconvert between inactive (R) and active (R*) conformations (see also Chapters 2 and 3). Agonists stabilize active receptor conformation, inverse agonists stabilize inactive receptor conformation, and neutral antagonists leave the equilibrium unchanged. The active receptor conformation binds G proteins with high affinity (recently confirmed for agonist occupied receptors; see [5]) and catalyze G protein activation. The extended ternary complex model has been further developed into a cubic model [6] and multiple active and inactive states are now believed to exist [7]; however, the basic principles of the extended ternary complex model still remain valid.

It follows from the extended ternary complex model that all GPCRs possess some degree of constitutive activity. In a practical sense, constitutive activity can be thought of as 'observable' agonist-independent activity which is dependent upon the assay system used (see also Chapter 8). The physiological and therapeutic implications of constitutive activity are not yet fully understood, but several diseases including male precocious puberty [8], retinitis pigmentosa [9], and thyroid adenomas [10] are at least in some cases caused by polymorphisms increasing the constitutive activation of GPCRs (see also Chapter 7). Besides mutant receptors, many examples of constitutively active wild-type GPCRs in native systems have been described.

The muscarinic receptor family is made up of five subtypes (M_1R–M_5R), of which M_1R, M_3R, and M_5R primarily couple to G_q to stimulate phospholipase C-catalyzed phosphatidyl inositol hydrolysis, whereas M_2R and M_4R primarily couple to pertussis toxin-sensitive G_i proteins to inhibit cAMP production [22, 23]. Here we review the

literature describing constitutive activity of muscarinic acetylcholine receptors in native and recombinant systems, and discuss the effect of constitutive activity on muscarinic pharmacology. Finally, we summarize the structure–function data on constitutive activity and the activation mechanism of muscarinic receptors obtained through mutagenesis experiments.

12.2 Constitutive Activity – Native Systems

The physiological relevance of constitutive activity for muscarinic receptors was first suggested by Hilf and Jakobs, who showed that atropine, a classic muscarinic receptor antagonist, inhibits M_2R-mediated activity in isolated heart tissue [11]. Specifically, atropine and several other muscarinic receptor antagonists reduced the binding of guanosine 5'-[γ-thio]triphosphate (GTPγS) in porcine atrial membranes below basal values by approximately 25%, both in the absence and in the presence of the agonist carbachol, a stable analogue of the naturally occurring muscarinic agonist acetylcholine. This inhibitory action of atropine was not due to antagonism of endogenous acetylcholine. Hilf and Jakobs proposed that unliganded muscarinic receptors interact with, and activate, G_i proteins in native cardiac membranes and that antagonist binding induces a conformational change in the receptor, preventing either G protein activation or turnover. Subsequently, atropine-sensitive constitutive activity was observed in transfected CHO cells [12], and inverse agonist-mediated suppression of basal muscarinic receptor activity in cardiomyocytes was shown to be stereospecific, confirming that this was a receptor-mediated effect [13].

All examples of constitutive activity of muscarinic receptors in native systems have used heart tissue. This may be because the measurement of constitutive activity is greatly dependent on receptor expression levels [14, 15] (see also Chapter 8). Heart tissue contains high levels of the M_2R subtype, and it is easy to obtain large quantities of material suitable for biochemical and pharmacological experiments.

12.3 Constitutive Activity – Recombinant Systems

Constitutive activity of all five muscarinic receptor subtypes has been observed with use of recombinant systems, in which high expression levels are easily attainable. Jakubik et al. demonstrated constitutive activity of muscarinic receptors in stably transfected CHO cell lines expressing recombinant M_1R, M_2R, M_3R, and M_4R [12]. The M_2R and M_4R subtypes were found to exert a tonic, antagonist-sensitive inhibition of cAMP production, while M_1R and M_3R mediated agonist-independent inositol phosphate production that was also reduced by antagonists. Cell lines expressing higher and lower (41 fmol vs. 6.6 fmol per million cells) levels of M_1R were tested to address whether or not the observed constitutive signaling was physiologically relevant. Though the magnitude of the effect was reduced, there was observable suppression of basal in-

ositol phosphate production in the cells expressing lower levels of M_1R, suggesting that constitutive activity may produce significant responses at physiologically relevant levels of receptor expression. More recently, constitutive activity up to 66% of the maximal GTPγS binding by carbachol was attained by use of baculovirus-expressed M_2R at very high expression levels (>1 pmol mg^{-1}) [16].

The M_5R was shown to be constitutively active in an assay system that used cellular proliferation as a measure of receptor activation [17–19]. The constitutive activity observed in these studies was low but consistent and amounted to approximately 5% of the maximum response to carbachol. The M_1R, M_2R, and M_3R subtypes were subsequently shown to display low levels of constitutive activity in the same cellular proliferation assay [15].

12.4
Constitutive Activation by G Proteins

The ternary complex model was originally proposed to explain the widely observed phenomenon that inclusion of non-hydrolyzable GTP analogues in binding assays promotes conversion of high-affinity agonist binding sites into a uniform population of low-affinity agonist-binding sites [2] (see also Chapter 8), and was extended to explain constitutive activity [4]. A feature of this model is that it predicts that the active (R*), but not the inactive (R), conformation of the receptor binds G proteins with high affinity. A further prediction of this model is that increasing the concentration of G protein should stabilize the active receptor conformation and thus increase constitutive activity. To test this hypothesis, we examined constitutive coupling of muscarinic receptors to different signaling pathways and the effects of varying the levels of transducing G proteins. Overexpression of the α subunit of G_q markedly enhanced the level of agonist-independent activity mediated by M_1R, M_3R, and M_5R in a cellular proliferation assay [20, 21]. Overexpression of $G\alpha_q$ also potentiated the responses to full agonists and increased the maximal responses to partial agonists, consistently with the extended ternary complex model [21]. These studies also demonstrated that atropine, pirenzepine, *N*-methyl scopolamine (NMS), quinuclidinyl benzilate (QNB), trihexyphenidyl, and 4-diphenylacetoxy-*N*-methylperidine (4-DAMP) are inverse agonists at muscarinic receptors. However, in contrast to its potentiating effect on agonists, overexpression of $G\alpha_q$ did not significantly affect the potency of inverse agonists in relation to their abilities to inhibit carbachol-induced activity.

Promiscuous coupling of receptors to multiple signal transduction pathways is well documented. Although M_1R, M_3R, and M_5R primarily stimulate phosphatidyl inositol hydrolysis and M_2R and M_4R primarily inhibit cAMP production [22, 23], all five subtypes, with varying degrees of efficiency, activate other signal transduction pathways including stimulation of cAMP production, stimulation of phosphatidyl inositol hydrolysis through pertussis toxin-sensitive G proteins, stimulation of arachidonic acid release, and activation of ion channels [12, 24–30]. To date, constitutive coupling of muscarinic receptors to less preferred pathways, whether naturally occurring or artificially created, has not been reported. In contrast to its effects on M_1R, M_3R,

and M_5R, there was little effect of $G\alpha_q$ overexpression on constitutive activity of M_2R, though $G\alpha_q$ did enable agonist-mediated responses to M_2R [31]. Over-expression of $G\alpha_{12}$, which is not known to couple muscarinic receptors endogenously, was only able to mediate slight agonist-dependent responses to M_2R, and did not affect the basal activity of M_2R or M_5R [31]. Similarly, the chimeric G protein with the five C-terminal amino acids of $G\alpha_q$ replaced by $G\alpha_{i2}$, which enables stimulation of phosphatidyl inositol hydrolysis by G_i/G_o-coupled receptors [32], could enable robust agonist-stimulated responses to the M_2R and M_4R, but only very weak constitutive responses [15]. Thus, it appears that observable levels of constitutive activity in most assay systems can be modulated by altering either receptor or G protein levels, although not enough to produce significant constitutive responses through artificial or less preferred signal transduction pathways.

Migeon and Nathanson used a cAMP-driven luciferase assay to show that both M_1R and M_4R could inhibit cAMP-production in an agonist-dependent manner, but that only M_4R could constitutively suppress reporter activity [24]. These results indicate that M_4R constitutively activates pertussis toxin-sensitive G proteins to suppress reporter activity (i.e., M_4R constitutively activates its preferred signaling pathway), while M_1R stimulation of reporter activity was not coupled efficiently enough to produce agonist-independent activity.

To study the relationship between receptor–effector coupling and receptor density, the effects of porcine M_2R receptor expressed at varying densities on three signal transduction outputs – inhibition of cAMP production, stimulation of cAMP production and stimulation of phosphatidyl inositol hydrolysis – were examined [30]. Muscarinic antagonists (QNB, NMS, hyoscyamine) increased agonist-independent production of cAMP, presumably by inhibiting tonic suppression of cAMP production through pertussis toxin-sensitive G proteins. No effect of these inverse agonists was reported on the other effector systems. Thus, it appears that constitutive activity was observable only for the preferred coupling pathway. Similarly, antagonists reduced basal cAMP inhibition by M_2R and M_4R and basal phosphotidyl inositol hydrolysis by M_1R and M_3R subtypes, but not basal stimulation of cAMP production [12]. This was true for both high and low levels of receptor expression.

12.5 Structure–Function Analysis of Receptor Activation

Structure–function analysis of muscarinic receptors has provided an understanding of the ligand binding sites in the receptors, the G protein coupling domains, the activation mechanisms, and the overall topology of the receptors [22, 33–34]. These studies have uncovered a wealth of ligand binding sites on the muscarinic receptors. Classic antagonists such as NMS and classic agonists such as acetylcholine appear to bind through an orthosteric binding site made up of resides located in the extracellular third of transmembrane (TM) domains 3, 5, 6, and 7, with contributions to binding affinity from TM4 (see [33, 34] for reviews). The binding sites overlap extensively around TM3, but differ slightly in TM5 and 6 [33, 35]. In contrast, the agonist activity of the M_1R-

Table 12.1 Summary of constitutively activating mutations in the muscarinic receptor family. This table summarizes residues in which single-point mutations caused an increase in constitutive activity or a greater than threefold increase in the potency of agonists. Agonist potencies measured in radioligand binding assays, corrected to account for radiolabel concentration by the method of Cheng and Prusoff (see [95]), were used where available. Other papers describe constitutively active receptors containing more than one mutation [15, 17, 45, 96]. A mutant M_1R with mutations at Ser[388(6.58)] and Thr[389(6.59)] that shows a markedly increased affinity for agonists has been described [54].

	Residue	To[a]	BW#[b]	Increase in constitutive activity		Increase in agonist potency			Reference
				Yes/no	Assay	Fold increase	Agonist	Assay	
M_1R	Asn[43]	Ala	1.50			10	ACh	[³H]-NMS[c]	65
M_1R	Asn[61]	Ala	2.40	No	PI[d]	7	ACh	[³H]-NMS	65
M_1R	Leu[64]	Ala	2.43	No	PI	5	ACh	[³H]-NMS	65
M_1R	Asp[71]	Asn	2.50			5	CCh	[³H]-QNB[c]	48
M_1R	Val[113]	Ala	3.40	Slight	PI	10	ACh	[³H]-NMS	49
M_1R	Leu[116]	Ala	3.43	Yes	PI	38	ACh	[³H]-NMS	49
M_1R	Ser[120]	Ala	3.47	Yes	PI	4	ACh	[³H]-NMS	49
M_1R	Asp[122]	Asn+[a]	3.49	No	PI	7	ACh	[³H]-NMS	79
M_1R	Tyr[124]	His	3.51	No	PI	3	ACh	[³H]-NMS	79
M_5R	Phe[130]	Gln+	3.52	Yes	R-SAT[e]	13	CCh	[³H]-NMS	17
M_5R	Arg[134]	Glu+	3.56	Yes	R-SAT	3	CCh	[³H]-NMS	17
M_5R	Thr[137]	Ile	3.59	Yes	R-SAT	10[f]	CCh	R-SAT	17
M_5R	Tyr[138]	Thr+	3.60	Yes	R-SAT	6	CCh	[³H]-NMS	17
M_5R	Arg[142]	His+	3.64	Yes	R-SAT	22	CCh	[³H]-NMS	17
M_1R	Ala[160]	Gly	4.60	Yes	PI	3	ACh	[³H]-NMS	62
M_1R	Phe[190]	Cys	5.40	Yes	PI	7	ACh	[³H]-NMS	64
M_5R	Thr[220]	Val+	5.65	Yes	R-SAT	0.9	CCh	[³H]-NMS	71
M_1R	Glu[360]	Ala	6.30	Yes	PI	11[g]	CCh	[³H]-NMS	76
M_3R	Gln[490]	Leu	6.35	Yes	PI	4	CCh	[³H]-NMS	99
M_2R	Ile[390/1]	Insertion	6.38/6.39	Yes	PI	0.7–1	ACh	[³H]-NMS	77
M_5R	Ile[447]	Ser	6.40	Yes	R-SAT	120	CCh	[³H]-NMS	98
M_5R	Ala[450]	Val	6.43	Yes	R-SAT	3[f]	CCh	R-SAT	98
M_5R	Phe[451]	Val+	6.44	Yes	R-SAT	9	CCh	[³H]-NMS	98
M_5R	Asn[459]	His+	6.52	Yes	R-SAT	1	CCh	[³H]-NMS	98
M_3R	Asn[507]	Ser	6.52	Yes	PI	0.7	ACh	[³H]-QNB	53
M_5R	Ser[465]	Phe+	6.58	Yes	R-SAT	50	CCh	[³H]-NMS	97
M_1R	Trp[405]	Ala	7.40	Yes	PI	2	ACh	[³H]-NMS	62
M_1R	Asn[410]	Ala	7.45	No	PI	4	ACh	[³H]-NMS	62
M_1R	Asn[414]	Ala	7.49	No	PI	17	ACh	[³H]-NMS	62
M_1R	Pro[415]	Ala	7.50	No	PI	21	ACh	[³H]-NMS	62
M_3R	Pro[540]	Ala	7.50			7	ACh	[³H]-NMS	51
M_1R	Tyr[418]	Ala	7.53	No	PI	14	ACh	[³H]-NMS	62
M_1R	Tyr[418]	His	7.53			15	ACh	[³H]-NMS	63

[a] Where more than one constitutively activating mutation was found at a single position in a single paper, a well characterized example is shown, plus the symbol "+".

[b] BW# represents the residue number according to the system of Ballesteros and Weinstein [37].

[c] [³H]-*N*-methylscopolamine and [³H]-quinuclidinyl benzilate radioligand inhibition binding assays.

[d] PI: Stimulation of phosphatidyl inositol degradation.

[e] R-SAT: Receptor Selection and Amplification Technology [97].

[f] Affinity shift represents change in EC_{50} for carbachol.

[g] Affinity shift represents change in IC_{50} for carbachol. Values cannot be corrected for radioligand concentration, as the K_d of the radioligand was not calculated. Carbachol showed 6.3-fold higher potency on the Gln[360(6.30)] to Ala mutant relative to wild type in phosphatidyl inositol assays. Expression levels were similar.

selective ligand AC-42 required the M_1R sequence in the N terminus/TM1 and third extracellular loop/TM7 of the receptor [36], although substitution of these regions with the M_5R sequence has no effect on the activity of carbachol, an acetylcholine analogue. Additionally, mutation of $Tyr^{381(6.51)*}$ to alanine in TM6 of M_1R abolished the stimulatory effects of both acetylcholine and carbachol, but had little effect on the agonist activity of AC-42 (4-*n*-butyl-1-[4-(2-methylphenyl)-4-oxo-1-butyl]-piperidine hydrogen chloride; see [36]) or *N*-desmethylclozapine, and massively potentiated the agonist activity of clozapine [38], clearly demonstrating differences in the activation sites of these ligands. Finally, allosteric modulators such as gallamine bind through residues located in the third extracellular loops [39, 40], and competition experiments have revealed that other modulators (analogues of WIN 62 577) interact through a distinct and as yet uncharacterized site [41]. G protein coupling to the muscarinic receptors is mediated through the internal loops [42, 43]. Mutagenesis studies have established the Arg in the highly conserved Asp-Arg-Tyr region at the base of TM3 and the N and C termini of intracellular loop 3 (Ni3 and Ci3) as crucial for mediating G protein activation [42–44].

There are numerous mutagenesis studies showing constitutive activation of muscarinic receptors (see Table 12.1). Increases in constitutive activity could potentially arise from mutations that alter the equilibrium between the inactive receptor conformation with active conformations (either by destabilizing the inactive conformation or by stabilizing an active conformation), increase G protein affinity, promote G protein activation, or increase receptor expression. Many mutations that increase the affinity of agonists such as acetylcholine have also been described (see Table 12.1). Changes in agonist potency are a predicted consequence of changing the equilibrium between the active and inactive receptor conformations [18, 45, 100], and so, although mutations could directly affect the strength of interaction between the receptor and the agonist, in most cases it is considered more likely that the observed increases are due to increased constitutive activity.

Here we have organized the data for both types of mutation by receptor region, starting with the extracellular and transmembrane domains, and followed by the cytoplasmic domains.

12.5.1 Transmembrane Domain 3

TM3 is a crucial region for agonist activation of muscarinic receptors. Acetylcholine mustard, a reactive analogue of acetylcholine, covalently bound to $Asp^{105(3.32)}$ in TM3 of the M_1R [46] and mutations to $Asp^{105(3.32)}$ abolish agonist activation of the receptor [47, 48]. Given its pivotal role in agonist activation, it is therefore not surprising that many

* Superscripts in parentheses represent the residue number according to the system of Ballesteros and Weinstein [37]. The digit before the dot represents the TM domain. The two digits after the dot show the position of the residue relative to the most conserved position in the helix, given the number '50'. For example, in M_5R, $Arg^{128(3.50)}$ in the DRY motif is the most conserved residue in TM3. The adjacent Asp is numbered $Asp^{127(3.49)}$, and the adjacent Tyr is $Tyr^{129(3.51)}$.

constitutively activating mutations in TM3 have been described. A scanning mutagenesis study of amino acid residues 100–121 in the M_1R, comprising much of TM3, was performed and confirmed the crucial role of $Asp^{105(3.32)}$ in receptor activation by acetylcholine and identified several other crucial residues [49]. Besides these loss of function mutants, three gain of function mutations were identified: $Val^{113(3.40)}$, $Leu^{116(3.43)}$, and $Ser^{120(3.47)}$. These mutations were characterized by significant increases in acetylcholine affinity, and in two cases ($Ser^{120(3.47)}$ and $Leu^{116(3.43)}$), agonist-independent activity that was reversible by atropine was also observed. Substitutions at each of these residues caused significant decreases in receptor expression, suggesting these mutants were less stable than the wild-type receptor. In addition, numerous substitutions in TM3 had little effect on ligand binding or signaling properties of M_1R. When mapped in a helical net representation, these residues formed one face of the TM3 helix, which probably faces the lipid bilayer. Residues important for receptor activation formed another face, which the authors suggested forms intramolecular and intermolecular (receptor–ligand or receptor–G protein) interactions [49]. The three residues showing "gain of function" mutations formed a small contiguous area believed to hold the receptor in an inactive conformation. It was proposed that acetylcholine binding strengthens the first set of intramolecular contacts while weakening the second, thereby switching the receptor conformation to an active configuration.

12.5.2
Transmembrane Domain 6

TM6 is also known to be a contact site for both muscarinic agonists and antagonists [35, 50–53]. To explore the functional role of this domain fully, we performed random saturation mutagenesis, using a PCR-based strategy (see [101] for technical details; see also [17, 19, 45, 69, 78]) on the M_5R, and coupled this with a functional screen to search for novel phenotypes [18, 19, 45]. Unlike in the scanning mutagenesis studies, all residues in the target region were randomly mutated to all amino acid substitutions, and most of the resultant receptors had multiple mutations. Only functional receptors were sequenced. We hypothesized that residues important for interactions with ligands or the activation of mechanism of the receptor would be identified because they would never be mutated in the functional receptors. Seven of these "functionally conserved" residues were found in TM6: $Thr^{454(6.47)}$, $Trp^{455(6.48)}$, $Pro^{457(6.50)}$, $Tyr^{458(6.51)}$, $Val^{462(6.55)}$, $Leu^{463(6.56)}$, and $Val^{464(6.57)}$. This was consistent with the results of Wess et al. [35, 50, 51] showing that mutation of $Tyr^{506(6.51)}$ in M_3R to Phe severely inhibited carbachol activity and binding, and that mutation of $Pro^{505(6.50)}$ to alanine strongly decreased receptor expression.

Numerous constitutively active receptors were seen in the random mutagenesis study, and five residues where mutations could cause constitutive activity ("constitutively activating residues") were identified. One of the most activated receptors contained two substitutions on the extracellular end of TM6, a region not conserved between the five muscarinic subtypes [45]. This receptor displayed strong, agonist-independent activity and markedly increased affinity for agonists. Atropine and all other

muscarinic antagonists tested displayed inverse agonism, reversing the agonist-independent activity of this receptor. We inferred that mutation of $Ser^{465(6.58)}$ conferred the constitutive activity relative to other mutant receptors. To confirm this, and to explore the mechanistic reasons for the observed constitutive activity, point mutations representing all classes of amino acids were introduced at position 465 [18]. Replacement of $Ser^{465(6.58)}$ with large (Phe and Val) or basic (Arg and Lys) residues increased the constitutive activity of the receptor to the greatest extent (55 and 110% of the maximum response of the wild-type receptor to the agonist carbachol). Other substitutions (e.g., Cys and Leu) increased the constitutive activity to an intermediate level (30%), while small and acidic residues (Gly, Asp, and Glu) caused small or insignificant increases, suggesting that steric effects at the top of the helix were causing the increased constitutive activity. The increase in the constitutive activity of each mutant receptor correlated with an increase in the potency of carbachol in both binding and functional assays, while the potency of the inverse agonist atropine did not change. A two-state model of receptor function was fitted to the data, and the observed changes in carbachol potency could be explained almost entirely by a shift in the equilibrium between the inactive receptor conformation and an active conformation bound by carbachol. Since multiple substitutions caused constitutive activity, substitutions to $Ser^{465(6.58)}$ probably destabilized the inactive conformation of the receptor, shifting the equilibrium to favor the active conformation. Subsequent studies showed that analogous mutations in the other muscarinic receptor subtypes had a similar effect [15, 54].

Four other residues in which point mutations caused constitutive activity were identified in TM6 [19]. Two of these, $Phe^{451(6.44)}$ and $Asn^{459(6.52)}$, were mutated to other residues. Most mutations to $Phe^{451(6.44)}$ increased constitutive activity, but in contrast to mutations at $Ser^{465(6.58)}$, many also decreased the potency of carbachol and atropine, suggesting that they also disrupted the orthosteric binding site.

An M_3R in which the residue analogous to $Asn^{459(6.52)}$ – $Asn^{507(6.52)}$ – was mutated to Ser was constitutively active and had dramatically lower affinity for antagonists, suggesting this residue directly contacted atropine, locking the receptor in an inactive conformation [53]. In M_5R, only mutation of $Asn^{459(6.52)}$ to Ser or His constitutively activated the receptor, and the mutations affected both atropine and carbachol potency [19].

The importance of $Tyr^{381(6.51)}$, $Asn^{382(6.52)}$, and $Leu^{386(6.56)}$ in agonist binding was confirmed by alanine scanning between $Tyr^{381(6.51)}$ and $Val^{387(6.57)}$ in M_1R [52]. No constitutively active mutants were identified in this study, which concentrated on the agonist-binding portion of TM6. When arranged on a helical net, the "constitutively activating" and "functionally conserved" residues identified in these studies lay on one face of the TM6 helix, while residues that tolerated radical substitutions lay on the other [19, 52]. The results suggested that the constitutively activating/functionally conserved face of TM6 was involved in intermolecular and intramolecular interactions, while the other face made nonspecific interactions with the membrane. Coupled with structural data from rhodopsin [55] and experiments demonstrating direct physical movements of TM6 in other receptors [56–60], the dual observation that TM6 is involved both in ligand binding and in stabilizing the inactive receptor confirmation suggests that TM6 is a switch that defines the activation state of the receptor, and that

ligand interactions with TM6 stabilize the receptor either in an active or in an inactive conformation. Experiments identifying a 'toggle switch' within TM6, which governs the transition back and forth between active and inactive conformations, also support these assertions [61].

12.5.3 Transmembrane Domain 7

A thorough scanning mutagenesis study of TM7 in M_1R identified one constitutively activating mutation (Trp$^{405(7.40)}$ to Ala) that increased constitutive activity to a level equivalent to 12–20 % of the carbachol response of the wild-type receptor. Additionally, four Ala mutations in the intracellular region of TM7 – Asn$^{410(7.45)}$, Asn$^{414(7.49)}$, Pro$^{415(7.50)}$, and Tyr$^{418(7.53)}$ – increased the potency of acetylcholine over threefold in binding assays [62]. Mutation of Asn$^{414(7.49)}$ to Ala abolished all signaling through phosphatidyl inositol hydrolysis even after atropine preincubation, which increased the expression of [^{3}H]-NMS binding sites to 117 % of the wild-type level. Similarly, His substitution of Asn$^{414(7.49)}$ and Tyr$^{418(7.53)}$ increased acetylcholine affinity but essentially abolished G_q-mediated phosphoinositide signaling [63]. Finally, replacement of Pro$^{540(7.50)}$ with Ala in the M_3R caused a 7–19-fold increase in affinity for carbachol, but severely impaired the receptor's ability to stimulate carbachol-induced phosphatidyl inositol hydrolysis (its maximum response was approximately 25 % of that of wild-type M_3R) and decreased expression [51].

Mutations in the extracellular part of the helix, including Ala substitutions of Tyr$^{404(7.39)}$, Cys$^{407(7.42)}$, and Tyr$^{408(7.43)}$, have been shown to decrease acetylcholine potency [35, 50, 62]. Again, these functionally conserved residues would align with the activating residues in TM7 on one side of an α helix. In this case, the activating residues are intracellular to the conserved residues.

Asn$^{414(7.49)}$, Pro$^{415(7.50)}$, and Tyr$^{418(7.53)}$ make up the Asp-Pro-x-x-Tyr motif conserved in the monoamine receptors and rhodopsin. They mark the intracellular end of TM7 at its transition to a short linking sequence followed by helix 8 [55]. Lu and Hulme created a high-affinity Zn^{2+} binding site by mutating Leu$^{116(3.43)}$, Phe$^{374(6.44)}$, and Asn$^{414(7.49)}$ to His [63]. This provided evidence that the residues lie in close proximity, at least in the inactive receptor, and it is possible that direct van der Waals contacts exist between their side chains. Asn$^{414(7.49)}$ and Tyr$^{418(7.53)}$ may resemble Leu$^{116(3.43)}$, Ser$^{120(3.47)}$, and Phe$^{374(6.44)}$ in making intramolecular contacts that stabilize the inactive ground state of the receptor. However, the loss of signal when they are mutated implies that they are also important for the formation of the agonist–receptor–Gq protein signaling complex. This is consistent with the high degree of conservation in this region. It has been proposed that residues in TM3, TM6, and TM7 may form part of a network of conserved intramolecular contacts that maintain the off state of the receptor [63].

12.5.4
Other Transmembrane Domains and Extracellular Domains

Constitutively activating mutations are not common in TM5. In general, mutation of residues in TM5 decreased acetylcholine affinity and potency, though the mutation $Phe^{190(5.40)}$ to Cys in M_1R displayed increased acetylcholine affinity and potency, and increased basal activity [64]. Several potential contact points for acetylcholine were identified, in agreement with previous studies identifying this domain as important for ligand binding.

Scanning mutagenesis of TM4 identified one constitutively activating mutation: $Ala^{160(4.60)}$ to Gly [62]. This receptor showed a slightly increased affinity for acetylcholine, but no other activating mutations were found in this domain. Surprisingly many mutations in TM4 were found to decrease acetylcholine affinity and potency, suggesting a greater role for TM4 in receptor activation than previously expected.

Scanning mutagenesis experiments in TM2 [65] showed that Ala substitution of $Asn^{61(2.40)}$ and $Leu^{64(2.43)}$ in M_1R caused increases in acetylcholine potency. Both residues have been proposed to fulfil a role in stabilizing the ground state of the M_1R [33]. Substitution of the highly conserved residue $Asp^{71(2.50)}$ in M_1R increased the affinity of carbachol fivefold, though the receptor could not be further stimulated by carbachol [48].

Mutation of $Asn^{43(1.50)}$ in TM1 of the M_1R increases acetylcholine affinity tenfold, but also causes a tenfold decrease in receptor expression and a tenfold decrease in signaling efficacy [65].

The N terminus and extracellular loops of muscarinic receptors have not yielded activating mutations, though constitutively activating mutations in these regions have been described for other receptor families [9, 66–68]. This could reflect that less testing of these regions has been performed, or that these regions do not play as dynamic a role in the transition between active and inactive states of muscarinic receptors. It was recently shown that the N terminus of the M_1R might contribute to an alternate activation domain for 'ectopic', or agonists allosterically acting outside the acetylcholine binding pocket [36].

12.5.5
Cytoplasmic Domains

The primary sites of muscarinic receptor/G protein interactions are thought to be the short cytoplasmic extensions of TM3, TM5, and TM6 [17, 42, 43, 69–72]. These extensions are referred to as the intracellular loop 2 (i2), the N terminus of the i3 loop (Ni3), and the C terminus of the i3 loop (Ci3) and they may form α-helical extensions of the corresponding TM domains above [17, 72]. Studies using chimeric receptors have shown that the Ni3 domain is a primary determinant of G protein coupling specificity, with the Ci3 domain also contributing [42, 43]. The i2 loop contains the highly conserved Asp-Arg-Tyr motif. Mutagenesis has shown that the Arg residue is absolutely essential for receptor signaling [44].

12.5.6
i3 Loop

From the structure of rhodopsin, the cluster of basic and acidic residues in the C-terminal end of the i3 loop (Ci3) face the residues in the i2 loop at the base of TM3, where they may form salt bridges with the residues in the highly conserved Asp-Arg-Tyr motif [55]. It has been postulated that these salt bridges may constitute one of the constraining interactions that stabilize the off state of the receptor, explaining why mutations in this region often cause constitutive activity [73, 74]. Indeed, the first examples of GPCRs constitutively activated by mutations were α-adrenoceptors mutated in this region [75]. To some degree, similar results have been observed with muscarinic receptors. A constitutively activating mutation ($Glu^{360(6.30)}$ to Ala) was described for the M_1R, and could be acting by destabilizing the receptor in the manner described above [76]. Insertion of one, two, three, or four additional Ala residues at the base of TM6 (i.e., above Ci3) at position 390(6.38) constitutively activates the M_2R [77]. In contrast, similar insertions at position 384(6.32) did not affect basal activity. Thus, it was concluded that altering the positioning of the region between residues 384 and 390 was sufficient to constitutively activate the M_2R. In contrast, analogous experiments with the M_3R resulted in inactivation of the receptor. Since M_2R and M_3R prefer different G proteins, one possible explanation is that the structures of M_2R and M_3R differ in this region. Alternatively, G proteins in the $G_{q/11}$ and $G_{i/o}$ classes may interact differently with the receptor. However, given that similar regions are constitutively activating in the G_q-coupled α_{1B}-adrenoceptor [75], this cannot completely explain the discrepancy. In a random mutagenesis study on the M_5R, over 300 mutant receptors containing mutations within a 20-residue stretch of the Ci3 were screened [69]. Of the 36 functional receptors isolated, most were either functionally impaired or behaved similarly to the wild-type receptor. The patterns of poorly and well tolerated mutations suggested that Ci3 does indeed form an α-helical cytoplasmic extension of TM6, although no activated receptors were isolated and only two of the receptors displayed more potent responses to carbachol. Thus the Ci3 region, while an important hotspot for constitutively activating mutations in many GPCRs, does not seem to be so for all the muscarinic subtypes.

The N terminus of the i3 loop is an important determinant of G protein coupling selectivity [42, 43, 70, 72], but few activating mutations have been identified in this region. A random mutagenesis study showed that, like Ci3, Ni3 forms a cytoplasmic α-helical extension of TM5 [78]. In a subsequent study, only two substitutions causing constitutive activity in this region were identified (both at $Thr^{220(5.65)}$), and the effects observed were quite modest [71]. These results are not surprising given that few strong constitutively active phenotypes had been seen in previous studies on TM5 (discussed above). Thus, TM5 – and its cytoplasmic extension Ni3 – may not undergo large dynamic shifts in conformation upon receptor activation as postulated for TM3, TM6, and TM7.

12.5.7
i2 Loop

As discussed above, TM3 plays a pivotal role in receptor activation. Random mutagenesis of the i2 loop of the M_5R provided evidence that the N-terminal half of the i2 loop forms a cytoplasmic α-helical extension of TM3 [17]. In this study, numerous mutant receptors displaying high constitutive activity were identified. Mutations to $Phe^{130(3.52)}$, $Arg^{134(3.56)}$, $Ile^{137(3.59)}$, $Tyr^{138(3.60)}$, and $Arg^{142(3.64)}$ were shown to increase constitutive activity substantially. Eight functionally conserved residues that resulted in functionally impaired receptors when mutated were also identified. These included $Asp^{127(3.49)}$, $Arg^{128(3.50)}$, $Tyr^{129(3.51)}$, $Ile^{132(3.54)}$, $Pro^{135(3.57)}$, $Leu^{136(3.58)}$, $Arg^{139(3.61)}$, and $Thr^{143(3.65)}$. It was previously shown in M_1R that mutations of $Asp^{122(3.49)}$ and $Tyr^{124(3.51)}$ decrease receptor expression, but increase the affinity of acetylcholine [79], suggesting that mutations at these positions might cause constitutive activity that was unmeasurable due to low receptor expression. This would be consistent with results from other receptors showing that mutations at the analogous Asp caused constitutive activity and a loss in structural stability [80–82].

When arranged in a helical wheel, the constitutively activating residues and the functionally conserved residues formed opposing faces. Point mutations in M_5R at $Phe^{130(3.52)}$, $Arg^{134(3.56)}$, $Tyr^{138(3.60)}$, and $Arg^{142(3.64)}$ showed that diverse substitutions spanning the range of amino acid classes caused constitutive activity and potentiated the actions of carbachol [17]. This suggested that the primary effect of mutating these residues was disruption of specific amino acid interactions. It was proposed that the functionally conserved residues might be involved in intra- or intermolecular interactions important for receptor expression or signaling. A model was proposed in which the face comprised of the constitutively activating residues forms interactions that constrain the receptor in the off state while the face comprised of the functionally conserved residues couples G proteins in the on state. A similar model was subsequently proposed for the function of TM3 [49].

Comparison of the data for i2 with the crystal structure of rhodopsin did not yield a clear model. The i2 loop of rhodopsin is not an α-helix, and the constitutively activating and functionally conserved residues from M_5R do not segregate when mapped on the rhodopsin structure [83]. It is possible that the structures of rhodopsin and the muscarinic receptors diverge in this area. For example, the TM3 helix may extend further, and the cytoplasmic regions of TM4 (which are highly tolerant of mutation; see [62]) may be unwound. Alternatively, the structure of the i2 loop may be highly influenced by the i3 loop (which is much longer in the muscarinic receptors than in rhodopsin) or by interactions with G proteins. These questions are unlikely to be definitively answered until the crystal structure of a muscarinic receptor is determined.

12.6 Structure–Function Model For Activation

Muscarinic receptors exhibit varying degrees of constitutive activity, both in native and recombinant systems. Structure–function analysis has yielded many examples of constitutively activated receptors and, when interpreted in the context of current structural models of GPCRs, provides insight into the activation mechanisms of muscarinic receptors. The extended ternary complex model predicts that constitutive activity can be induced either by affecting the intrinsic equilibrium constant governing the transition between active and inactive receptor conformations (denoted as J) or by improving G protein coupling [4] (see also Chapter 6). Constitutively activating mutations could affect the equilibrium between active and inactive receptor conformations either by mimicking active conformations of the receptor or by disrupting key interactions needed to stabilize the inactive conformation of the receptor. Given that overexpression of G proteins can also enhance constitutive activity of muscarinic receptors, mutations that improve G protein coupling also have the potential to induce constitutive activity. Mathematically, in the extended ternary complex model (see [4]), this would be represented by an increase in the affinity constant M governing association of R* and the G protein. Increases in M could account for the effects of constitutively activating mutations found in the intracellular loops of muscarinic receptors. However no studies have yet demonstrated constitutive activity that arises primarily from an effect on M. Recently, methods for direct determination of receptor/G protein affinity that would allow this have been described [5].

We have summarized many mutations in muscarinic receptors that cause increased constitutive activity (see Figure 12.1 and Table 12.1). From examination of the distribution of constitutively activating mutations across the receptor it is immediately apparent that the majority of mutations are clustered in TM3, the i2 loop, and TM6. The prevalence of constitutively activating mutations in these regions suggests they have the greatest involvement in the dynamic switching between inactive and active receptor conformations. This assertion is supported by studies showing that restricting the movement of these domains inhibits receptor activation in M_1R and rhodopsin [63, 84]. Similarly, in adrenoceptors, receptor activation changes the environment around TM6 [58] and access of polar ligands to TM6 [80], indicating dynamic conformational changes occur in TM6. Furthermore, changes in the relative positions of TM3 and TM6 have been directly shown in other receptor systems [56, 57, 59, 60].

12.7 Conclusions

The physiological importance of constitutive activity for muscarinic receptors is unclear at this time. Targeted deletion of each of the five muscarinic subtypes has yielded insights into their probable physiological roles [85–87] and indicates that basal tone may play a part in normal muscarinic physiology, though this has not been investigated in detail. For example, M_2R was shown to regulate heart rate and smooth muscle

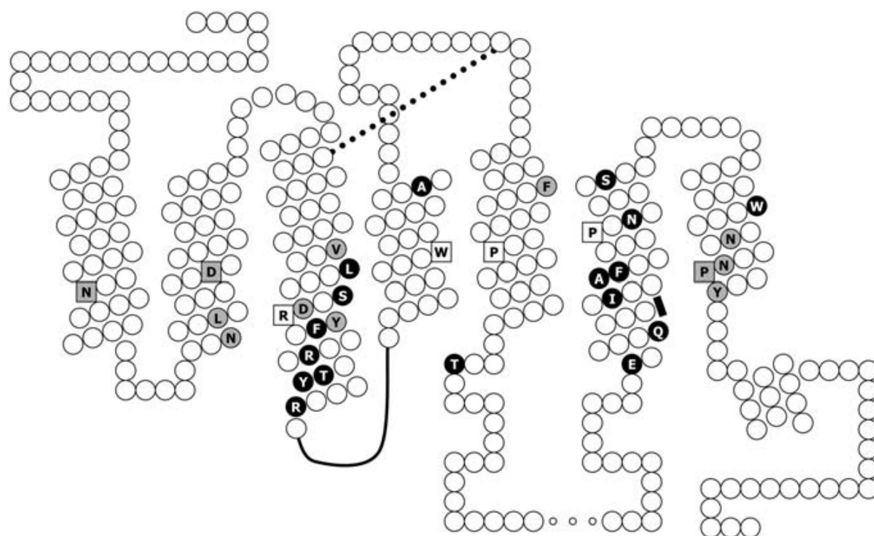

Fig. 12.1 Snake diagram showing the positions of constitutively activating mutations in the muscarinic receptor family. Circles and squares represent individual amino acids. Letters indicate amino acids in the wild-type M_5R by the single letter code. Black fill indicates positions at which mutations have increased constitutive activity. Gray fill indicates positions at which mutations have increased the affinity of agonists by over threefold. Squares indicate highly conserved residues (M_5R: $Asn^{48(1.50)}$, $Asp^{76(2.50)}$, $Arg^{128(3.50)}$, $Trp^{155(4.50)}$, $Pro^{205(5.50)}$, $Pro^{457(6.50)}$, and $Pro^{492(7.50)}$) used in the Ballesteros and Weinstein numbering system [37]. Positions of the helices are modeled after [55] with the alignment published previously [83], with the exception of TM3, which is extended into the cytoplasm as described [17]. Extension of this helix "uses up" residues that form a loop between TM3 and TM4 in rhodopsin. An analogous loop must exist in the muscarinic receptors, so either or both of TM3 and TM4 are shorter than is shown in this figure. Since we are unsure of the receptor structure in this region, a solid line was drawn connecting TM3 to TM4. Small white circles stand for approximately 100 residues in the i3 loop omitted for clarity; no constitutively activating mutations have been identified in this region. Dotted line represents a disulfide bond linking $Cys^{103(3.25)}$ to $Cys^{183(5.28)}$ [22, 98].

contraction in the trachea, ileum, and bladder [88, 89], though only agonist-mediated activity was measured in these studies. However, the original observations by Hilf and Jakobs showing atropine-sensitive GTPγS binding in cardiac membranes suggest that basal activity of M_2R probably contributes to the control of heart rate [11]. Knockout of M_4R enhances both basal and agonist-stimulated dopamine D_1-receptor-mediated locomotor stimulation, indicating that M_4R exerts inhibitory control [90]. Given that basal locomotor activity was increased, and that muscarinic inverse agonists are used to treat patients with Parkinson's disease, it is possible that constitutive activity of M_4R plays a part in maintaining appropriate levels of motoric activity. Roles for M_5R in maintenance of cerebral vascular tone (see [91]), M_1R in hippocampal neuronal activity [92], and M_3R in control of smooth muscle tone in bladder, lung, and stomach [93] and in food intake [94] have been described. It would be of interest to determine the contribution, if any, of constitutive activity to these functions. The use of available inverse agonists with these muscarinic receptor knockout models should help clarify this

point. Ultimately, the development of subtype-selective inverse agonists and neutral antagonists will be necessary for full understanding of how the constitutive activity of muscarinic receptors regulates normal physiological functions in humans.

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13
Constitutively Active Histamine Receptors

Remko A. Bakker and Rob Leurs

13.1
Introduction

Much of the information cells receive is transduced by a membranous signaling system that uses heterotrimeric guanine nucleotide binding proteins (G proteins) for functional coupling of cell surface receptors (G protein coupled receptors, GPCRs) to a variety of effectors. During recent years it has been shown that receptors, G protein α, β, and γ subunits, and effectors involved in this signaling system exhibit a remarkable structural diversity and that the interactions of these components display a bewildering complexity. Even though many questions remain to be answered, it is becoming obvious that G proteins form the basis of a complex membranous signaling network that allows the cell to coordinate and to process incoming signals already at the level of the plasma membrane [1–7].

Whereas receptor studies were rather tedious in the early days, nowadays, by use of various molecular biological techniques, we can induce the expression of our gene of interest to a varying degree in more or less any kind of cellular system we would like. It is therefore not surprising that information on receptors such as the GPCRs is rapidly growing. Although Peters *et al.* had already investigated the mechanisms of enhanced agonist-induced signaling through the β-adrenoceptor (βAR) after pretreatment of the cells with βAR antagonists [8], a phenomenon now closely associated with constitutive GPCR receptor activity [9–14], the first clear description of constitutive GPCR activity was reported by Costa and Hertz in 1989 [15]. Through the use of GTPase assays (see also Chapter 8), Costa and Hertz showed that the endogenously expressed δ opioid receptor (DOP(δ)R) displayed a remarkable activity in the absence of any agonist. Moreover, this basal activity could be inhibited by a variety of antagonists that henceforth were known as pure antagonists of the DOP(δ)R. Such compounds have negative efficacy and have been described either as negative antagonists or as inverse agonists. The term was originally devised to describe the behavior of compounds acting at benzodiazepine receptors that allosterically inhibited signaling at γ-aminobutyric acid (GABA) receptors [16]. Obviously, these antagonists were able to elicit a response opposite to the response induced by agonists and so they were referred to as inverse

agonists. As these ligands are able to induce a response they also possess intrinsic activity; their intrinsic activity ranges from –1 for full inverse agonists to 0, in analogy to the agonists, for which the intrinsic activity ranges from 0 to +1 for a full agonist. This observation has resulted in the reclassification of a large variety of ligands: many antagonists were found to possess negative intrinsic activity, while only a small number of ligands failed to display any intrinsic activity. The latter set of ligands we now refer to as neutral antagonists to distinguish them from the formerly known antagonists, although in principle the term antagonist would suffice.

Constitutive, basal, or spontaneous activity of the receptor, in the context of receptor pharmacology, is signaling by the receptor in the absence of agonist. It is most commonly seen in systems with high levels of receptor expression. Initially, the finding of constitutive activity of G protein-coupled receptors was met with skepticism, as the prerequisite for such signaling is the absence of agonist. Ligand-independent receptor activity is now seen not as a curious property that may only prevail in a rather limited number of receptors but rather as a general phenomenon [17–22].

Several potentially nonbeneficial aspects of inverse agonists have been recognized, among them receptor upregulation, which may render such compounds unfavorable as therapeutics (see also Chapter 7) [12]. Although the mechanistic details remain open to debate, the existence of inverse agonists is now widely accepted and many existing therapeutics have been reclassified as inverse agonists. There seem to be conditions under which application with inverse agonists is more promising than treatment with neutral antagonists: in the case of a highly constitutively active receptor, for instance. Nowadays constitutive receptor signaling is generally studied in heterologous expression systems although, as evidence of constitutive receptor signaling in physiological systems accumulates, its presence is now also recognized as a part of the possible physiological roles of GPCRs [23] (see also Chapter 7).

Members of the family of histamine receptors have played an important role in the recognition of the importance of constitutive GPCR signaling, including the potential drawbacks of the use of inverse agonists as therapeutics [12] and the occurrence of constitutive activity *in vivo* [23]. In this chapter we review the constitutive activity of the family of histamine receptors and the specific ligands currently available to assay the receptors, and also discuss convenient assay systems for study of the constitutive activity of these receptors.

13.2
The Histamine Receptors

The monoamine 2-(1*H*-imidazol-4-yl)ethylamine was first synthesized and identified as an endogenous biologically active molecule about one century ago [24]. Histamine is now recognized as a chemical messenger that regulates a wide variety of physiological responses; it acts as a neurotransmitter in the central nervous system [25–27] and also as a major inflammatory mediator released from mast cells after irritation or allergic provocation [25, 28]. As such, histamine has been implicated in various (patho)physiological conditions. Histamine exerts its effects by binding to histamine receptors,

classified on the basis of pharmacological studies and gene cloning into four receptor subtypes, all of which belong to the superfamily of G protein-coupled receptors [25, 29–34].

Initially, research in the histamine field focussed completely on this role of histamine in allergic diseases and this intensive research resulted in the development of several potent "antihistamines", useful in inhibiting certain symptoms of allergic conditions. H_1R antagonists were thought to inhibit the action of histamine on the histamine H_1 receptor (H_1R). The observation that these "antihistamines" could not antagonize all effects of histamine led Ash and Schild in 1966 to hypothesize the existence of at least two distinct receptor subtypes [35]. In 1972 this hypothesis became generally accepted when Black and his co-workers succeeded in the synthesis of a series of new compounds (e.g., burimamide: 1-[4-(1*H*-imidazol-4-yl)butyl]-3-methylthiourea dihydrobromide) that selectively antagonized the effects of histamine on the stomach and the heart [36]. These H_2-receptor (H_2R) antagonists proved to be very useful in the therapy of gastric ulcers.

In various peripheral tissues histamine is stored in mast cells, basophils, enterochromaffin cells, and probably also in specific neurons. Histamine release after mast cell degranulation results in various of the well known symptoms of allergic conditions in skin and airway preparations, whereas histamine release from enterochromaffin cells in the gastric mucosa stimulates gastric acid secretion by parietal cells [25, 28].

In the CNS, histamine is synthesized in a restricted population of neurons located in the tuberomammillary nucleus of the posterior hypothalamus. These neurons project diffusely to most cerebral areas and have been implicated in various brain functions (e.g., sleep/wakefulness, hormonal secretion, cardiovascular control) in mammalian species [25–27]. The identification of the presynaptic H_3 receptor (H_3R) as a new receptor subtype in 1983 by Arrang and colleagues [37] gave rise to a new field of interest. The cloning of the H_3R gene in 1999 [29] confirmed the existence of this receptor at the molecular level, and the H_3R is now regarded as a general regulatory system in the CNS and a potential target for new therapeutics [38].

The use of genomic databases has recently resulted in the identification of a new histamine receptor: the histamine H_4 receptor (H_4R) [30–34]. In view of its expression profile this receptor has been suggested as a new target in the regulation of immune function.

13.2.1 The H_1R

The H_1R is involved in many of the symptoms of allergic reactions. Histamine H_1R antagonists are widely used to relieve these symptoms and have become one of the safest and most prescribed drug families in western countries [39].

13.2.1.1 H_1R-mediated Signaling

Histamine-induced contractions in airway smooth muscle are mediated through the $G_{q/11}$-coupled [40] histamine H_1Rs, stimulation of which affects the inositol phospholipid signaling systems, resulting in formation of inositol-1,4,5-triphosphate (IP_3) and 1,2-diacylglycerol (DAG) from phosphatidylinositol-4,5-biphosphate hydrolysis (see Figure 13.1). In addition, several other signaling pathways can be activated, including the activation of protein kinase C (PKC) secondary to changes in intracellular concentrations of calcium and DAG [40], and also the stimulation of phospholipase D by monomeric GTP-binding proteins [41] and of phospholipase A_2 (PLA_2) resulting in arachidonic acid release [40]. Furthermore, the H_1R was recently shown to promote transcription of genes under the control of nuclear factor kappa B (NF-κB) [42–44], a ubiquitous transcription factor involved in the regulation of a wide variety of genes

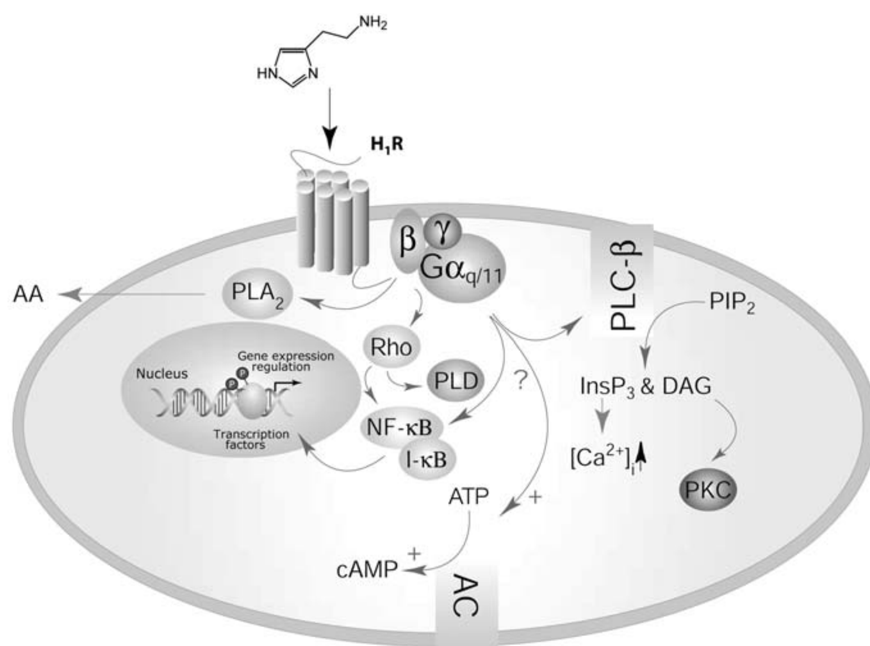


Fig. 13.1 Major signaling pathways of the H_1R. The H_1R activates members of the family of $G_{q/11}$ proteins to modulate cellular signaling. Activated $G_{q/11}$ proteins stimulate phospholipase C (PLC) to induce the formation of 1,2-diacylglycerol (DAG) and inositol-1,4,5-triphosphate (IP_3) from phosphatidylinositol-4,5-biphosphate (PIP_2). IP_3 subsequently increases intracellular Ca^{2+} concentrations, while DAG activates protein kinase C (PKC). In addition, H_1R activation also leads to the activation of adenylyl cyclase (AC) and the formation of cyclic AMP (cAMP), the activation of phospholipase A_2 (PLA_2), which releases arachidonic acid (AA), as well as activation of the transcription factor nuclear factor kappa B (NF-κB) by several mechanisms. Inactive NF-κB, which is complexed together with inhibitory kappa-B (I-κB) in the cytosol, is activated by activated $G\alpha_{q/11}$ proteins or released $G\beta\gamma$ subunits, as well as through the monomeric GTP-binding protein Rho, which may also activate phospholipase D (PLD). Activated NF-κB subunits dissociate from the complex and subsequently modulate gene transcription in the nucleus.

coding for molecules involved in the immune function and inflammation responses [45]. In addition, the H1R may modulate other signaling pathways, including the activation of mitogen-activated protein kinase (MAPK) [46, 47], protein kinase B (PKB, also known as Akt) [48], tyrosine hydroxylase [49], and the canonical β-catenin pathway [50].

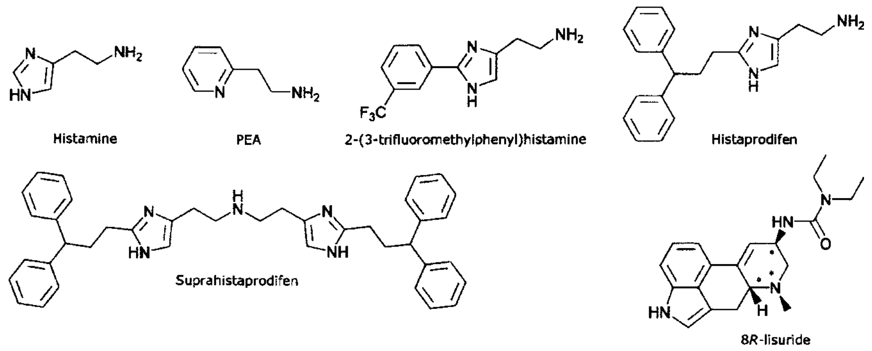
13.2.1.2 H_1R Ligands

Although many pharmacological tools for the study of H_1Rs are available, most effort has been directed towards the development of H_1R antagonists. For many years the substituted 2-phenylhistamines (e.g., 2-(3-trifluoromethylphenyl)histamine; Figure 13.2) have been the best choice as H_1R agonist. These agonists show relatively high H_1R affinity, but appear to possess only limited efficacy [51]. Recently, however, the 'histaprodifens' have been introduced as a new class of highly potent H_1R agonists [52]. The histaprodifens interact with both agonist and antagonist binding sites of the H_1R [53], and are clearly more potent than histamine (Figure 13.2). Several histaprodifens, however, have been shown to possess species selectivity towards the guinea pig H_1R relative to the human H_1R [54]. Also, (8*R*)-lisuride has recently been identified as a potent stereospecific H_1R agonist [55]. (8*R*)-Lisuride (Figure 13.2) is the most potent H_1R agonist known to date, but as a partial agonist it may act as an antagonist in assay systems with low H_1R expression levels.

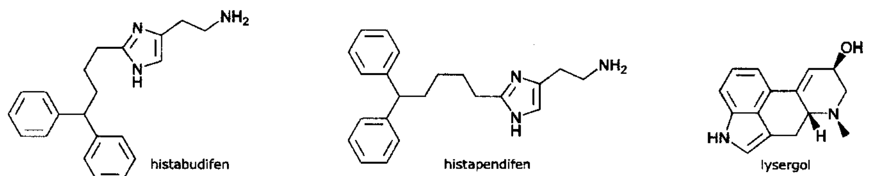
Many potent and selective receptor antagonists for the study of H_1Rs are available [56]. However, one should be aware of the possible antagonistic properties at muscarinic acetylcholine receptors and 5-HT receptors and of the local anesthetic properties of several classical H_1R antagonists at concentrations that are usually much higher than those needed for blocking the H_1Rs [57]. Currently, mepyramine (pyrilamine; Figure 13.2), with a nanomolar H_1R affinity, is the most commonly used H_1R antagonist for pharmacological studies, while the D and L enantiomers of chlorpheniramine are also very effective for receptor classification. These compounds easily penetrate the brain and so can be used for *in vivo* CNS studies. Recently, many new H_1R antagonists that hardly pass the blood–brain barrier (e.g., cetirizine and loratadine; Figure 13.2) have been developed, and are of therapeutic importance in allergic disorders [56]. The classification of H_1R antagonists has recently been altered. H_1Rs exhibit considerable constitutive activity, which can be inhibited by most H_1R antagonists, thus acting as inverse agonists [58].

Since all examined H_1R antagonists, including various therapeutically used substances (Figure 13.2), turned out to exhibit negative intrinsic activity, we searched for neutral antagonists for the human H_1R [59]. Newly synthesized H_1R ligands structurally related to H_1R agonists were screened for their affinity in radioligand displacement studies, and their functional activities were assessed by a NF-κB-driven reporter gene assay (see Chapter 8) that allows for the detection of both agonistic and inverse agonistic responses [44]. By this approach [59] we ultimately identified two neutral H_1R antagonists: 2-[2-(4,4-diphenylbutyl)-1*H*-imidazol-4-yl]ethylamine (histabudifen; Figure 13.2) ($pK_i = 5.8$) and 2-[2-(5,5-diphenylpentyl)-1*H*-imidazol-4-yl]ethylamine (histapendifen; Figure 13.2) ($pK_i = 5.9$). In addition, we have recently identified lysergol ($pK_i = 5.6$), a ligand structurally related to lisuride, as a potential lead structure for the synthesis of additional neutral H_1R antagonists [55].

A



B



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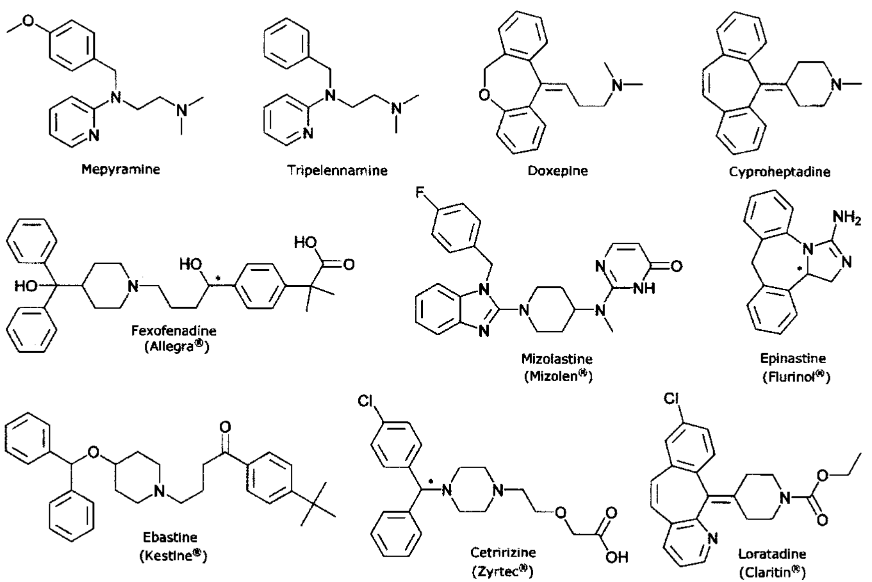


Fig. 13.2 Structures of various H_1R agonists (A), neutral H_1R antagonists (B), and inverse H_1R agonists (C).

13.2.2 The H_2R

The H_2R is known for its role in the regulation of gastric acid secretion and is a target for H_2R antagonists that prevent the production of gastric acid. These substances are used to heal ulcers and to relieve the symptoms and pain associated with gastroesophageal reflux disease [60].

13.2.2.1 H_2R-mediated Signaling

The H_2R is a prototypical GPCR coupled to the G_s family of G proteins. Activation of the receptor therefore results in the activation of adenylyl cyclase (AC) and subsequent formation of cyclic AMP (cAMP) in the cell (see Figure 13.3 and [61] for review). The second messenger cAMP subsequently activates protein kinase A (PKA), which in turn modulates the activity of a variety of signaling pathways, including the activation of MAPK in the brain [62] as well as the activation of the transcription factor cAMP responsive element-binding (CREB) protein to modulate gene transcription (see Chapter 8). In addition to the activation of AC, the H_2R may also couple to PLC and trigger intracellular Ca^{2+} signaling through the activation of $G\alpha_q$ proteins in certain cell systems [63–68].

13.2.2.2 H_2R Ligands

Both agonists and antagonists for the histamine H_2R are available for proper pharmacological characterization. Amthamine (2-amino-5-(2-aminoethyl)-4-methylthiazole;

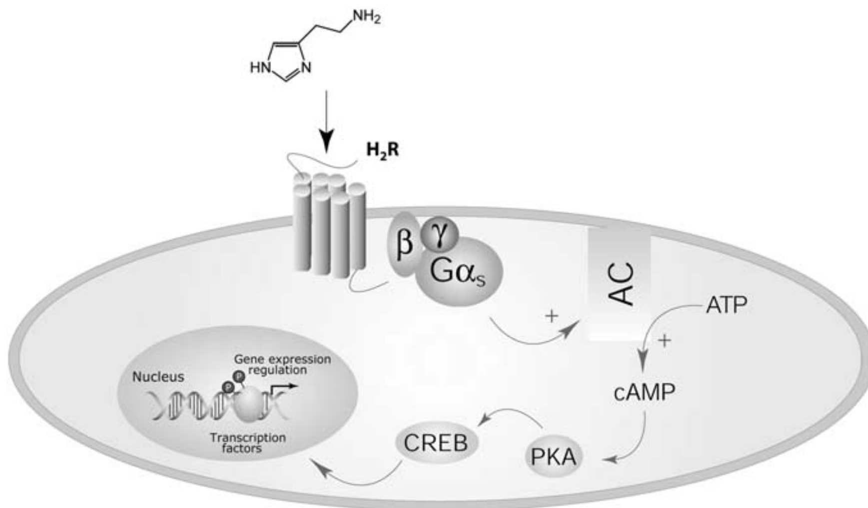
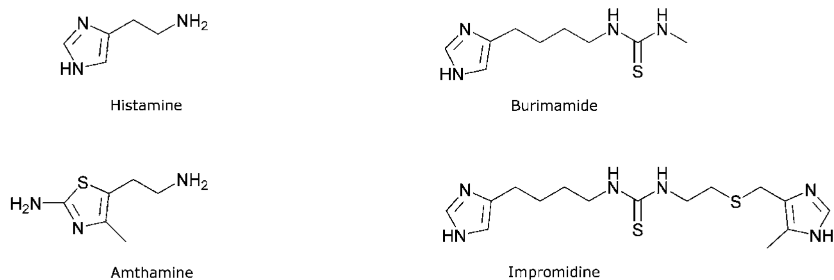


Fig. 13.3 Major signaling pathways of the H_2R. The H_2R activates members of the family of G_s proteins to modulate cellular signaling. Activated G_s proteins stimulate AC, the enzyme that produces cAMP, which in turn results in the activation of protein kinase A (PKA) and consequently cAMP-responsive element binding protein (CREB) to modulate gene transcription.



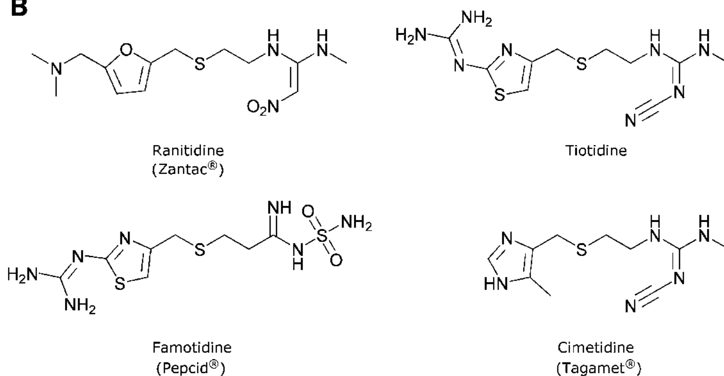


Fig. 13.4 Structures of various H_2R agonists (A) and inverse H_2R agonists (B).

Figure 13.4) is the best choice for a selective H_2R agonist, as it combines a high H_2R selectivity with a slightly higher potency than histamine [69]. Many compounds with potent H_2R antagonistic properties have been described (see [70–72] for extensive reviews). Nowadays compounds such as cimetidine, ranitidine, and tiotidine (Figure 13.4) are usually applied as selective tools for functional H_2R studies. As in the case of the H_1R antagonists, the recognition of constitutive H_2R activity resulted in the reclassification of cimetidine, ranitidine, and tiotidine as inverse agonists and of burimamide as a neutral antagonist [12] or a weak partial agonist [73].

13.2.3 The H_3R

While the H_3R was identified pharmacologically in 1983, by Arrang *et al.*, and found to modulate histamine release in the brain [37], its gene coding was identified in 1999 by Lovenberg *et al.* [29]. Cloning of the H_3R gene subsequently resulted in the identification of another gene coding for the histamine H_4R (see Section 13.2.4). The identifica-

tion of the H_3R at the molecular level has greatly facilitated the study of the pharmacology of the receptor; however, it is now apparent that a large variety of H_3R isoforms with different pharmacological profiles may exist (see [74] for a review).

Currently, the H_3R has attracted interest as a potential drug target for the treatment of a variety of disorders/ailments, including diabetes and obesity [74–76], attention-deficit hyperactivity disorder (ADHD) [77, 78], Alzheimer's disease [79], epilepsy [79–81], and schizophrenia [81, 82], as well as for treatment of myocardial ischemia [83, 84] and inflammatory and gastric acid-related diseases [85–89]. Many interesting activities in various preclinical models of important human diseases have been reported both for H_3R agonists and for H_3R inverse agonists/antagonists (see [90] for a review).

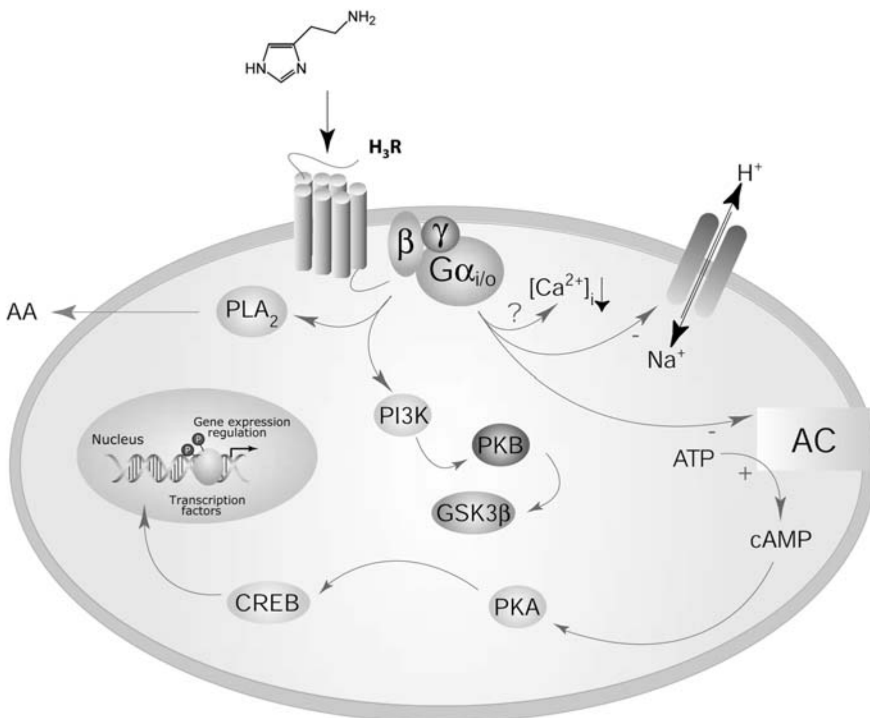


Fig. 13.5 Major signaling pathways of the H_3R. The H_3R activates members of the family of $G_{i/o}$ proteins to modulate cellular signaling. Activated $G_{i/o}$ proteins inhibit AC, resulting in turn in reduced levels of cAMP and a reduced activation of PKA and CREB-mediated modulation of gene transcription. In addition, other effector pathways, including the activation of MAP and PI-3 kinases and the activation of PLA_2 to induce the release of AA may be activated, as well as the inhibition of the Na^+/H^+ exchanger and the lowering of intracellular Ca^{2+} levels. Subsequent activation of the MAPK and PI-3K pathways results in the phosphorylation of ERKs and protein kinase B (PKB, also known as Akt), respectively. Activated PKB subsequently phosphorylates and thereby inhibits the action of glycogen synthase kinase 3β (GSK3β), a major tau kinase in the brain.

13.2.3.1 H_3R-mediated Signaling

The H_3R couples to members of the G_i family of G proteins, such as $G\alpha_i$ and $G\alpha_o$. As such, the receptor is negatively coupled to adenylyl cyclase, and activation of the receptor results in the inhibition of cAMP formation (see Figure 13.5). In addition, a variety of other effector pathways can be activated by the H_3R, including the activation of MAPK [62, 91] and PI-3 kinase (PI3K), the activation of PLA_2, inhibition of the Na^+/H^+ exchanger [86], and the lowering of intracellular Ca^{2+} levels by an unidentified mechanism [85].

The negative modulation of AC also results in the inhibition of downstream events such as CREB-dependent gene transcription by lowering of cAMP levels. Activation of the MAPK and PI3K pathways induces the phosphorylation of ERKs and PKB/Akt, respectively [92]. PKB subsequently phosphorylates, and thus inhibit the action of, glycogen synthase kinase 3β (GSK3β) [92], a major tau kinase in the brain [93].

13.2.3.2 H_3R Ligands

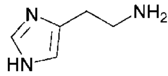
Soon after the initial description of the H_3R [37], Arrang *et al.* described highly potent and selective H_3R ligands [94]. The agonists (*R*)-α-methylhistamine, immepip (4-(1*H*-imidazol-4-ylmethyl)piperidine), and imetit ((*S*)-[2-(1*H*-imidazol-4-yl)ethyl]isothiourea), and the inverse agonist thioperamide (*N*-cyclohexyl-4-(1*H*-imidazol-4-yl)piperidine-1-carbothioamide) have become valuable H_3R ligands (Figure 13.6) [95–98]. Most H_3R ligands, however, also interact with the H_4R (see below) and are therefore not selective. The use of (*R*)-α-methylhistamine in combination with its less potent enantiomer (*S*)-α-methylhistamine is very effective for the characterization of H_3R-mediated effects, while the dimethylated histamine analogue (*R*)-α,β-dimethylhistamine has also been shown to be a potent H_3R agonist [99]. In contrast to immepip, (*R*)-α-methylhistamine shows some agonistic effects at α_2-adrenoceptors and H_1Rs, whereas imetit shows reasonable agonistic activity at 5-HT_3Rs [100, 101].

Recently, immethridine (4-(1*H*-imidazol-4(5)-ylmethyl)pyridine; Figure 13.6) has been described as a potent H_3R selective agonist, exhibiting a 300-fold selectivity relative to the H_4R [102], and appears to be a promising pharmacological tool for investigation of the H_3R. Methimepip (4-((1*H*-imidazol-4-yl)methyl)-1-methylpiperidine), a close analogue of immethridine (Figure 13.6), possesses an even greater H_3R *versus* H_4R selectivity [103].

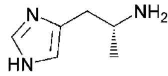
Only a few neutral H_3R antagonists have thus far been described. VUF 4904 ([5-(1*H*-imidazol-4-yl)pentyl]-isopropyl-amine, $pK_i = 7.9$; Figure 13.6) [104] and VUF 5681 (4-(3-(1*H*-imidazol-4-yl)propyl)piperidine, $pK_i = 8.4$; Figure 13.6) [105] are two available neutral H_3R antagonists.

A wide variety of potent H_3R antagonists have been described [106]. Thioperamide (Figure 13.6, $pA_2 = 8.4$) [94] and clobenpropit (2-[3-(1*H*-imidazol-4-yl)propyl]-1-(4-chlorobenzyl)isothiourea; Figure 13.6, $pA_2 = 9.9$), both of which are now classified as inverse H_3R agonists, were developed in the early years [96] and are often regarded as the standard H_3R antagonists despite their affinity for the H_4R. Interestingly, the inverse H_3R agonist A-331440 (4'-[3-((3*R*)-3-dimethylaminopyrrolidin-1-yl)propoxy]biphenyl-4-carbonitrile; Figure 13.6) appears to possess a much greater negative intrin-

A



Histamine



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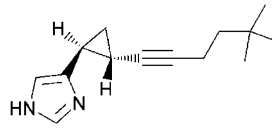

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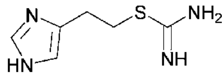

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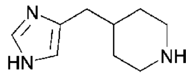

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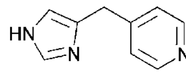
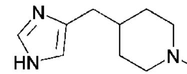


Immethridine



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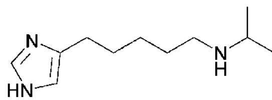
B



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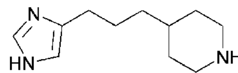

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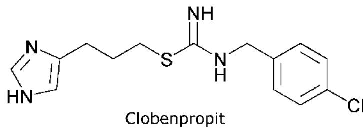
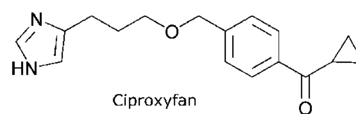
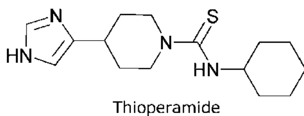
C



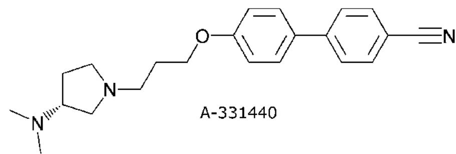
Clobenpropit



Ciproxyfan

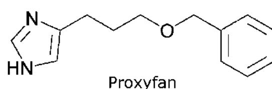


Thioperamide



A-331440

D



Proxyfan

Fig. 13.6 Structures of various H_3R agonists (A), neutral H_3R antagonists (B), inverse H_3R agonists (C), and the protean H_3R agonist proxyfan (D).

sic activity than other inverse H_3R agonists as measured by [^{35}S]guanosine 5'-[γ-thio]-triphosphate ([^{35}S]GTPγS) binding assays (see Chapter 8) [75]. If it is proven that the observed response of A-331440 is selectively due to its inhibition of constitutive H_3R activity, many of the heretofore identified inverse H_3R agonists, including thioperamide and clobenpropit, should be reclassified as partial inverse H_3R agonists accordingly.

Proxyfan (4-[3-(benzyloxy)propyl]-1*H*-imidazole; Figure 13.6) is a peculiar H_3R ligand in that it exhibits H_3R agonistic, neutral H_3R antagonistic, and inverse H_3R agonistic properties, depending on the signaling assay used to assay H_3R activity [107, 108]. As such, proxyfan is a protean agonist that exhibits H_3R agonistic behavior when assayed for inhibition of cAMP formation, MAPK activation, and in [^{35}S]GTPγS binding assays, while it displays inverse H_3R agonistic behavior when assayed in calcium ionophore-induced arachidonic acid release assays (see also Chapter 8) [107]. The protean agonistic behavior of proxyfan should also be taken into account when comparing H_3Rs from different species; proxyfan acts, for instance, as a neutral H_3R antagonist on [^{35}S]GTPγS binding in rat brain tissues [108], whereas it acts as a partial H_3R agonist on [^{35}S]GTPγS binding on heterologously expressed human H_3Rs [23].

13.2.4
The H_4R

The completion of the human genome sequencing project has identified various genes encoding proteins that belong to the GPCR superfamily [109, 110]. One of these genes was identified simultaneously by several researchers as encoding a novel histamine receptor, and was thus characterized as the fourth histamine receptor (H_4R) on the basis of the sequence homology of the orphan receptor with the H_3R [30–34].

To elucidate the role of the H_4R *in vivo*, the H_4R was subsequently cloned from various species, including mouse, rat, guinea pig [111], and pig [112]. The H_4Rs share 65–72 % sequence homology with the human H_4R and exhibit substantial pharmacological species variation both for binding affinity and for signal transduction response [111, 112], the human H_4R having the highest affinity for histamine.

From the expression profile of the H_4R, which is most abundant in leukocytes [30], including eosinophils, dendritic cells, and tonsil B cells, as well as in bone marrow and spleen [33, 111], the receptor was suggested to be involved in immunological functions. Such as role for the H_4R has recently been confirmed by studies indicating critical roles of the receptor in leukotriene B_4 production and neutrophil recruitment [113, 114], chemotaxis and cytoskeletal changes of eosinophils [115–117] and mast cells [118], and interleukin-16 release from human $CD8^+$ T cells [119], as well as from studies using selective H_4R antagonists [117, 120, 121]. In addition to its expression in leukocytes, bone marrow, and spleen, the H_4R is also expressed in various parts of the brain [32, 33, 122, 123] and lung [119, 122].

13.2.4.1 H_4R-mediated Signaling

Similarly to the H_3R, the H_4R couples to members of the G_i family of G proteins. H_4R activation thus gives rise to a decrease in the formation of cAMP and the inhibition of downstream events such as CREB-dependent gene transcription (see Figure 13.7). In addition, a variety of other effector pathways can be activated by the H_4R, including the activation of MAPKs [34] and increases in intracellular calcium from intracellular stores upon receptor activation in, for instance, mast cells [118]. The H_4R-mediated calcium signaling is induced through a PTX-sensitive (PTX = pertussis toxin) activation of PLC, suggesting the involvement of $G\beta\gamma$ subunits dissociated from $G\alpha_{i/o}$ proteins. Pathways downstream of H_4R-mediated calcium mobilization, which may include PKC as well as pathways causing mast cell chemotaxis toward histamine, are currently unknown.

13.2.4.2 H_4R Ligands

Heterologous expression of the H_4R in cells confers the ability to bind [^{3}H]histamine with high affinity ($K_D = 5$ nM). Pharmacologically, the H_4R resembles the H_3R to some

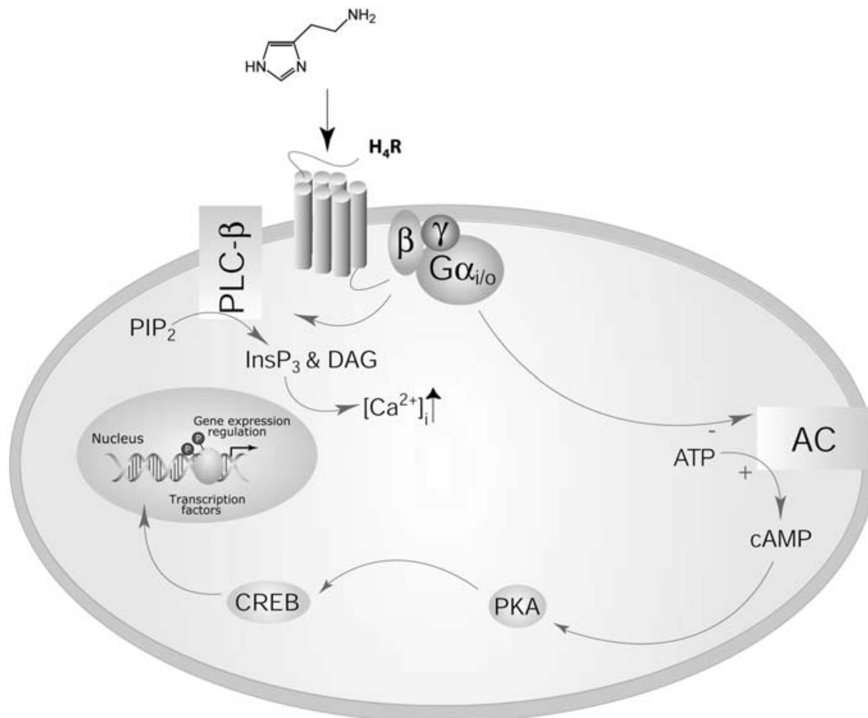


Fig. 13.7 Major signaling pathways of the H_4R. The H_4R activates members of the family of $G_{i/o}$ proteins to modulate cellular signaling. Activated $G_{i/o}$ proteins inhibit AC, resulting in turn in reduced levels of cAMP and a reduced activation of PKA and CREB-mediated modulation of gene transcription. In addition, the H_4R activates intracellular Ca^{2+} mobilization through the activation of PLC by $G\beta\gamma$ subunits released upon activation of $G_{i/o}$ proteins.

extent in that it binds many of the known H_3R agonists and antagonists, albeit with a different rank order of affinity and potency. Oda and colleagues have shown that the histamine H_4R can bind and be activated not only by histamine, but also by H_3R ligands such as (*R*)-(α)-methylhistamine, *N*-(α)-methylhistamine, clozapine (8-chloro-11-(4-methyl-piperazin-1-yl)-5*H*-dibenzo[*b*,*e*][1,4]diazepine), imetit, immepip, and – interestingly – also by the H_2R antagonist burimamide and the H_3R antagonist clobenpropit [34]. Recently, we have described OUP-16 ((–)-2-cyano-1-methyl-3-[(2*R*,5*R*)-5-(1*H*-imidazol-4(5)-yl)tetrahydrofuran-2-ylmethyl]guanidine; Figure 13.8) as the first full H_4R agonist exhibiting specificity for the H_4R relative to the H_3R [124]. The H_4R does not bind H_1- and H_2R antagonists such as diphenhydramine, loratadine, ranitidine, and cimetidine, but has modest affinity for the H_2R agonists dimaprit [33] and impromidine [33, 34].

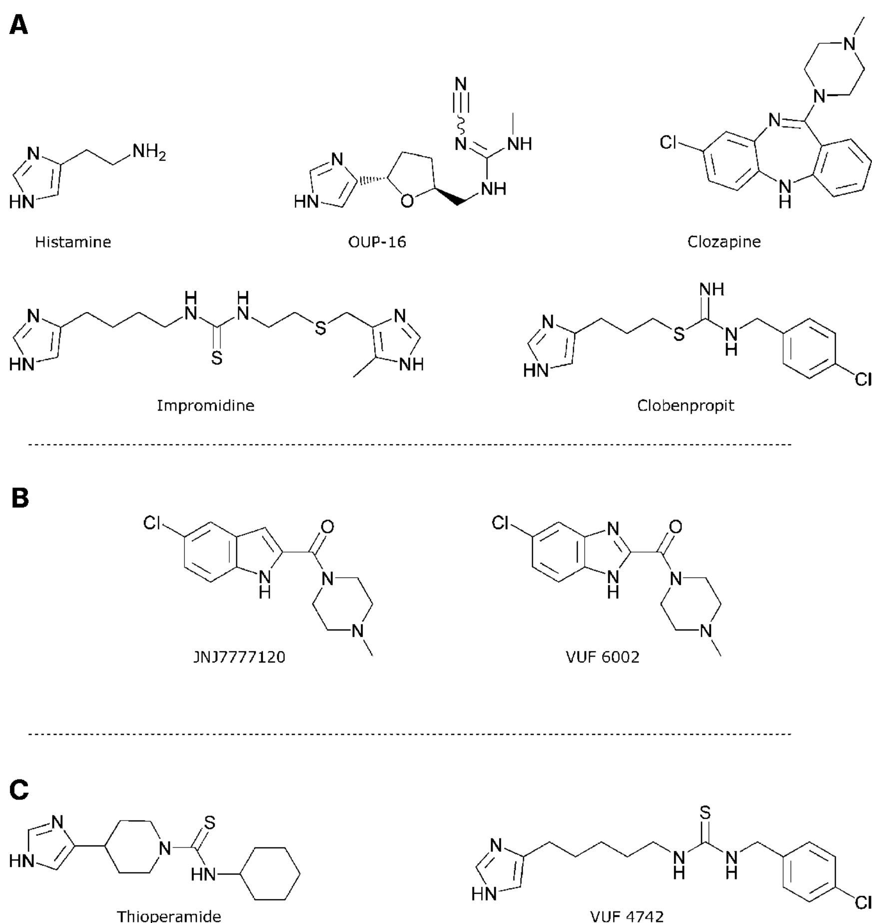


Fig. 13.8 Structures of various H_4R agonists (A), neutral H_4R antagonists (B), and inverse H_4R agonists (C).

Interestingly, thioperamide acts not only as an inverse H_3R agonist, but also as an inverse H_4R agonist [30, 33, 34]. Only one ligand other than thioperamide has so far been reported to possess inverse H_4R agonistic properties: VUF 4742 (1-[5-(imidazol-4-yl)pentyl]-3-(4-chlorophenylmethyl)thiourea; Figure 13.8). VUF 4742 has a potency and efficacy for the H_4R very similar to that thioperamide [125].

In contrast to the rather limited number of inverse H_4R agonists found so far, the identification of neutral H_4R antagonists appears to be relatively easy in comparison to the identification of neutral H_1R antagonists. The potent and selective H_4R ligand JNJ7777120 (1-[(5-chloro-1*H*-indol-2-yl)carbonyl]-4-methylpiperazine; Figure 13.8), as well as its benzimidazole analogue (VUF 6002, (5-chloro-1*H*-benzo[d]imidazol-2-yl)-(4-methylpiperazin-1-yl)methanone, see Figure 13.8) and the H_3R inverse agonist iodophenpropit (*S*-[3-(4(5)-imidazolyl)propyl]-*N*-[2-(4-iodophenyl)ethyl]isothiourea), act as neutral H_4R antagonists (unpublished results).

13.3 Assay Systems for Detection of Constitutive Activity of Histamine Receptors

Because of the diversity of the coupling capabilities of the four histamine receptors it is no surprise that a wide variety of assay systems for assaying (constitutive) histamine receptor activity are available. In this section we briefly discuss the major assay systems that have been used to assay histamine receptor activity and their suitability for monitoring constitutive receptor activity. A more detailed discussion of methods available for the assessment of constitutive GPCR activity is presented in Chapter 8.

13.3.1 Histamine Receptor Expression and the Detection of Constitutive Activity

GPCRs exhibit varying levels of constitutive activity [17]. Detection of (constitutive) receptor activity is facilitated by overexpression of the receptor in heterologous expression systems. Histamine receptors have been successfully expressed in various heterologous expression systems, including mammalian cell lines such as HEK293, CHO, and COS-7 cells. The receptor has also been overexpressed in insect cells (Sf-9), but these cells may be less suited for functional studies due to low levels of endogenous G proteins in them [126].

The heterologously expressed H_2R exhibits considerable constitutive activity, resulting in elevated levels of cAMP [12, 127]. High levels of H_2R expression, to facilitate the detection of constitutive receptor activity, can be achieved by applying viral expression systems such as the Semliki forest virus [127], which can be used to infect a large variety of host cells [128, 129]. Moreover, the Semliki forest virus system has been successfully used for the detection of constitutive activity of mutant H_2R receptors that can be expressed only at low levels by use of more conventional transfection methods [127].

The H_3R controls histaminergic neuron activity, and also exhibits high constitutive activity. In fact, it is the only histamine receptor for which constitutive activity has been demonstrated *in vivo* [23]. Various H_3R isoforms differing in the lengths of their third intracellular loops have been described. Investigations into the constitutive activity of H_3R isoforms have shown that the longer H_3R isoform (H_{3L}), consisting of 445 amino acids, exhibits slightly more pronounced constitutive activity than the shorter H_3R isoform, which consists of 413 amino acids due to a 32-amino acid deletion in its third intracellular loop (H_3S) [23]. Rat H_3R isoforms also differ in the lengths of their third intracellular loops, and these receptors also differ in their effectiveness in activating signaling pathways, including CRE-dependent transcription and MAPK activation [91].

Constitutive H_4R activity has been detected in several heterologous expression systems, including HEK 293 and SK-N-MC neuroblastoma cells [30, 33, 34].

Prolonged treatment of constitutively active receptors with inverse agonists is thought to stabilize the receptor and will subsequently give rise to receptor upregulation. For histamine receptors, these kinds of effects were first recognized for heterologously expressed H_2Rs upon treatment with cimetidine [12], and have been linked to the occurrence of tolerance. Interestingly, one described allelic variant of the histamine H_2R exhibits low constitutive activity and is resistant to receptor upregulation upon prolonged treatment with inverse H_2R agonists [130]. The mutation, a replacement of adenine 649 with guanine ($A^{649}G$), results in a change of Asn^{217} to Asp in the third intracellular loop of the receptor (H_2R $Asn^{217}Asp$). Interestingly, this allelic variant has been reported to be more frequent in Caucasian schizophrenics [131, 132].

The human H_1R is endogenously expressed in some cell lines, such as HeLa cells, at fairly low levels of expression [59]. Prolonged treatment of these cells with an inverse H_1R agonist results in H_1R upregulation, which is also accompanied by increased responsiveness to histamine-induced H_1R-mediated IP_3 accumulation. Although these data suggest the H_1R is constitutively active in these cells, this constitutive H_1R activity is not detected directly by IP_3 measurements.

13.3.2 Changes in Intracellular Ca^{2+}

Measurements of changes in intracellular Ca^{2+} upon H_1R activation have been routinely used to evaluate H_1R activation [25] and are well suited to measure H_1R desensitization. The H_2R, H_3R, and H_4R have also been shown to mediate agonist-induced changes in intracellular Ca^{2+} [65, 68, 85, 118, 120]. However, the assay does not allow the detection of constitutive H_1R activity (see Chapter 8) and, moreover, the highly potent partial H_1R agonist (8*R*)-lisuride behaves as an antagonist in this assay, demonstrating the limitations of intracellular calcium measurements as a sensitive readout of H_1R activity [55].

Measurement of guinea pig (gp) ileum contractions has been the assay system of choice for measuring H_1R activity for many years. Although this assay is still regularly used to obtain functional data for H_1R ligands, it is also not suited for evaluation of the potential negative intrinsic activity of ligands for the gpH_1R.

The carboxy terminus of the Gα protein is a key domain in the GPCR–G protein interaction [133, 134]. Chimeric G proteins, in which the last few amino acids have been replaced by corresponding amino acids present in other families of G proteins, have been widely used in screening assays to facilitate functional coupling of receptors, allowing functional screening for ligands modifying the receptor activity [135, 136]. These approaches have also been used for the H_3R [75] and H_4R [32, 34, 111, 137] in calcium mobilization assays.

13.3.3
[^{35}S]GTPγS Binding Assays (see also Chapter 8)

By substitution of [^{35}S]GTPγS for GTP, Gα protein activation results in the incorporation of the nonhydrolyzable and radioactively labeled [^{35}S]GTPγS, allowing the quantification of activated Gα proteins. [^{35}S]GTPγS binding assays thus monitor the activation of the receptor-associating G proteins and are particularly suitable for measurement of the activation of G_i- and G_o-coupled receptors, including the H_3R [23, 138] and H_4R [34].

[^{35}S]GTPγS binding assays have also been used to demonstrate constitutive H_3R activity in rat brain [108]. Constitutive [^{35}S]GTPγS binding was inhibited by various inverse H_3R agonists, and this inhibition was blocked by proxyfan, which acts as a neutral antagonist in this assay [108].

The pharmacological characterization of the human H_4R by [^{35}S]GTPγS binding assays on stably transfected HEK293 cells indicated a high level of constitutive H_4R activity [34], and resulted in the identification of thioperamide as the first inverse H_4R agonist.

Although the [^{35}S]GTPγS binding assay is particularly suitable for G_i- and G_o-coupled receptors, it was recently demonstrated that the assay is also applicable to GPCRs coupling to the G_q family of G proteins, including the H_1R [139] and H_2R [140], following an immunoprecipitation procedure [141]. Although this assay would be suited to direct determination of the amount of G proteins constitutively activated by the H_1R, there are no currently available data on constitutive H_1R activity as measured by [^{35}S]GTPγS binding assays by such an approach. Measurement of [^{35}S]GTPγS binding to the H_2R fused to either the long or the short splice variants of Gα_s, however, indicated that both receptor-fusion proteins exhibit similar [140], but only moderate, levels of constitutive activity .

In accordance with the reported moderate level of constitutive H_1R activity in IP_3 (see Section 13.3.4) [58, 143] and reporter-gene assays (see Section 13.3.7 and Chapter 8) [44, 143], low levels of constitutive H_1R activity and inverse agonistic activity of first- and second-generation H_1R antagonists were also detected by GTPase assays (see Chapter 8) in insect Sf9 cells [54].

13.3.4
IP_3 Formation (see also Chapter 8)

The H_1R couples to members of the G_q family of heterotrimeric G proteins to activate PLC and subsequently to generate IP_3. A significant increase in the basal level of [^{3}H]inositolphosphates production is observed upon overexpression of the wild-type human H_1R [58], indicating the H_1R is constitutively active. All tested H_1R antagonists potently and selectively inhibited the observed constitutive H_1R activity with potencies that correlate well with their respective affinities, which resulted in the reclassification of several H_1R antagonists as inverse H_1R agonists at the human H_1R [58]. Importantly, and in accordance with the known H_1R stereospecificity [144], the enantiomers of cetirizine display stereospecific inhibition of the constitutive histamine H_1R activity.

Although the measurement of IP_3 provides a sensitive measurement of agonist-induced H_1R responses, the assay is not very sensitive for the detection of constitutive H_1R activity. The assay is therefore less well suited for the evaluation of constitutive H_1R activity, as more sensitive assay systems for the detection of this have been described (see Section 13.3.7).

Although the coupling specificity of the H_2R is more efficient for G_s than for G_q proteins [145], activation of the H_2R is also reported to induce the formation of IP_3 and changes in intracellular calcium [63–68]. In addition, activation of the H_4R is suggested to result in the activation of PLCβ in mast cells [118]. As yet, however, no evidence for constitutive H_2R- or H_4R-mediated formation of IP_3 has been reported.

13.3.5
cAMP Assays (see also Chapter 8)

Because cAMP is one of the most important second messengers for control of various metabolic pathways, a number of different assays to measure the formation of cAMP from ATP by AC upon receptor stimulation have been developed over the years. The AC effector enzyme is activated by G_s proteins and inhibited by G_i proteins, so these assays can be used to monitor the activation of a wide variety of GPCRs, and have been routinely used to assay H_2R, H_3R [29], and H_4R activity [34].

The G_s-coupled H_2R potently stimulates AC to induce formation of cAMP and is suitable for the detection of constitutive H_2R activity [12]. The detection of constitutive H_2R activity, and the inverse agonistic properties of H_2R inverse agonists, however, are more readily detected when the cells are stimulated with the diterpene forskolin [146]. Forskolin directly stimulates AC, allowing for enhanced sensitivity of the detection of the inverse H_2R agonist-induced inhibition of AC.

Detection of the modulation of AC activity by the G_i-coupled H_3R and H_4R also requires the use of the AC stimulant forskolin [104]. Constitutive activity of H_3Rs is readily detected with this assay system [104, 147].

13.3.6 Measurements of Arachidonic Acid (AA) Release

Activation of heterologously expressed H_3Rs in cells that have been treated with the calcium ionophore A23187 by various H_3R agonists such as histamine and imetit has been shown to result in a $G_{i/o}$-dependent release of AA [23]. Heterologous expression of the H_3R in CHO cells, for instance, also results in a pronounced constitutive H_3R modulation of A23187-induced AA release, which can be inhibited by inverse H_3R agonists such as ciproxifan and thioperamide [23]. Interestingly, proxyfan may act as a partial agonist, a neutral antagonist, and a partial inverse agonist in this assay system [107].

13.3.7 Reporter Gene Assays (see also Chapter 8)

Changes in the levels of intracellular second messengers may also result in alteration in the expression of various genes due to modulation of the activity of transcription factors. These transcription factors may bind to specific transcription factors present in the promoter regions of genes to enhance or repress gene expression. Reporter gene assays for monitoring the activation of various transcription factors include CRE, 12-*O*-tetradecanoate-13-acetate response element (TRE), serum response element (SRE), NF-κB, and activator protein-1 (AP-1), mediated transcription of, for instance, luciferase and β-galactosidase, for which the enzyme activity can be assayed in the cell lysate, and green fluorescent protein. Reporter gene technology is now widely used to monitor the cellular events associated with signal transduction and gene expression. The principal advantage of these assays is their high sensitivity, reliability, convenience, and adaptability to large-scale measurements [148, 149]. The disadvantages of the use of reporter gene assays are the longer incubation times in the presence of ligands and the distal read-out of the assay. Reporter gene assays have been used to assist the deorphanization of various orphan GPCRs, including the H_4R [32, 33].

NF-κB reporter gene assays can be used as a very sensitive and dynamic readout for H_1R activity, well suited for the detection of constitutive H_1R activity [44]. This assay has been used to show that the therapeutically used H_1R antagonists all have inverse agonistic H_1R activity. Sensitivity can be enhanced in this assay by co-expression of appropriate Gα and Gβγ subunits (see also Section 13.3.9) [44].

cAMP-dependent reporter genes usually provide sensitive assays that have been successfully applied to assay the activity of the various histamine receptors. The G_s-coupled H_2R stimulates the expression of reporter genes under the transcriptional control of CREB [127], while the G_i-coupled H_3 [29] and H_4 receptors inhibit the expression of such reporter genes [32, 33]. As with direct cAMP assays (see Section 13.3.5), the detection of constitutive H_2R activity and inverse H_2R agonism can be enhanced by using forskolin, for instance, to stimulate AC directly; such direct activation of AC is required for the detection of H_3- and H_4-receptor-induced modulation of CREB-mediated gene transcription.

In addition, reporter genes may be used as marker genes to quantify, for instance, cell proliferate responses. In these cases the reporter gene is not under direct transcriptional control as is the case for reporter gene assays [150]. A cell-based technology platform for the functional evaluation of gene families that are potential drug targets, including GPCRs, is called Receptor Selection and Amplification Technology (R-SAT) and makes use of such a marker gene as a functional readout [151]. We used this assay system successfully for the identification of the potent H_1R agonist properties of lisuride [55], as well as for the evaluation of the negative intrinsic activity of various drugs for the H_1R (Bakker *et al.*, unpublished).

13.3.8 Activation of Kinases

All four histamine receptors have been shown to activate MAPK and related kinases [34, 46–49, 91, 92, 107, 152]. The ability of the histamine receptors to activate these pathways should be of importance in (patho)physiology. The activation of MAPK by H_2R and H_3R agonists in the brain, for instance, enhances fear memory [62]. Interestingly, different rat H_3R isoforms have been shown to differ in their effectiveness in activating MAPK pathways [91], suggesting potential differential roles for the H_3R isoforms. Constitutive activation of these pathways, however, has not yet been described.

13.3.9 Effects of the Cellular Environment on Histamine Receptor Activity

Receptors are promiscuous in their coupling specificity towards their binding to and activation of G proteins. As a result, a receptor may activate G proteins that belong to various G protein families. Although some GPCRs couple to members from all families of heterotrimeric G proteins [153], most GPCRs couple with some specificity to various G proteins. Additionally, other expressed receptors may also influence histamine receptor signaling. The cellular context is therefore critically important in determining which G proteins are ultimately activated upon receptor activation.

13.3.9.1 Co-expression of G Proteins

Appropriate G proteins can be co-expressed to enhance the detection of constitutive receptor activity [44, 154–156]. For the H_1R, co-expression with members of the G_q family of G proteins has been shown to facilitate detection of constitutive H_1R activity both in IP_3 accumulation and in NF-κB reporter gene assays, as well as in the detection of inverse agonism [44]. Specific combinations of Gβ and Gγ subunits (which dimerize to form Gβγ subunits) can also be co-expressed to enhance constitutive H_1R-mediated activation of NF-κB [44].

13.3.9.2 Co-expression of GPCRs

The activity of the H_1R may also be modulated by the co-expression of unrelated GPCRs [143]. Co-expression of unrelated $G_{i/o}$-coupled receptors may enhance constitutive H_1R activity, while inverse H_1R agonists may completely reduce the constitutive activity observed under the co-expression conditions. Although preliminary experiments suggest that the co-expression of such $G_{i/o}$-coupled receptors also affects the potency of inverse H_1R agonists, the stereospecific action of the enantiomers of cetirizine as inverse H_1R agonists is retained under the co-expression conditions. There is a prominent role for the constitutive H_1R activity in allowing co-expressed receptors to signal through H_1R-activated pathways.

In addition to homodimerization of the H_1R [157], it has recently also been shown that the H_1R may form a heterodimeric complexes together with the α_{1B}-adrenoceptor, and that these dimeric receptors function through >transactivation of associated G proteins [139]. It is not yet known how the formation of dimeric receptor alters the (constitutive) activity and signaling properties of the H_1R.

13.3.10 Construction and Expression of Constitutively Active Mutant Receptors

Heterologous expression of GPCRs may result in relatively high expression levels and often allows detection of agonist-independent or constitutive receptor activity. Alternatively, the receptors can be engineered such that constitutively active mutant (CAM) receptors are generated by an alteration in receptor conformation induced by amino acid substitution. The CAM receptors are usually generated on the basis of analogy with the effects on receptor activity found upon mutation of specific residues in other GPCRs. A sequence often considered when a CAM receptor is desired is the GPCR signature triplet amino acid sequence Asp-Arg-Tyr (DRY) found downstream of the third transmembrane domain (TM3) of most GPCRs and is involved in the interaction of the receptor with G proteins. Another domain that can be considered to generate a CAM is TM6 [158–161]. The relative orientation of TM3 and TM6 is thought to be important for receptor activity; the change from an inactive to an active GPCR is thought to be accompanied by a change in the relative orientation of these two TM domains, with a concomitant rotation of TM6 [162].

Mutation of the aspartate in the DRY motif in the H_2R (Asp^{115}) has resulted in the generation of two highly constitutively active mutant H_2Rs (H_2R $Asp^{115}Ala$ and H_2R $Asp^{115}Asn$) that also possess an increased affinity for histamine [163]. The mutant H_2R $Asp^{115}Ala$ and H_2R $Asp^{115}Asn$ receptors were found to be highly structurally unstable. In addition, mutation of the arginine in the DRY motif in the H_2R (Arg^{116}) has resulted in the generation of two additional structurally unstable mutant H_2Rs (H_2R $Arg^{116}Ala$ and H_2R $Arg^{116}Asn$ [163]. Although the unstable mutant H_2R $Arg^{116}Ala$ and H_2R $Arg^{116}Asn$ receptors could still couple functionally to G_s proteins upon histamine stimulation, these effects were only observed with pretreatment of the cells with an inverse H_2R agonist. Both H_2R agonists and inverse H_2R agonists were found to stabilize the unstable H_2R mutant proteins [163].

A highly constitutively active mutant mouse H_3R has been generated by conversion of Ala^{357} in the bottom of TM6 to a Lys (H_3R $Ala^{357}Lys$) [147]. Interestingly, the carboxy terminus of the third intracellular loop of the H_3R contains a stretch of eight amino acids very similar to the corresponding sequence in a CAM β_2R [164] and also critical for constitutive activity in other GPCRs [158, 159, 165]. Residue 357 falls within this stretch of eight amino acids. Surprisingly, however, mutation of $Ala^{357}Lys$ in the H_3R results in a H_3R receptor mutant that has a Lys at a position that corresponds to a Lys that is present in the wild-type β_2AR, but which is mutated, along with other residues, into an Ala in the CAM β_2R [164]. The mutant H_3R $Ala^{357}Lys$ receptor exhibits a dramatically enhanced cAMP accumulation response to the inverse H_3R agonist thioperamide and has an increased affinity for the agonist imetit and the protean agonist proxyfan, together with decreased affinities for inverse agonists such as thioperamide [147].

13.3.11
Contamination with Endogenous Histamine

There has previously been some debate regarding potential contamination with agonist as an explanation for constitutive GPCR signaling (see also Chapter 1) [166, 167]. As we were unable to identify a neutral H_1R antagonist, we were concerned about potential contamination with histamine. However, experiments with cells cultured in histamine-free medium or tests in which treatment with (*S*)-(+)-α-fluoromethylhistidine (α-FMH), a suicide inhibitor of histidine decarboxylase (HDC) [168] is applied still reveal constitutive H_1R activity and inverse agonism with H_1R antagonists.

In addition, we have determined the levels of histamine in the cell culture medium, but were unable to measure detectable levels of histamine (less then 1 nM) [12].

13.4
Conclusions

The four histamine receptors have quite different physiological roles and are linked to diverse signaling pathways. All four histamine receptors have been shown to exhibit constitutive activity by a variety of assay systems. Although difficult to assess, the levels of constitutive activity are different for the four histamine receptors, the H_2 and H_3 receptor appearing to display high levels of constitutive activity, whereas levels of constitutive activity for the H_1 and H_4 receptors are more moderate. Apparent levels of constitutive receptor activity, however, may also depend on the assay system used. The H_2R fused to G_s, for instance, exhibits only moderate levels of constitutive activity in Sf9 cell membranes when GTPase activity is assayed [17, 142]. As yet the H_3R is the only histamine receptor for which constitutive activity has been shown *in vivo*.

A large variety of ligands for the different histamine receptors, possessing various degrees of receptor affinity and specificity, are becoming available. Surprisingly, the therapeutically used H_1R and H_2R antagonists all possess negative intrinsic activity. While potent neutral H_1R and H_2R antagonists are still lacking, it appears that potent

neutral H_3R and H_4R antagonists can be more easily discovered. Such potent neutral histamine receptor antagonists should be useful in evaluation of the importance of constitutive activity in physiology. As yet the importance of the intrinsic activity of histaminerigic ligands for their therapeutic application is not known. With the discovery of proxyfan as a protean H_3R agonist it is becoming evident that the next challenge will be to obtain histamine receptor ligands with tailor-made properties in terms not only of receptor affinity and specificity, but also of intrinsic receptor activity under appropriate assay conditions.

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14
Constitutively Active Serotonin Receptors

Katharine Herrick-Davis

14.1
Introduction

Serotonin (5-HT) produces its physiological actions by interacting with a highly diversified family of plasma membrane receptors widely distributed throughout the brain and peripheral tissues. To date, 14 different 5-HT receptors (5-HTRs) have been identified. They have been classified into separate families, designated $5\text{-}HT_1R$ through $5\text{-}HT_7R$, on the bases of radioligand binding, second messenger activation, and amino acid sequence homology. In addition, there are multiple splice variants and isoforms that further enhance the molecular diversity of 5-HTR subtypes. Thirteen of the 5-HTRs are G protein-coupled receptors (GPCRs) and one, the $5\text{-}HT_3R$, is a ligand-gated ion channel. 5-HTRs couple to four different families of G proteins: G_s, $G_{i/o}$, $G_{q/11}$, and $G_{12/13}$. Within a given 5-HT receptor family the overall amino acid sequence homology ranges from 40–63 %, while the sequence homology between different families of 5-HTRs is lower and ranges from 25–39 % [1]. 5-HTRs are targets for many different classes of drugs currently prescribed for treating anxiety, depression, schizophrenia, migraine, eating disorders, emesis, and irritable bowel syndrome [1], so it is of great importance to understand the molecular mechanisms involved in 5-HTR activation.

5-HTR mutagenesis studies are beginning to elucidate receptor regions involved in 5-HT binding as well as receptor activation of G proteins. Studies with both native and mutant 5-HTRs have demonstrated that 5-HTRs can exist in a conformation that mimics the agonist-bound or active state of the receptor in the absence of 5-HT, indicating that 5-HTRs display constitutive activity. Recent studies have shown that genetic mutations producing constitutively active thyroid-stimulating hormone receptors (TSHRs), luteinizing hormone receptors (LHRs), parathyroid hormone–parathyroid hormone-related peptide receptors (PTH1Rs), and rhodopsin receptors are associated with diseases in humans (reviewed in section 1.7). Therefore, drugs that reverse or turn off receptor constitutive activity (i. e., inverse agonists) represent an important class of therapeutic agents.

Native and mutant forms of $5\text{-}HT_1Rs$, $5\text{-}HT_2Rs$, $5\text{-}HT_4Rs$, $5\text{-}HT_6Rs$, and $5\text{-}HT_7Rs$ display constitutive activity. RNA splicing produces multiple splice variants of 5-

HT_4Rs and $5\text{-}HT_7Rs$ that differ in the lengths of their C-terminal tails and their degrees of constitutive activity (reviewed in Chapter 4 on GPCR splice variants and constitutive activity). There have been two reports suggesting that $5\text{-}HT_6Rs$ display constitutive activity: mouse $5\text{-}HT_6Rs$ displayed constitutive activity when examined by a cAMP-based reporter gene assay (see Chapter 8) [2], and mutagenesis of the third intracellular loop (i3) of the human $5\text{-}HT_6R$ has been reported to enhance constitutive activity [3]. This chapter therefore focuses on $5\text{-}HT_1R$ and $5\text{-}HT_2R$ subtypes that display constitutive activity (1A, 1B, 1D, 2A, 2C) and the available literature on native and mutant receptors and inverse agonists is reviewed. To allow a general comparison of amino acids between different GPCRs, the Ballesteros and Weinstein numbering scheme [4] is provided as a superscript in parenthesis following the amino acid number given for an individual receptor. Results of mutagenesis studies are described in view of current molecular models of receptor/G protein activation (see Chapters 2, 3, and 9) and potential physiological roles for constitutively active 5-HTRs are discussed (see also Chapter 8).

14.2
5-HT_{1A} Receptor (5-HT_{1A}R)

$5\text{-}HT_{1A}Rs$ are expressed in many brain regions including the raphe, hippocampus, amygdala, and cortex. In the raphe they function as somatodendritic autoreceptors producing hyperpolarization and inhibition of 5-HT neuronal firing [1]. In other brain regions they have a postsynaptic localization where they stimulate K^+ channels, inhibit or stimulate adenylyl cyclase (AC), and inhibit Ca^{2+} channels [1]. These effects are mediated through interaction with the G_i family of G proteins. $5\text{-}HT_{1A}Rs$ are potential targets for drugs used to treat anxiety, depression, and schizophrenia.

Native human $5\text{-}HT_{1A}Rs$ have been demonstrated to exhibit constitutive activation of $[^{35}S]$guanosine-5'-[γ-thio]triphosphate ($[^{35}S]GTP\gamma S$) binding to G_i proteins when expressed in recombinant cell systems [5–11]. $[^{35}S]GTP\gamma S$ binding is a common assay used to monitor constitutive activity of receptors that couple to G_i (see Chapter 8). $[^{35}S]GTP\gamma S$ binds to G proteins after activation of the G protein by a receptor. A comparison of basal $[^{35}S]GTP\gamma S$ binding in untransfected and GPCR transfected cells followed by inhibition of $[^{35}S]GTP\gamma S$ binding by inverse agonist is used to measure receptor constitutive activity. Moderate to high levels of constitutive activity have been reported for $5\text{-}HT_{1A}Rs$ expressed from 0.18 to 10 pmol mg^{-1} in HEK293, COS-7, CHO, Hela, and Sf9 insect cells [5–11]. The degree of constitutive activation of $[^{35}S]GTP\gamma S$ binding and the ability to measure inverse agonist activity are regulated by NaCl concentration: decreasing the NaCl concentration and ionic strength of the assay medium increases basal $[^{35}S]GTP\gamma S$ binding and inverse agonist efficacy [8, 9]. Methiothepin, spiperone, and butaclamol consistently displayed inverse agonist activity in these systems, while *N*-{2-[4-(2-methoxyphenyl)-1-piperazinyl]ethyl}-*N*-2-pyridinyl-cyclohexanecarboxamide (WAY-100635) displayed weak or no inverse agonism [5–11]. Studies evaluating antipsychotic drugs for activity in $[^{35}S]GTP\gamma S$ binding assays in cells trans-

fected with 5-HT_{1A}Rs have identified clozapine, ziprasidone, quetiapine, and tiospirone as partial agonists [8, 9]. Risperidone, sertindole, haloperidol, pimozide, raclopride, and chlorpromazine were neutral antagonists in [^{35}S]GTPγS binding assays at 5-HT_{1A}Rs expressed in CHO cells [8], but were inverse agonists when the [^{35}S]GTPγS binding assays were performed in the presence of 100 mM NaCl [9]. While studies have suggested a role for 5-HT_{1A}Rs in the pathology of schizophrenia, it remains to be determined whether partial agonism and/or inverse agonism at 5-HT_{1A}Rs contributes to the clinical efficacy of antipsychotic drugs (see also Chapter 7).

Co-expression of G_i proteins with 5-HT_{1A}Rs, individually or as fusion proteins, produces constitutive activation of [^{35}S]GTPγS binding and high-affinity GTP hydrolysis [10, 11]. Interestingly, a single amino acid mutation in the G_i protein can alter the level of constitutive activity of the 5-HT_{1A}R [10, 11]. These studies suggest that mutations in G proteins may regulate GPCR constitutive activity and, if found in the human population, may play a role in human diseases. For a more detailed discussion of the impact of G proteins on receptor constitutive activity, see Chapter 6.

Radioligand binding studies using [^{3}H]WAY-100635 to label 5-HT_{1A}Rs expressed in CHO cells were performed in the absence and in the presence of GTPγS to determine the affinity of agonists and inverse agonists for G protein-coupled and -uncoupled forms of the receptor. Agonist competition curves were shifted to the right and inverse agonist curves to the left in the presence of GTPγS (see also Chapters 8 and 9) [12]. The changes in agonist and inverse agonist binding affinities in the presence of GTPγS correlated closely with their efficacies as determined in [^{35}S]GTPγS binding assays [12]. These results indicate that 5-HT_{1A}R agonists exhibit higher affinity for the G protein-coupled form of the receptor, while inverse agonists have higher affinity for the G protein-uncoupled form of the receptor (see also Chapter 2).

In addition to mediating inhibition of AC, the 5-HT_{1A}R can stimulate AC in brain regions that express AC isoform II. In HEK293 cells, 5-HT_{1A}R stimulation of ACII is mediated by $G_{\beta\gamma}$ subunits associated with G_{i2} [13]. This effect usually requires co-stimulation of ACII with $G_{\alpha s}$. Co-expression of ACII with the 5-HT_{1A}R and G_{i2} in HEK293 cells revealed a 5-HT_{1A}R-mediated constitutive activation of cAMP production that was 40–50% inhibited by the anxiolytic drugs buspirone and flesinoxan. From these results it was suggested that anxiolytic activity may involve inhibition of the constitutive activity of the 5-HT_{1A}R [13].

Previous mutagenesis studies have identified highly conserved domains in the i2 loop that regulate G protein coupling and receptor constitutive activity [14]. The C-terminal region of i2 of the 5-HT_{1A}R contains a Thr residue, Thr$^{149(4.38)}$, that is highly conserved among GPCRs that couple to G_i proteins. Mutation of Thr$^{149(4.38)}$ to Ala in the 5-HT_{1A}R completely abolished constitutive activity [13], highlighting the importance of i2 in coupling the 5-HT_{1A}R to G_i.

Mutations in the BBXXB$^{(6.31\text{-}6.35)}$ motif (B = basic amino acid; X= any amino acid) in the C-terminal region of i3 enhance the constitutive activity of many, but not all, GPCRs (reviewed in [15]). Mutations within this region of the 5-HT_{1A}R, Val$^{344(6.34)}$Glu or Thr$^{343(6.33)}$Ala and Val$^{344(6.34)}$Glu, did not give rise to enhanced constitutive activity. Instead, these mutations switched the agonist-dependent coupling of the receptor from G_i to G_s [16]. Additional studies are required to determine if other mutations

of $Val^{344(6.34)}$ or of other residues within this region enhance 5-HT_{1A}R constitutive activity. Mutation of $Ala^{293(6.34)}$ of the α_{1B}-adrenoceptor (α_{1B}AR) to any other amino acid enhanced constitutive activity [17, 18]. The differences in results obtained with the 5-HT_{1A}R and α_{1B}AR may reflect structural differences related to G protein preference (G_i and G_q, respectively) or constitutive activity of the native receptors. Native 5-HT_{1A}Rs have significant constitutive activity, while native α_{1B}ARs have little or no constitutive activity [17, 18]. While these studies highlight the importance of i3 in G protein activation for both 5-HT_{1A}Rs α_{1B}ARs, they also demonstrate that the exact mechanism and amino acids involved in receptor activation may differ among members of class A GPCRs.

Naturally occurring polymorphisms located in regions of a GPCR critical for G protein activation may alter receptor constitutive activity, thereby producing hypofunctioning or hyperfunctioning receptors *in vivo*. To date, seven different polymorphisms producing amino acid substitutions in the 5-HT_{1A}R have been identified in the human population (reviewed in [19, 20]). Three of the polymorphisms (Pro^{18}Leu, Gly^{22}-Ser, Ile^{28}Val) are located in the N terminus of the 5-HT_{1A}R, a region of the receptor not implicated in 5-HT binding or receptor activation [20]. However, the Arg^{219}Leu, Val^{267}-Met, and Gly^{273}Asp polymorphisms are located in the N and C termini of i3, regions of the receptor known to be important for G protein activation. It remains to be determined if these mutations alter the G protein coupling and constitutive activity of the 5-HT_{1A}R. The Asn^{418}Lys polymorphism is located in the C-terminal end of the receptor. While the C-terminal tail regulates the constitutive activity of 5-HT_4Rs and 5-HT_7Rs [21, 22], it remains to be clarified whether the Asn^{418}Lys polymorphism alters the constitutive activity of 5-HT_{1A}Rs *in vivo*.

Many studies have reported that native 5-HT_{1A}Rs display constitutive activity in recombinant cell systems, but there have been no reports of 5-HT_{1A}R constitutive activity in intact tissues or *in vivo*. [^{35}S]GTPγS binding studies can be performed in brain slices [23], so it may be possible to test the hypothesis that 5-HT_{1A}Rs present in brain tissue are constitutively active. 5-HT_{1A}R constitutive activity, if present in the dorsal raphe, may play an important role in regulating the tonic/basal firing rate of 5-HT neurons and 5-HT release.

14.3 5-HT_{1B} and 5-HT_{1D} Receptors (5-HT_{1B}R and 5-HT_{1D}R)

Human 5-HT_{1B}Rs (formerly 5-$HT_{1D\beta}$) and 5-HT_{1D}Rs (formerly 5-$HT_{1D\alpha}$) have high structural homology and similar distribution in the CNS. They are located in basal ganglia, striatum, raphe, hippocampus, nucleus accumbens, and cortex [1]. In these regions, 5-HT_{1B}Rs and 5-HT_{1D}Rs function as terminal autoreceptors on 5-HT neurons to control 5-HT release and as terminal heteroreceptors on non-5-HT neurons to control the release of norepinephrine, acetylcholine, and γ-aminobutyric acid (GABA) [1]. Both receptors have been identified in the cerebral vasculature and in the trigeminal ganglia, where they are likely targets for anti-migraine drugs (e. g., sumatriptan, a 5-HT_{1D}R agonist).

Like 5-HT$_{1A}$Rs, 5-HT$_{1B}$Rs and 5-HT$_{1D}$Rs couple to the G$_i$ family of G proteins to mediate inhibition of AC. Human 5-HT$_{1B}$Rs and 5-HT$_{1D}$Rs display constitutive activity when expressed in recombinant cell systems. [^{35}S]GTPγS binding assays in membranes prepared from transfected CHO cells reveal moderate to high levels of 5-HT$_{1B}$R and 5-HT$_{1D}$R constitutive activity [24–33]. Methiothepin, 1'-methyl-5-{[2'-methyl-4'-(5-methyl-1,2,4-oxadiazol-3-yl)biphenyl-4-yl]carbonyl}-2,3,6,7-tetrahydrospiro[furo[2,3-f]indole-3,4'-piperidine] oxalate, and 1'-ethyl-5-[2'-methyl-4'-(5-methyl-1,3,4-oxadiazol-2-yl)biphenyl-4-carbonyl]-2,3,6,7-tetrahydrospiro[furo[2,3-f]indole-3,4'-piperidine] (SB-224289) decrease basal [^{35}S]GTPγS binding, indicating that they are inverse agonists, while *N*-[1-(2,3-dihydro[1,4]dioxin-5-yl)piperid-4-yl]indan-2-ylamine (S18127) is a neutral antagonist [24–32]. Typical antipsychotics (i.e., haloperidol and chlorpromazine) and atypical antipsychotics (i.e., clozapine, olanzapine, risperidone, and ziprasidone) have been tested for inverse agonist activity at cloned human 5-HT$_{1B}$Rs and 5-HT$_{1D}$Rs [33]. All of these drugs are inverse agonists at 5-HT$_{1B}$Rs and 5-HT$_{1D}$Rs, except for ziprasidone, which was a partial agonist at 5-HT$_{1B}$Rs. Inverse agonist potencies for these drugs are in the micromolar range, except for risperidone and ziprasidone, which are potent inverse agonists at 5-HT$_{1D}$Rs. From these results, it was concluded that inverse agonist activity at 5-HT$_{1B/1D}$Rs is unlikely to be a common property of antipsychotic drugs *in vivo* [33].

Mutations within the BBXXB$^{(6.31\text{-}6.35)}$ motif in the C-terminal region of i3 of the 5-HT$_{1B}$R have been reported to increase the constitutive activity of native 5-HT$_{1B}$Rs. Mutagenesis of Thr$^{313(6.34)}$ to Lys, Arg, or Glu in i3 of the 5-HT$_{1B}$R increases basal [^{35}S]GTPγS binding in transfected CHO cells [34]. The basal activity of the mutant receptors represents 30% of the maximal stimulation produced by 5-HT and is reversed by the inverse agonists methiothepin and 3-[3-(dimethylamino)propyl]-4-hydroxy-*N*-[4-(4-pyridinyl)phenyl]benzamide dihydrochloride (GR-55562) [34]. In the same study, mutation of Thr$^{313(6.34)}$ to His eliminated 5-HT$_{1B}$R constitutive activity. These results demonstrate the importance of i3 in regulating 5-HT$_{1B}$R constitutive activity. While i3 may physically associate with the G protein to regulate G protein activation, conformational changes in transmembrane domain (TM) V and VI, which are linked together by i3, may influence the ability of i3 to regulate G protein activation. In support of this hypothesis, mutation of a highly conserved Ser in TM V (Ser$^{212(5.42)}$) or a highly conserved Phe in TM VI (Phe$^{331(6.52)}$) to Ala significantly decreased 5-HT$_{1B}$R constitutive activity [35]. Similarly, mutations in TM IV may alter the ability of i2 to interact with G proteins. Mutation of Phe$^{185(4.61)}$ to Ala or Met in TM IV of the 5-HT$_{1B}$R decreases the number of receptors in the G protein-coupled conformation and decreases the binding affinity of 5-HT for the uncoupled conformation [36]. These results indicate that 5-HT$_{1B}$R constitutive activity can be altered by mutations in regions that are directly involved in G protein coupling (such as i3) or indirectly by mutations in TM regions that influence the ability of intracellular regions to interact with G proteins.

A naturally occurring polymorphism that results in an amino acid substitution of Phe$^{124(3.27)}$ for Cys has been identified in the 5-HT$_{1B}$R with a prevalence of 2% in the Caucasian population [37, 38]. Phe$^{124(3.27)}$Cys is located near the extracellular end of TM III. While 5-HT$_{1B}$ agonists display higher affinity for the Cys$^{124(3.27)}$ variant, basal

levels of [^{35}S]GTPγS binding are the same for Phe$^{124(3.27)}$ and Cys$^{124(3.27)}$ expressed in rat C6 glioma cells, suggesting that the polymorphism does not alter 5-HT$_{1B}$R constitutive activity [38].

Studies using SB-224289 and S18127 were performed to determine whether 5-HT$_{1B}$Rs display constitutive activity *in vivo* [30]. Three different assays were used to address this issue: 1) [^{35}S]GTPγS binding in substantia nigra and caudate, 2) microdialysis measurements of extracellular 5-HT levels in the frontal cortex, and 3) changes in core body temperature. While SB-224289 and S18127 blocked the actions of agonists in all three assays, neither drug given alone had any effect on basal levels in these assays. Therefore, these studies failed to detect 5-HT$_{1B}$R constitutive activity *in vivo*. However, two other studies reported that SB-224289 and SB-236057 increased extracellular 5-HT levels in the dentate gyrus of the freely moving guinea pig [39, 40], consistent with the hypothesis that 5-HT$_{1B}$Rs may be constitutively active *in vivo*. Unfortunately, no experiments with neutral antagonists (to determine if the observed effect of the inverse agonist could be blocked) were performed, so it is not possible to determine if endogenous 5-HT influenced the results of these experiments. When reserpine was used to deplete endogenous 5-HT in guinea pigs, SB-224289 had no effect on 5-HT synthesis or 5-HIAA (5-hydroxyindoleacetic acid) production, indicating that no constitutive activity of 5-HT$_{1B}$Rs was detectable *in vivo* under these experimental conditions [41]. Clearly, additional studies to determine whether 5-HT$_{1B}$Rs display constitutive activity *in vivo* are required.

14.4
5-HT$_{2A}$ Receptor (5-HT$_{2A}$R)

5-HT$_{2A}$Rs are widely distributed throughout the human body. In peripheral tissues, 5-HT$_{2A}$Rs are found in the gastrointestinal tract, uterus, vasculature, and platelets, where they mediate physiological responses such as muscle contraction, vasoconstriction, and platelet aggregation [1]. In the brain, 5-HT$_{2A}$Rs are found in the cortex, basal ganglia, hippocampus, and hypothalamus, where they are located postsynaptically to 5-HT neurons and regulate dopamine, adrenaline, GABA, and glutamate neurotransmission [1]. 5-HT$_{2A}$Rs are believed to play important roles in cognition, mood, hallucinations, and neuroendocrine regulation, and are targets for drugs used to treat depression and schizophrenia.

Mutagenesis and molecular modeling studies have been performed in order to identify key amino acid residues involved in agonist binding and agonist-mediated 5-HT$_{2A}$R activation. Highly conserved Asp residues in TMs II and III, Ser in TMs III and V, and Phe and Try residues in TMs VI and VII have been identified as important for agonist binding and receptor activation [42–46]. In addition, i3 has been identified as an important region for 5-HT$_{2A}$R/G$_q$ protein coupling [47]. Inositol phosphate (IP) production is an assay commonly used to measure constitutive activity of receptors coupled to G$_q$ (see Chapter 8). Native 5-HT$_{2A}$Rs display little or no constitutive activity in IP production when expressed in recombinant systems [48, 49].

The first evidence for 5-HT$_{2A}$R constitutive activity was reported following mutagenesis of Cys$^{322(6.34)}$ in the C-terminal BBXXB$^{(6.31-6.35)}$ region of i3 [48]. This study was

designed on the basis of results obtained with α_{1B}ARs demonstrating that the C-terminal region of i3 is a critical determinant of G_q activation and receptor constitutive activity [17, 18]. When expressed in COS-7 cells at 0.2 to 0.3 pmol mg^{-1} protein, mutant 5-HT$_{2A}$Rs (Cys$^{322(6.34)}$Arg, Cys$^{322(6.34)}$Lys, Cys$^{322(6.34)}$Glu) displayed the hallmark characteristics of constitutively active receptors: increased agonist binding affinity, agonist potency, and basal IP production [48]. In this system, spiperone and ketanserin were potent inverse agonists and decreased receptor constitutive activity by 60–80 %. These results confirm that the C-terminal region of i3 plays a critical role in maintaining the 5-HT$_{2A}$R in an inactive conformation.

The binding affinities of a several competitive 5-HT$_{2A}$R antagonists at native and Cys$^{322(6.34)}$Lys 5-HT$_{2A}$Rs were compared [48]. Methysergide, clozapine, mianserin, and haloperidol had significantly lower affinity for ^{3}H-ketanserin-labeled Cys$^{322(6.34)}$Lys 5-HT$_{2A}$Rs than for native 5-HT$_{2A}$Rs, consistently with the hypothesis that inverse agonists have lower affinity for the active state of the receptor [50, 51]. However, in the same study, ketanserin, spiperone, chlorpromazine, and risperidone were found to have similar affinities for the native and mutant 5-HT$_{2A}$Rs. These results, which are not unique to the 5-HT$_{2A}$R, indicate that drugs from different chemical classes interact with different amino acids within the binding pocket to stabilize different conformations of the receptor (see also 2.1).

From the crystal structure of bovine rhodopsin, a strong ionic interaction between the highly conserved Arg$^{(3.50)}$ in the Glu/Asp, Arg, Tyr motif of the TM III/i2 junction and Glu$^{(6.30)}$ in the C-terminal region of i3 is predicted to play an important role in maintaining the receptor in an inactive state [52]. This hypothesis was recently tested at the 5-HT$_{2A}$R following mutagenesis of Arg$^{173(3.50)}$ to Glu and Glu$^{318(6.30)}$ to Arg [53]. In this study, native and mutant 5-HT$_{2A}$Rs were expressed in HEK293 cells and monitored for changes in 5-HT affinity, potency, and constitutive activity. The Glu$^{318(6.30)}$Arg mutation resulted in a significant increase in 5-HT affinity, 5-HT potency, and constitutive activity. On the other hand, 5-HT potency and constitutive activity were significantly reduced in the Arg$^{173(3.50)}$Glu mutant, and the double reverting mutant (Arg$^{173(3.50)}$Glu/Glu$^{318(6.30)}$Arg) displayed an intermediate phenotype. While the results with the Arg$^{173(3.50)}$Glu mutant may seem inconsistent with the proposed hypothesis it should be noted that previous studies have demonstrated the critical role of Arg$^{(3.50)}$ in receptor activation. Additionally, mutation of this amino acid results in diminished receptor expression [54] and decreases the ability of the receptor to couple to G proteins [55, 56]. The results observed with Glu$^{318(6.30)}$Arg mutant 5-HT$_{2A}$Rs are consistent with those observed with adrenoceptors demonstrating significant increases in constitutive activity following mutation of Glu$^{298(6.30)}$ in α_{1B}ARs (see Chapter 11) [57, 58] and Glu$^{268(6.30)}$ in β_2ARs [59]. These results are consistent with the hypothesis that the ionic interaction between Arg$^{(3.50)}$ and Glu$^{(6.30)}$ helps to stabilize the receptor in an inactive conformation and that disruption of this interaction may play an important role in receptor activation [52, 53, 57–59]. While the hypothesis predicts that the double revertant mutant Arg$^{173(3.50)}$Glu/Glu$^{318(6.30)}$Arg should reverse the constitutive activity of the Glu$^{318(6.30)}$Arg mutant back to wild-type levels (through hydrogen bond formation between Glu$^{(3.50)}$ and Arg$^{(6.30)}$), computer modeling studies of the α_{1B}AR have shown that the hydrogen bond predicted to exist between Arg$^{(3.50)}$ and Glu$^{(6.30)}$ in

the wild-type receptor cannot be reestablished in the $Arg^{(3.50)}Glu/Glu^{(6.30)}Arg$ mutant receptor, due to changes in the spatial orientation of $Glu^{(3.50)}$ and $Arg^{(6.30)}$ in the double mutant receptor [58].

If, as proposed, an ionic interaction between $Arg^{(3.50)}$ and $Glu^{(6.30)}$ is a critical determinant for constraining the receptor in an inactive conformation, it may seem odd at first glance that the first mutations reported to produce constitutive activity were located several amino acids distally to $Glu^{(6.30)}$. While substitution of any amino acid for $Ala^{293(6.34)}$ increased the constitutive activity of the $\alpha_{1B}AR$, it is noteworthy that amino acids with long, charged side chains (such as Lys, Arg, Glu) produced greater constitutive activity than the smaller, neutral amino acids [17]. In molecular modeling studies of the $\alpha_{1B}AR$, mutation of $Ala^{293(6.34)}$ to Glu is predicted to disrupt the hydrogen bond between $Arg^{(3.50)}$ and $Glu^{(6.30)}$. It is possible that substitutions at $Ala^{293(6.34)}$ that produce the greatest regional alteration in structure will be more capable of disrupting the ionic interaction between $Arg^{(3.50)}$ and $Glu^{(6.30)}$, thereby producing the greatest degree of constitutive activity.

In addition to providing the first evidence that $5\text{-}HT_{2A}Rs$ can be mutated to a constitutively active state, studies with the $Cys^{322(6.34)}Lys$ $5\text{-}HT_{2A}R$ were the first to reveal a novel property of antipsychotic drugs. While antipsychotic drugs are known to exhibit high affinity for $5\text{-}HT_{2A}Rs$ [61], studies with the constitutively active $Cys^{322(6.34)}Lys$ $5\text{-}HT_{2A}R$ identified antipsychotic drugs as $5\text{-}HT_{2A}R$ inverse agonists [48]. In COS-7 cells transfected with the $Cys^{322(6.34)}Lys$ $5\text{-}HT_{2A}R$, clozapine, risperidone, chlorpromazine, and loxapine were potent inverse agonists, capable of reversing 70–80% of the $5\text{-}HT_{2A}R$-mediated constitutive activation of IP production [48]. These results were confirmed and significantly extended in NIH3T3 cells by use of a modified β-galactosidase assay (receptor selection and amplification or *R*-SAT) (see Chapter 8) in which native $5\text{-}HT_{2A}Rs$ have low but measurable levels of constitutive activity [62]. In this system, atypical and typical antipsychotic drugs were identified as $5\text{-}HT_{2A}R$ inverse agonists. Twenty-six of these drugs were potent inverse agonists, with EC_{50} values of 100 nM or less. The results of both of these studies demonstrate that atypical and typical antipsychotic drugs are potent $5\text{-}HT_{2A}R$ inverse agonists and suggest a potential role for increased serotoninergic tone in the pathophysiology of schizophrenia [48, 62]. A transgenic mouse expressing the constitutively active $Cys^{322(6.34)}Lys$ $5\text{-}HT_{2A}R$ would provide an interesting tool with which to study the neurochemical and behavioral consequences of increased serotoninergic tone and may provide a novel animal model of schizophrenia.

While it has been suggested that increased serotoninergic tone and or constitutive activity of $5\text{-}HT_{2A}Rs$ may play a role in psychiatric diseases, $5\text{-}HT_{2A}R$ mutations that alter receptor constitutive activity have not been reported in patients with mental illness. Five different polymorphisms resulting in amino acid substitutions in the $5\text{-}HT_{2A}R$ have been identified in the human population: $Thr^{25}Asn$, $Ile^{197}Val$, $Ser^{412}Phe$, $Ala^{447}Val$, and $His^{452}Tyr$ (reviewed in [63]). While the $Ile^{197}Val$, $Ala^{447}Val$, and $His^{452}Tyr$ polymorphisms are reported to decrease maximal IP response to 5-HT, the effect of these polymorphisms on $5\text{-}HT_{2A}R$ constitutive activity remains to be determined [63].

14.5 5-HT_{2C} Receptor (5-HT_{2C}R)

5-HT_{2C}Rs are present only in the CNS. Since 5-HT_{2C}Rs were first identified in high density in the choroid plexus and were suggested to play a role in cerebral spinal fluid regulation, less attention has been paid to investigating potential roles for 5-HT_{2C}Rs in the regulation of cognition, mood, and mental illness. However, we now know that 5-HT_{2C}Rs are widely distributed throughout the brain and are found in many regions where 5-HT_{2A}Rs are located. 5-HT_{2C}R mRNA is present in the choroid plexus, cortex, basal ganglia, hypothalamus, and hippocampus, as well as in other brain regions [1]. 5-HT_{2C}Rs are located postsynaptic to serotoninergic neurons and have an inhibitory influence on dopaminergic and adrenergic neurotransmission [1]. Biochemical and behavioral studies have provided evidence for 5-HT_{2C}R regulation of cognition, mood, appetite, neuroendocrine function, and locomotor activity. In addition, certain antipsychotics, antidepressants, and anxiolytic drugs bind with high affinity to 5-HT_{2C}Rs, suggesting a role for 5-HT_{2C}Rs in the pathophysiology of mental illnesses.

The 5-HT_{2C}R couples to the G proteins G_q and G_{13}, and stimulates phospholipases C, A_2, and D [64–66]. 5-HT_{2C}Rs display significant constitutive activation of G_q when expressed in mammalian cells. The first studies were performed in transfected NIH3T3 cells and reported moderate levels of 5-HT_{2C}R constitutive activity in IP production [51, 67]. In this system, mianserin, mesulergine, clozapine, ketanserin, and spiperone displayed inverse agonist activity by reversing 5-HT_{2C}R constitutive activity. Methysergide and 2-bromolysergic acid diethylamide (BOL) were classified as neutral antagonists because they did not alter basal levels of IP production but were capable of blocking inverse agonist activity [67]. Radioligand binding studies performed in the presence of guanine nucleotides (to uncouple constitutively active 5-HT_{2C}R from G proteins) demonstrated that inverse agonists display higher affinity for the uncoupled form of the receptor, agonists have higher affinity for the coupled form, and neutral antagonists have equal affinity for both forms of the receptor [51].

Mutagenesis studies have been performed to identify regions of the 5-HT_{2C}R involved in constitutive activation of G proteins. The C-terminal region of i3 in the 5-HT_{2C} receptor contains the highly conserved $BBXXB^{6.31\text{-}6.35}$ motif. In the α_{1B}AR, $Ala^{293(6.34)}$ was identified as a critical amino acid required for maintaining the receptor in an inactive conformation [17]. Mutation of the analogous amino acid in i3 of the 5-HT_{2C}R, $Ser^{312(6.34)}$ to Phe or Lys, enhanced 5-HT_{2C}R constitutive activity [60, 68]. The mutant receptors displayed the hallmark characteristics of constitutively active mutant receptors with an increase in 5-HT affinity and potency, increased basal IP production and reversal of constitutive activity with the inverse agonists mianserin and mesulergine [60, 68]. Saturation experiments demonstrated a decrease in mesulergine binding affinity, and guanine nucleotides had no effect on 5-HT binding affinity at the $Ser^{312(6.34)}$Lys 5-HT_{2C}R [60]. These results indicate that the mutant receptor is in an agonist high-affinity conformation even in the absence of G protein coupling. These results are similar to those observed with constitutively active mutant adrenoceptors that display an increase in agonist binding affinity in the absence of receptor G protein coupling [50, 69]. On the basis of the studies with mutant adrenoceptors, the ternary

complex model was revised to the extended ternary complex model to include a spontaneous isomerization step from the inactive (R) to the active (R^*) state that can occur in the absence of agonist and promotes spontaneous coupling of receptor with the G protein (see Chapters 2 and 3) [50]. While it is likely that there are multiple agonist high-affinity conformations, or R^* states (see Chapter 9), studies with the Ser$^{312(6.34)}$Lys 5-HT$_{2C}$R indicate that a revised ternary complex model of receptor activation is applicable to 5-HT$_{2C}$Rs.

5-HT$_{2C}$R constitutive activity is modified not only by mutations in i3, but also by mutations in the highly conserved Asn/Pro/X/X/Try$^{(7.49\text{-}7.53)}$ region in TM VII. Mutation of Tyr$^{(7.53)}$ to Ala or Cys increased 5-HT$_{2C}$R constitutive activity in IP production in COS-1 cells, while mutation to Phe eliminated constitutive and agonist-stimulated IP production [70]. Interestingly, all three mutant receptors displayed increased affinity for 5-HT, suggesting that the Phe mutant was in a high-affinity state for agonists even though it was not able to couple to G proteins and stimulate IP production. Mutation of Tyr$^{(7.53)}$ to Asn resulted in high constitutive activity in IP production that was not increased by agonist or blocked by inverse agonist, but behaved as a receptor that was “locked-on“ [71]. Computational modeling of the light receptor rhodopsin suggested that Tyr$^{(7.53)}$ may interact with Tyr$^{(7.60)}$ in the recently proposed TM VIII [52]. Consistent with this hypothesis, a double reverting mutation of Tyr$^{(7.60)}$ in the inactive Tyr$^{(7.53)}$Phe 5-HT$_{2C}$R restored the wild-type phenotype of the inactive receptor [71]. Based on these results it was suggested that Tyr$^{(7.53)}$ and Tyr$^{(7.60)}$ participate in a functional microdomain, connecting TMs VII and VIII, that may play a role in regulating the conversion between active and inactive receptor conformations [71].

Naturally occurring amino acid substitutions within i2 of the 5-HT$_{2C}$R are produced as a result of RNA editing [72] and regulate the degree of receptor constitutive activity [73,74]. Complimentary base pairings between nucleotides in the exon containing i2 and the downstream intron result in the formation of double-stranded RNA [72]. Adenosine deaminases (ADAR1 and ADAR2) convert adenosine into inosine at five different editing sites (designated A–E) in the double-stranded RNA, changing the coding potential of amino acids 156, 158, and 160 in i2 of the 5-HT$_{2C}$R [72, 74]. Fourteen different isoforms of the 5-HT$_{2C}$R have been identified in human brain [74–76], ranging from unedited (Ile$^{156(3.54)}$, Asn$^{158(3.56)}$, Ile$^{160(3.58)}$; INI) to fully edited (Val$^{156(3.54)}$, Gly$^{158(3.56)}$, Val$^{160(3.58)}$; VGV). RNA editing from INI to VGV decreases agonist binding affinity and potency at rat and human 5-HT$_{2C}$R [72–76].

The RNA editing site is located within the highly conserved Asp/Arg$^{(3.50)}$/Try/X/X/(Val/Ile)/X/X/X/L sequence at the junction of TM III and i2. Amino acid substitutions in this region have been shown to produce both inactive and constitutively active M-cholinoceptors (see Chapter 12) and adrenoceptors (see Chapter 11) [13, 57, 59, 77–79]. Computational modeling studies of the α_{1B}AR have suggested that the position of Arg$^{(3.50)}$ relative to Asp$^{(2.50)}$ in TM II [79] and Glu$^{(6.30)}$ in i3 [57] is important in maintaining the receptor in an inactive conformation. Therefore, it seemed possible that RNA editing may alter the constitutive activity of the 5-HT$_{2C}$R. The first study designed to test this hypothesis was performed in COS-7 cells transfected with seven different isoforms of the 5-HT$_{2C}$R [73]. When expressed at physiologically relevant receptor expression levels (2.6–3.4 pmol mg^{-1} protein), the unedited INI isoform displayed the

highest level of constitutive activity in IP production, the fully edited VGV isoform displayed little or no constitutive activity, and the partially edited isoforms (ISV, VSI, INV, VNV, and VSV) displayed intermediate levels of constitutive activity [73]. When receptor expression levels were decreased by approximately 50%, the 5-$HT_{2C}R$ constitutive activity was decreased by a similar magnitude, indicating that constitutive activity is related to receptor expression level. In these studies, 5-HT had higher affinity and greater potency at the unedited isoform, which displayed the highest basal activity, than at the fully edited receptor with no constitutive activity [73, 74]. These results are consistent with previous studies demonstrating that mutations in i3 increase 5-$HT_{2C}R$ constitutive activity and increase 5-HT affinity and potency [60].

It is particularly interesting to note that the 5-$HT_{2A}R$, which does not undergo RNA editing, has the same $Ile^{(3.54)}$, $Asn^{(3.56)}$, $Ile^{(3.58)}$ amino acid sequence in i2 as the unedited isoform of the 5-$HT_{2C}R$. This is intriguing because the 5-$HT_{2A}R$ has low levels of constitutive activity relative to the INI isoform of the 5-$HT_{2C}R$ [49]. In fact, the amino acid sequence of i2 in the 5-$HT_{2A}R$ is the same as the 5-$HT_{2C}R$, with the exception of two amino acids located within the RNA editing site: $Gln^{(3.55)}$ and $His^{(3.59)}$. It would be informative to determine if mutation of $Gln^{(3.55)}$ and $His^{(3.59)}$ to the corresponding amino acids in the 5-$HT_{2C}R$ ($Arg^{(3.55)}$ and $Glu^{(3.59)}$) would increase 5-$HT_{2A}R$ constitutive activity. Mutagenesis studies involving amino acids at positions 3.55 and 3.59 may provide useful information regarding the molecular determinants of 5-$HT_{2A/2C}R$ activation.

While RNA editing alters 5-$HT_{2C}R$ constitutive activity, it does not alter the maximal levels of IP produced in response to 5-HT stimulation. The same maximal level of agonist-stimulated IP production is observed for all 5-$HT_{2C}R$ isoforms [73, 74]. 5-HT produced a twofold increase in IP production over basal levels for INI and an eightfold increase in IP production over basal levels for VGV, but the maximal level of 5-HT-stimulated IP production was the same for both isoforms. These results indicate that while RNA editing alters agonist-independent receptor/G_q protein coupling, it does not alter the ability of 5-HT to promote receptor coupling to G_q proteins. From these results it was concluded that the unedited isoform has a greater ability to adopt an active conformation spontaneously (transition from $R \rightarrow R^*$) than the fully edited receptor [73, 74]. Therefore, an important consequence of RNA editing is to silence receptor constitutive activity by producing isoforms that are constrained in the inactive conformation and are less likely to adopt an active conformation spontaneously in the absence of 5-HT.

As stated previously, 5-HT has lower affinity and potency for the fully edited VGV isoform than the unedited INI isoform [72–76]. Similar results were observed with 1-(3-chlorophenyl)piperazine (*m*-CPP), 4-iodo-2,5-dimethoxyphenylisopropylamine (DOI), α-methyl 5-HT, and oxymetazoline [74, 75]. Surprisingly, lysergic acid diethylamide (LSD) and lisuride have high affinity for both INI and VGV isoforms [75, 80]. While LSD is a full agonist at the INI isoform, it is totally devoid of agonist activity at the VGV isoform [75, 80]. These results indicate that brain regions containing the unedited or partially edited isoforms would be more sensitive to 5-HT released from presynaptic nerve terminals, but the magnitude of the response elicited by 5-HT would be significantly less due to high basal constitutive activity. Since some drugs

have different efficacies for different isoforms of the 5-HT_{2C}R, RNA editing may alter the response to drug therapy (see also Chapter 7).

Another important consequence of RNA editing involves the preferential coupling of different 5-HT_{2C}R isoforms to different G proteins. Since RNA editing occurs in a region of the receptor critical for G protein coupling, studies were performed to determine if RNA editing alters the ability of the 5-HT_{2C}R to interact with different types of G proteins. Agonists have been shown to promote coupling of the unedited INI isoform to both IP production and arachadonic acid (AA) release with equal efficacies [81]. However, agonist efficacies were reversed for IP production and AA release at the fully edited VGV isoform, demonstrating that agonist trafficking of receptor stimulus is altered by RNA editing [81]. Studies using a β-galactosidase reporter gene assay (*R*-SAT) (see Chapter 8) demonstrated that both fully edited and unedited isoforms interact with G_q proteins [82]. However, the edited and unedited isoforms differed in their ability to activate G_{13}. The unedited isoform stimulated a G_{13}-mediated rearrangement of the actin cytoskeleton in CHO cells that was not detected in cells expressing the fully edited isoform [82]. In a subsequent study, it was shown that the 5-HT_{2C}R activates RhoA by a G_{13}- and PLD-dependent pathway [83]. This pathway is activated by the unedited isoform and not by the fully edited isoform [83]. These studies demonstrate that RNA editing regulates 5-HT_{2C}R-mediated coupling to different G proteins and activation of specific effector pathways in mammalian cells.

RNA editing of the 5-HT_{2C}R to produce multiple isoforms with varying degrees of constitutive activity and decreased agonist affinity and potency has important implications for serotoninergic signal transduction *in vivo*. Different 5-HT_{2C}R isoforms have different regional distributions in the brain [74–76]. For example, VSV is the most prominent isoform in human brain, accounting for almost 40% of the total population of 5-HT_{2C}Rs, while the unedited isoform represents 10% and the fully edited isoform represents 5% of all 5-HT_{2C}R isoforms [74]. In the human hypothalamus, VNV [75] and VSV [76] are the most prominent isoforms. In the rat choroid plexus, the INI and INV (isoforms with the greatest constitutive activity) are the most prominent isoforms [74, 76]. These results imply that individual brain regions may express different 5-HT_{2C}R isoforms with different levels of constitutive activity and differential sensitivity to 5-HT. Therefore, it is possible that altered patterns of RNA editing and 5-HT_{2C}R isoform expression in the brain may play a role in the pathophysiology of mental illness.

Several studies have looked for changes in RNA editing patterns in post mortem brain samples from patients with schizophrenia, major depression, and/or suicide. Four studies have examined RNA editing patterns in the prefrontal cortex of patients with schizophrenia. Three of these studies, each involving 15 control and 15 schizophrenic samples, reported no differences between the two groups in RNA editing patterns [84–86]. The fourth study, which reported an increase in INI and decease in VSV and VNV isoforms in the schizophrenic samples, included five subjects in each group [87]. In two studies, no differences in prefrontal cortical RNA editing patterns were observed in controls versus patients with major depression [84, 86]. However, three studies have reported increases in RNA editing in the prefrontal cortexes of patients with major depression who committed suicide: two of these studies reported an in-

crease in the VNI isoform [84, 86] and the other reported an increase in the VGI isoform [88]. Chronic treatment of mice with fluoxetine resulted in a small increase in the percentage of the ISV isoform [88]. Interestingly, 5-HT depletion following 1-(1-phenylcyclohexyl)piperidine (PCP) injections in the neocortexes of mice resulted in a decrease in RNA editing and subsequent increase in the expression of isoforms with higher sensitivity to 5-HT [89]. In the same study, four-day treatment with DOI (a 5-$HT_{2A/2C}$R agonist) increased RNA editing, resulting in the expression of isoforms with lower sensitivity to 5-HT [89]. While studies of this nature are difficult to perform, due to the large number of subjects required and the large number of clones that must be sequenced to provide an accurate representation of all isoforms, they suggest that RNA editing may play a role in major depression with suicide, and that brain 5-HT levels and drug therapy may alter patterns of RNA editing in the brain.

Expression of constitutively active 5-HT_{2C}Rs in mammalian cell lines provides a model system for identifying inverse agonists. Several commonly prescribed antipsychotic drugs exhibit high-affinity binding to dopamine D_2 receptors (D_2Rs), 5-HT_{2A}Rs, and 5-HT_{2C}Rs [90]. A current hypothesis on the mode of action of atypical antipsychotic drugs is that their unique ability to ameliorate the negative symptoms of schizophrenia while producing minimal extrapyramidal symptoms depends on their interaction with 5-$HT_{2A/2C}$Rs, while also occupying brain D_2Rs [91]. Previous studies have demonstrated that both typical and atypical antipsychotic drugs are potent inverse agonists at 5-HT_{2A}Rs [48, 62]. Additional studies were performed to determine whether there is a relationship between the classification of an antipsychotic drug as typical or atypical and inverse agonist activity at 5-HT_{2C}Rs [92]. In COS-7 cells expressing the unedited isoform of the human 5-HT_{2C}R, atypical antipsychotic drugs with high affinity (1–10 nM: clozapine, olanzapine, ziprasidone, sertindole, zotepine) or with moderate affinity (25–50 nM: risperidone, fluperlapine) for 5-HT_{2C}Rs, and thus likely to occupy brain 5-HT_{2C}Rs at clinically relevant concentrations, displayed inverse agonist activity and decreased basal IP production by 60–70 % [92]. In the same study, of the 13 typical antipsychotic drugs examined, only one exhibited high affinity (1–10 nM: chlorpromazine) and two had moderate affinity (15–50 nM: loxapine, thioridazine) for human 5-HT_{2C}Rs. All 13 of the typical antipsychotic drugs studied were devoid of inverse agonist activity, with the exception of loxapine. Chlorpromazine and thioridazine blocked both 5-HT-stimulated and inverse agonist activity. Similar results were obtained with a constitutively active mutant form of the rat 5-HT_{2C}R stably expressed in NIH3T3 cells [92]. These results suggest that the ability of atypical antipsychotics to improve cognitive function and negative symptoms in patients with schizophrenia may be related to 5-HT_{2C}R antagonism/inverse agonism *in vivo* [92].

Two subsequent studies reported no association between atypical antipsychotics and 5-HT_{2C}R inverse agonism [62, 93]. However, these studies examined many drugs that 1) have not been approved by the Federal Drug Administration (FDA) of the United States of America for treating schizophrenia, 2) do not have high affinity for 5-HT_{2C}Rs, and 3) have insufficient clinical trial data to be adequately classed as atypical or typical. Studies designed in this fashion could easily obscure important results obtained for drugs that have high affinity for 5-HT_{2C}Rs and would occupy brain 5-HT_{2C}Rs at clinically relevant concentrations. In fact, upon close inspection of the data presented in the

two subsequent studies, one finds the exact same trend as reported in the original study [92]. In the second study, all of the tested atypical antipsychotic drugs that display high affinity for 5-HT_{2C}Rs (i.e., clozapine, olanzapine, ziprasidone, sertindole, zotepine, and risperidone) were more potent inverse agonists than the 11 typical antipsychotic drugs studied (with the exception of loxapine) at 5-HT_{2C}Rs expressed in HEK293 cells [93]. The third study included 40 drugs (many of which do not have clinically proven antipsychotic activity), and only six of the drugs tested have high affinity (1–10 nM) for 5-HT_{2C}R [62]. In this study, atypical antipsychotics with high affinity for 5-HT_{2C}Rs were identified as inverse agonists (with the exception of risperidone), while typical antipsychotics displayed weak or no inverse agonist activity (except for loxapine and amoxapine) at 5-HT_{2C}Rs expressed in NIH3T3 cells [62]. The results of all three studies support the hypothesis that atypical antipsychotic drugs that bind with high affinity to 5-HT_{2C}Rs are inverse agonists and that typical antipsychotic drugs have less potent or no inverse agonist activity. Presently, five atypical antipsychotic drugs are FDA-approved: clozapine, ziprasidone, olanzapine, risperidone, and quetiapine. Large-scale clinical trials have provided evidence that sertindole, zotepine, and fluperlapine may be effective atypical antipsychotics [94–96]. With the exception of quetiapine (which has low affinity for 5-HT_{2A}Rs and 5-HT_{2C}Rs), all of these atypical antipsychotic drugs bind with moderate to high affinity at 5-HT_{2C}Rs and are inverse agonists at this receptor [92]. These results clearly indicate that 5-HT_{2C}R inverse agonism should be investigated as a possible mechanism of action for atypical antipsychotic drugs.

Consistently with the hypothesis that 5-HT_{2C}R inverse agonism may play a role in the clinical efficacy of atypical antipsychotics [92], several studies have reported that 5-HT_{2C}R inverse agonists increase prefrontal cortical dopamine release (a commonly used measure of atypical antipsychotic activity [97–99]). In these studies, systemic administration of the selective 5-HT_{2C}R inverse agonist 6-chloro-5-methyl-1-[6-(2-methylpyridin-3-yloxy)pyridin-3-ylcarbamoyl]indoline (SB-242084), increased dopamine release in the prefrontal cortexes of freely moving rats, while selective 5-HT_{2C}R agonists decreased dopamine levels in the prefrontal cortex [100, 101]. Similar results were obtained with nonselective 5-$HT_{2B/2C}$R and 5-$HT_{2A/2C}$R antagonists [91, 101, 102]. In addition, systemic administration of the selective 5-HT_{2A}R antagonist 1-[2-(4-fluorophenyl)ethyl]-4-piperidinemethanol) (MDL100907) had no effect on prefrontal cortical dopamine levels [91, 101]. Chronic, systemic administration of the selective 5-HT_{2C}R inverse agonist 5-methyl-1-{2-[(2-methyl-3-pyridyl)oxy]-5-pyridyl]carbamoyl}-6-trifluoromethylindoline hydrochloride (SB-243213) ($10\,mg\ kg^{-1}$) produced the same changes in dopamine neuronal firing rate in the vental tegmental area as chronic treatment with $20\,mg\ kg^{-1}$ clozapine [103]. Clozapine and risperidone, but not haloperidol, block the inhibitory effect of the selective 5-HT_{2C}R agonist (*S*)-2-(6-chloro-5-fluoroindol-1-yl)-1-methylethylamine hydrochloride (Ro 60-0175) on dopamine release, indicating that clozapine and risperidone occupy brain 5-HT_{2C}Rs following systemic administration [104]. All of these studies concluded that blockade of 5-HT_{2C}Rs may play an important role in atypical antipsychotic efficacy.

There are several lines of evidence suggesting that 5-HT_{2C}R constitutive activity may have biological significance:

1) In transfected NIH3T3 cells, 5-HT_{2C}Rs are mitogenic and promote oncogenic transformation [105]. In a cell-based functional assay using NIH3T3 cells, 5-HT_{2C}Rs demonstrated constitutive activation of [^{3}H]thymidine incorporation (increased cell division) that was blocked by inverse agonists, while neutral antagonists had no effect on their own but blocked the effect of inverse agonists [106].
2) Studies performed in primary choroid plexus epithelial cells endogenously expressing 5-HT_{2C}Rs demonstrated that chronic treatment with a 5-HT_{2C}R inverse agonist promotes 5-HT_{2C}R down-regulation, while treatment with a neutral antagonist had no effect [67]. In separate studies, 5-HT_{2C}R inverse agonists decreased basal IP production in choroid plexus epithelial cells [107, 108].
3) Two studies reported that selective 5-HT_{2C}R inverse agonists increased prefrontal cortical dopamine release *in vivo* in microdialysis studies [100, 101]. It would be interesting to determine if similar results were obtained in 5-HT-depleted rats or if the inverse agonist effect was blocked by pretreatment with neutral antagonists.
4) In an *in vivo* assay involving the motor performance of a cranial nerve reflex (the nictitating membrane reflex of the rabbit) mianserin, but not ketanserin, decreased the magnitude of the reflex, while BOL had no effect alone but reversed the effect of mianserin [109].
5) RNA editing, which occurs in the human brain, alters the constitutive activity of 5-HT_{2C}Rs expressed in recombinant cell systems at physiological receptor expression levels [73, 74].
6) In a recent study, systemic administration of the 5-HT_{2C}R inverse agonist 5-methyl-1-(3-pyridylcarbamoyl)-1,2,3,5-tetrahydropyrrolo[2,3-f]indole hydrochloride (SB-206553) in rats elicited a dose-dependent increase in dopamine release in the striatum and nucleus accumbens that was insensitive to reduction of 5-HT neuronal function induced by the 5-HT_{1A}R agonist 8-hydroxy-2-dipropylaminotetralin (8-OH-DPAT) or intra-raphe injections of 5,7-dihydroxytryptamine neurotoxin [110]. These results suggest that 5-HT_{2C}R constitutive activity may inhibit the tonic activity of mesencephalic dopaminergic neurons *in vivo*.

The studies described above suggest that 5-HT_{2C}R constitutive activity may have physiological significance *in vivo*, and that 5-HT_{2C}R inverse agonists may represent an important class of therapeutic agents.

14.6 Conclusion

Native and mutant forms of 5-HT_{1A}Rs, 5-HT_{1B}Rs, 5-HT_{1D}Rs, 5-HT_{2A}Rs, and 5-HT_{2C}Rs display constitutive activity when expressed in recombinant cell systems. Drugs used to treat anxiety, depression, and schizophrenia display inverse agonist activity at 5-HT_1Rs and/or 5-HT_2Rs expressed *in vitro*. Mutagenesis studies producing inactive and constitutively active 5-HT_1Rs and 5-HT_2Rs have identified the i2 and i3 loops as critical regions involved in receptor/G protein coupling. While 5-HT_1R and 5-

HT_2R polymorphisms have been identified in the human population, and some of these polymorphisms alter 5-HT affinity or potency, there is little evidence to suggest that these polymorphisms alter 5-HTR constitutive activity. Two questions still remain: are 5-HTRs constitutively active *in vivo*, and do inverse agonists possess greater therapeutic efficacy than neutral antagonists?

A common criticism of receptor constitutive activity studies is that many studies, particularly those performed in Sf9 insect cells, use very high and possibly nonphysiological receptor expression levels to measure constitutive activity. However, native 5-HT_{1A}Rs display constitutive activity when expressed at levels as low as 0.18 pmol mg^{-1} in HEK293 cells [13] or 0.5 pmol mg^{-1} protein in HeLa cells [9]. In addition, 5-HT_{2C}Rs display constitutive activity in NIH3T3 and COS-7 cells at receptor densities similar to those of endogenous 5-HT_{2C}Rs in rat choroid plexus tissue [67, 73]. These results indicate that 5-HTRs are constitutively active in recombinant cell systems when expressed at physiological levels.

Additional studies performed with primary neuronal cultures and *in vivo* techniques, such as microdialysis and electrophysiology, are needed to test the hypothesis that 5-HTRs are constitutively active *in vivo*. These types of studies are difficult to perform and the presence of endogenous 5-HT makes it difficult to interpret the results of inverse agonist studies *in vivo*. Studies using neutral antagonists are required to demonstrate that the observed effects of inverse agonists are not due to blockade of endogenous 5-HT, but actually reflect inhibition of receptor constitutive activity (see also Chapter 1). Neutral antagonists have been identified for the 5-HT_1Rs and 5-HT_2Rs discussed in this chapter. These drugs should provide essential tools for investigation of the role of constitutively active 5-HTRs in future studies.

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15
Virally Encoded Constitutively Active Chemokine Receptors

Barbara Moepps and Peter Gierschik

15.1
Introduction

15.1.1
Viral Strategies to Evade the Host Immune System

In the last few years, it has become evident that viruses capable of infecting mammalian cells have evolved multiple strategies to evade the surveillance of the host immune system [1, 2]. These strategies include changes in the expression of specific cellular and viral genes, the latter genes comprising homologues of cellular genes presumably acquired by gene transfer from the host [2, 3]. Cellular genes affected by viral infection include genes involved in regulation of cell cycle, apoptosis, and immune functions [4–6]. The viral genes induced upon infection encode molecules i) mimicking proteins that play important roles in the control of the immune system, or ii) interfering with the functions of these proteins (e.g., major histocompatibility class (MHC) molecules, cytokines, chemokines, and their receptors) [2, 3, 6]. Interestingly, the genes adopted from the host have been modified by the viruses in order to optimize the encoded proteins to serve their own purposes, such as prevention of detection of infected cells by the host immune system or for support of the propagation and dissemination of the virus in the host [2, 3]. One important viral strategy to manipulate the host immune system is to induce changes in the expression of cellular and/or viral genes encoding chemokines and chemokine receptors or homologues of these proteins, respectively [3, 7–11]. A summary of viral proteins currently known to exploit the chemokine system is presented in Table 15.1.

15.1.2
Chemokines and Chemokine Receptors

Chemokines belong to a growing family of structurally related, low molecular mass (8–11 kDa) cytokines with chemotactic activities. Chemokines regulate hematopoiesis and orchestrate leukocyte trafficking by controlling chemotaxis, adhesion, and trans-

Table 15.1 Viral chemokines, chemokine receptors, and chemokine binding proteins.

Virus family	Gene/protein	Virus	Known or proposed function	Ref.
Viral chemokines				
α-herpes virus	MDV003/vIL-8	MDV	CXC chemokine, virulence factor, dissemination	138
β-herpes virus	UL146/vCXC1	HCMV/HHV5	CXC chemokine, CXCR2 agonist, neutrophil chemoattractant	139
	UL147/vCXC2	HCMV/HHV5	Putative CXC chemokine	139
	U83/vCCL4	HHV6	CC chemokine, monocyte chemoattractant	140, 141
	MCK1/2	MCMV	CC-, CXCR antagonist, TH2 chemoattractant, angiogenic activity	142, 143
γ-herpes virus	K6/vMIP1	KSHV/HHV8	CC chemokine, CCR8 agonist, TH2 chemoattractant, angiogenic activity	144, 145
	K4/vMIP2	KSHV/HHV8	CC-, CXC-,CX3C, C-antagonist, TH2 chemoattractant, angiogenic activity	144, 146
	K4.1/vMIP3	KSHV/HHV8	CC chemokine, CCR4 agonist TH2 chemoattractant, angiogenic activity	146, 147
pox virus	MC148/vMCC1	MCV	CC chemokine, CCR8 antagonist	148, 149
lentivirus	Tat	HIV	Similarity with chemokines, monocyte attractant	150
paramyxovirus	Glycoprotein-G	RSV	Similarity with chemokines, CX_3CL1-like activity	61
Viral chemokine receptors				
β-herpes virus	US27	HCMV/HHV5	Putative CCR receptor, located on viral envelope	9, 62
	US28	HCMV/HHV5	Binding of CCL1, CCL5, CX_3CL1 and vMIP2, stimulation of cell migration, constitutive activity	52, 63, 64, 85
	UL33	HCMV/HHV5	Constitutive activity	151, 152
	U12	HHV6, HHV7	Binding of CCL2, CCL3, CCL4, CCL5, CCL19	153
	U51	HHV6, HHV7	Binding of CCL2, CCL3, CCL4, CCL5, CCL11, vMIP2	38
γ-herpes virus	ORF74/ KSHV-GPCR	KSHV/HHV8	Binding of CXCL1, CXCL4, CXCL7, CXCL8, CCL1, CCL2, CCL4, CCL5	96, 110
	E1	EHV2	Binding of CCL11, stimulation of chemotaxis	154
pox virus	SPV146/K2R	SPV	CXCR1 homologue	155
	145R	YLDV	CCR8 homologue	156, 157
	Q2/3L	CaPV	CC chemokine receptor homologue	158
	LSD011	LSDV	CC chemokine receptor homologue	159

Table 15.1 continued.

Virus family	Gene/protein	Virus	Known or proposed function	Ref.
Viral chemokine binding proteins				
pox virus	vCKBP1	MV	Binding of CC, CXC, C chemokines, modulation of leukocyte migration	160
	vCKBP2	VV, CPV, MV	Binding of CC chemokines, anti-inflammatory effects	161, 162
	A41L	VV	vCKBP2 homologue, anti-inflammatory effects	163
γ-herpes virus	vCKBP3	MHV68	Binding of CC, CXC, C, CX_3C chemokines	164, 165

MDV, Marek's disease virus; HCMV, human cytomegalovirus; MCMV, mouse cytomegalovirus; KSHV, Kaposi's sarcoma virus; MCV, molluscum contagiosum virus; HHV5, human herpes virus 5; HHV6, human herpes virus 6; HHV7, human herpes virus 7; HHV8, human herpes virus 8; EHV2, equine herpes virus 2; SPV, swine pox virus, YLDV, Yaba-like disease virus; CaPV, capri pox virus; LSDV, lumpin skin disease virus; MV, myxoma virus; VV, vaccinia virus; CPV, cow pox virus; MHV68, mouse herpes virus 68.

endothelial migration [12, 13]. More recently it has become evident that chemokines function as regulators not only of hematopoietic cells, but also of many other cell types, including cells of the central nervous system as well as endothelial and epithelial cells. By controlling various functions of these cells, chemokines play fundamental roles as regulators of embryogenesis, neurogenesis, and angiogenesis, and also as mediators of tumor cell invasion and metastasis [14–18]. In addition, chemokines block the entry of human immunodeficiency viruses (HIV) into their target cells [7, 19, 20].

Chemokines are defined by conserved cysteines and can be subdivided into four families – CC, CXC, CX_3C, and C – by the relative positions of these cysteines, some of which form intramolecular disulfide bonds [19, 21]. According to their role as regulators of immune cell functions, chemokines have been divided into i) homeostatic chemokines, which are constitutively expressed in lymphoid cells and tissues and are involved in regulating lymphocyte homing, and ii) inflammatory chemokines, which are produced by many different cells and tissues after challenge with bacterial toxins and proinflammatory cytokines [19]. CXC chemokines involved in modulation of angiogenesis have been grouped into angiogenic chemokines (bearing a Glu-Arg-Leu (ERL) motif preceding the first cysteine, as in CXCL2 and CXCL8) and angiostatic chemokines without this motif (such as CXCL9 and CXCL10) [19, 21].

Transmembrane signaling of chemokines is mediated by chemokine receptors, members of the superfamily of heterotrimeric G protein-coupled receptors (GPCRs) classified according to their interactions with chemokine ligands [22, 23]. Various leukocytes, including T cells and B cells, neutrophils, monocytes, and mast cells, as well as platelets, endothelial and epithelial cells, smooth muscle cells, and several cell types of the brain and the liver, express chemokine receptors [22, 23]. To date, 18 genes encoding chemokine receptors have been identified in humans [23, 24]. In general, cellular CXC and CC chemokine receptors specifically interact with CXC and with CC

chemokines, respectively [23, 24]. The receptors XCR1 and CX_3CR1 are specifically activated by the C chemokine XCL1 and the CX_3C chemokine CX_3CL1, respectively [24]. Only a few CC and CXC chemokine receptors interact with only a single chemokine agonist. Examples are the receptors CCR6 and CXCR4, with their ligands CCL20 and CXCL12, respectively [24]. The majority of CC and CXC chemokine receptors interact with several chemokines, and many chemokines interact with several chemokine receptors. This promiscuity increases not only the diversity, but also the complexity of the chemokine–chemokine receptor network [19, 24]. Most leukocytes express only a subset of the chemokine receptors and thus respond to only a subset of chemokine ligands depending on their state of differentiation and activation [22, 25]. Chemokine receptors interact either with soluble chemokines or with chemokines bound to macromolecules, such as glycosaminoglycans (GAGs). Binding of chemokines to GAGs, such as on the surface of endothelial cells, i) facilitates the generation of chemokine gradients that direct leukocyte migration, and ii) allows the specific sequestration of chemokines at sites of inflammation [19, 26]. The CX_3C chemokine CX_3CL1 is a cell surface-bound molecule, from which a soluble chemokine fragment can be released [19].

In most cells, chemokine receptors are coupled to PTX-sensitive (PTX = pertussis toxin) G proteins, which regulate the activity of a number of intracellular effectors, including phospholipase C-β isozymes (PLCβs), inositol phospholipid 3-kinases (PI3-Ks) [27], and mitogen-activated protein (MAP) kinases [27, 28]. These functions are known to be mediated either by the activated Gα and/or by free Gβγ subunits. Furthermore, chemokine receptors have been reported to regulate the functions of integrins [29], Janus kinase/signaling transducer and activator of transcription (Jak/STAT) [30, 31], and signaling components involved in mediating apoptosis [32]. Moreover, coupling of chemokine receptors to the PTX-insensitive $G\alpha_q$ family members $G\alpha_{14}$ and $G\alpha_{16}$ resulting in PLCβ activation has been described [33, 34]. A summary of signaling pathways regulated by chemokine receptors is given in Figure 15.1.

15.1.3
Viral Homologues of Chemokines and Chemokine Receptors and Viral Chemokine-binding Proteins

Genes encoding viral homologues of chemokines and chemokine receptors mimicking the functions of their mammalian counterparts have mainly been found in the genomes of three virus families: herpes viruses, pox viruses, and lentiviruses (cf. Table 15.1) [3, 8, 11]. At least 11 genes encoding homologues of chemokines and 11 genes encoding chemokine receptors have been identified in the genomes of theses viruses (cf. Table 15.1) [3, 11]. In addition, viruses have also evolved chemokine-binding proteins with no homology to known mammalian proteins [35]. These viral proteins specifically interact with chemokines to interfere with their binding to receptors, to glycosaminoglycans, or to both [3, 35]. As one example, the chemokine-binding protein (CKBP) vCKBP1 of myxoma virus specifically interacts with the GAG-binding

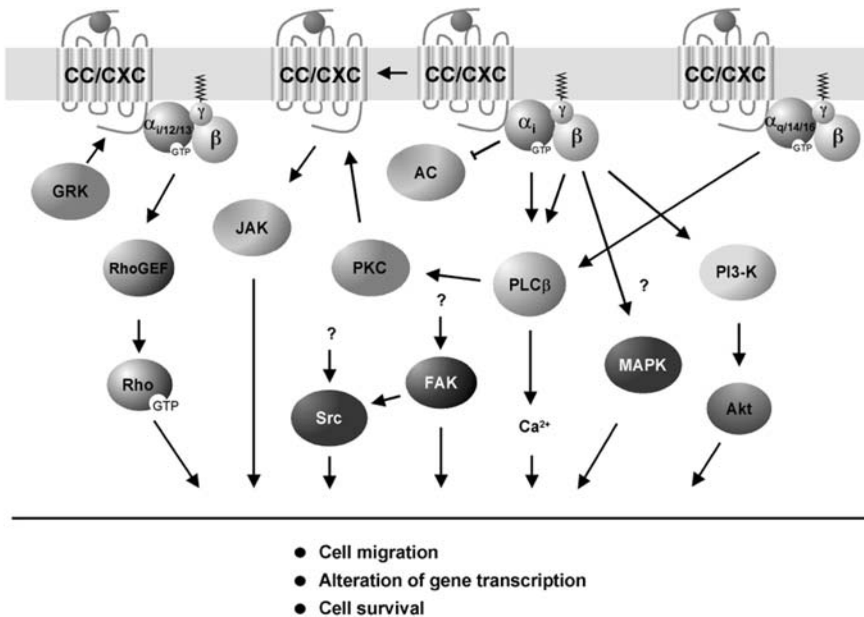

Fig. 15.1 Model of signaling pathways activated by chemokine receptors. Signal transduction by CC and CXC chemokine receptors is mediated by various intracellular effector proteins, including PLCβ isoforms, adenylyl cyclases, and Rho GTPases, as well as by lipid and protein kinases such as PI3-K, p38, ERK, and JNK. Receptor activation is mediated by G_i, G_q, and $G_{12/13}$ proteins. Receptor dimerization may result in activation of the JAK/STAT pathway. Receptor responsiveness is regulated through phosphorylation by members of the GRK and/or the PKC families. Chemokine receptor-dependent regulatory inputs into downstream signal transduction components with unknown signaling intermediates or unclear relationship to other pathways are marked in Figures 15.1 to 15.3 with question marks.

domain of the CXC chemokine CXCL8. The CKBPs vCKBP2 and vCKBP3 – of pox and herpes viruses, respectively – interact with many chemokines to block their interactions with chemokine receptors and/or GAGs [3].

The viral chemokine homologues are broad-spectrum chemokines that bind to several cellular chemokine receptors and viral chemokine receptor homologues and may act as agonists, inverse agonists, or antagonists [3, 8]. Some of the viral chemokine receptor homologues in turn display nonselective binding of cellular CC, CXC, and CX_3C chemokines as well as viral chemokines. The majority of the viral chemokine receptor homologues, however, are made up of orphan receptors for which no ligands have yet been identified. Interestingly, some viral chemokine receptor homologues show intrinsic activity independent of ligand binding, which is referred to as constitutive activity [36].

Since chemokines and chemokine receptors are key regulators of innate and acquired immune functions and play important roles in the control of invading microorganisms, including viruses [7, 20], interfering with chemokine and/or chemokine receptor functions may help the virus to evade and/or to exploit the host immune

system [9]. Viral chemokines interfere with the functions of cellular chemokines by, for example, acting as antagonists at cellular chemokine receptors, and it has been suggested that internalization of viral chemokine receptor homologues together with their bound ligands results in a depletion of chemokine in the environments of infected cells [36, 37]. Both mechanisms may prevent detection of infected cells by the host immune system and may allow the virus to persist in the host. Expression of viral chemokine receptor homologues may also facilitate the dissemination of the virus in the host by, for example, directing infected cells to specific organs [36].

Several viral chemokine receptor homologues have been identified in the genomes of β- and γ-herpes viruses, including those of human cytomegalovirus (HCMV/HHV5), human herpes virus 6 (HHV6), human herpes virus 7 (HHV7), and Kaposi's sarcoma-associated virus (KSHV/HHV8) (cf. Table 15.1) [36, 38, 39]. This review focuses on the signaling properties of two of these viral chemokine receptor homologues, pUS28 and KSHV-GPCR, which are encoded by the open reading frames (ORFs) US28 of HCMV and ORF74 of KSHV, respectively [9, 36], and summarizes the current knowledge about their functions and potential regulatory roles in infected cells. Both proteins are constitutively active and may alter the activity of host cell signaling pathways in a ligand-independent manner [36].

15.2 The Human Cytomegalovirus-encoded Chemokine Receptor Homologue pUS28

15.2.1 Characteristics of Human Cytomegalovirus Infection

Human cytomegalovirus (HCMV) is a member of the β-herpes virus family and is one of several related species-specific viruses that cause similar diseases in various animals [40]. HCMV is a slowly growing virus and HCMV infection rarely causes symptomatic disease in immunocompetent individuals. HCMV has evolved several mechanisms to evade detection by the host immune system and can establish lifelong latency after primary infection. HCMV infection or HCMV reactivation in immunocompromised patients can result in disseminated disease characterized by fever and leukopenia, hepatitis, pneumonia, esophagitis, gastritis, colitis, and retinitis [40]. HCMV infection has been recognized as an independent risk factor for vascular diseases such as atherosclerosis and arterial restenosis [41].

In vivo, HCMV appears to replicate in a variety of cell types, including fibroblasts, epithelial cells, endothelial cells, and smooth muscle cells [42]. HCMV infection has long been known to be associated with changes in the activity of many cellular signaling pathways [43, 44]. The cellular responses triggered by HCMV infection include increased hydrolysis of phosphatidylinositol 4,5-bisphosphate by PLCβ within minutes after exposure of cells to HCMV [45]. Other alterations observed upon HCMV infection include sustained activation of MAP kinases [46, 47], changes in the host cell cycle [44, 48], modulation of interferon signaling [44], and the initiation of cellular and/or viral gene expression through the activation of host transcription factors, in-

cluding nuclear factor kappa B (NF-κB), cAMP response element-binding protein (CREB), and serum response factor (SRF) [46, 49, 50].

15.2.2
Functional Characteristics of pUS28

Four ORFs with homology to the genes of mammalian chemokine receptors have been identified in the HCMV genome: US27, US28, UL33, and UL78 [36]. No viral or cellular ligands for the proteins encoded by US27, UL33, and UL78 have yet been described. In contrast, the protein (p) encoded by US28 (pUS28) binds a number of human CC chemokines, including CCL2, CCL3, CCL5, and the CX_3C chemokine CX_3CL1 with high affinity [51–55]. CX_3CL1 is structurally peculiar in that its chemokine domain is located on top of a mucin-like stalk that is connected to a transmembrane domain [19]. In contrast to other chemokines, CX_3CL1 functions not only as potent chemotactic cytokine, but also as an adhesion molecule. To fulfil these functions, CX_3CL1 is either cleaved between the chemokine portion and the membrane anchor, to release the former as a soluble protein, or remains cell surface-bound to serve as an adhesion molecule for cells expressing chemokine receptors [56]. The observation that the soluble chemokine portion of CX_3CL1 binds to pUS28 with subnanomolar affinity and shows a significantly slower off-rate from this receptor than CCL5 [55, 57] has prompted the suggestion that binding of pUS28 to membrane-associated CX_3CL1 might play a role in increasing the affinity and/or duration of the contact between HCMV-infected, pUS28-expressing cells and cells displaying CX_3CL1 on their surfaces, which might in turn increase the efficiency of cell-to-cell transfer of HCMV *in vivo*. Recently, Haskell et al. have shown that such a mechanism is operative, at least in transfected cells, even under shear flow conditions [57]. However, whether or not the cell-to-cell interaction mediated by pUS28 and CX_3CL1 contributes to the entry of HCMV into CX_3CL1-expressing cells remains to be determined. pUS28 also acts as a cofactor for the entry of several types of human immunodeficiency viruses and causes cell-to-cell-fusion by interacting with several types of viral envelope proteins, including HIV viral envelope proteins and a glycoprotein from vesicular stomatitis virus, VSV-G [58–60]. Interestingly, the G glycoprotein of respiratory syncytial virus (RSV) (cf. Table 15.1) is structurally similar to CX_3CL1 and specifically binds to CX_3CR1 [61]. These observations suggest that pUS28 may function to support HCMV dissemination by mediating cell-to-cell-transfer of the virus as well as facilitating secondary infection of HCMV-infected cells by other viruses *in vivo*.

15.2.3
Signaling Pathways Regulated by pUS28

Binding of CC chemokines such as CCL5 to cells expressing pUS28 was shown to cause increases in the concentration of cytosolic free Ca^{2+} [52, 62] and in the activity of extracellular signal-regulated protein kinase 1/2 (ERK1/2) by stimulation either of endogenous PTX-sensitive G_i proteins or of exogenous PTX-insensitive G_{16} [53]. How-

ever, we and others recently found that pUS28 is also capable of mediating a constitutive, chemokine-independent activation of PLCβ, apparently mediated by PTX-insensitive members of the G_q family. This effect was shown for transiently transfected COS-7 cells [63] as well as for HCMV-infected human fibroblasts [64]. Interestingly, addition of CX_3CL1 caused a reduction in constitutive inositol phosphate formation, while addition of CCL5 and CCL2 had no effect [63, 64]. Since infection of fibroblasts with HCMV caused increases in the extracellular concentrations of certain chemokines, including CCL5 and CCL2 [54], an autocrine loop made up of the secreted pUS28 ligands CCL5 and/or CCL2 and of the surface-exposed pUS28 had to be considered as the cause of the observed constitutive inositol phosphate formation. However, readdition of conditioned medium of pUS28-expressing HCMV-infected cells to thoroughly washed cells, or addition of CCL5- or CCL2-neutralizing antibodies to the extracellular medium of HCMV-infected fibroblasts did not affect inositol phosphate formation [64]. Furthermore, a mutant of pUS28 lacking the 21 amino terminal residues failed to bind [^{125}I]-CCL5 in transiently transfected COS-7 cells, but still constitutively stimulated inositol phosphate formation [64]. Taken together, these observations exclude the possibility that binding of CC chemokines is involved in constitutive activation of PLCβ. The results also suggest that CX_3CL1, unlike CCL5 and CCL2, acts as an inverse agonist at pUS28 [63, 64]. The physiological relevance of the latter activity of CX_3CL1 at pUS28 remains to be determined.

15.2.4
Regulation of Transcriptional Activity by pUS28

The activity of several cellular transcription factors, including NF-κB, CREB, and SRF, has been reported to be modified upon HCMV infection [46, 49, 50]. Activation of these transcription factors is required for the expression of viral immediate-early (IE) genes, and consensus sites for the binding of NF-κB, CREB, and SRF have been identified in the major immediate-early promoter [65]. Induction of HCMV IE genes is crucial for productive infection as well as for reactivation of the virus from latency [65]. Interestingly, transient expression of pUS28 activates these three transcription factors in a constitutive manner [63, 66, Moepps et al., unpublished; see Chapter 8].

NF-κB is a ubiquitously expressed transcription factor that plays an important role in inflammation by inducing several proinflammatory genes, including those of chemokines [67]. The constitutive activity of NF-κB induced by transient expression of pUS28 in COS-7 cells was not altered by CC chemokines, while it was partially inhibited by CX_3CL1 [63]. Activation of NF-κB by pUS28 was not sensitive to treatment of cells with PTX. Coexpression of $G\alpha_q$ and $G\alpha_{11}$ together with pUS28 caused a further increase in NF-κB activity [63]. Thus, members of the $G\alpha_q$ family are involved in mediating this effect of pUS28. In addition, G protein βγ dimers also appear to play a stimulatory role [63]. Interestingly, the constitutive NF-κB activation induced by pUS28 was not sensitive to inhibitors of p38 or ERK1/2 MAP kinases [66].

CREB activates the transcription of its target genes in response to a diverse array of stimuli, including peptide hormones and growth factors [68]. Activation of CREB is mediated by a variety of protein kinases, including cAMP-dependent protein kinase (PKA), MAP kinases, and Ca^{2+}/calmodulin-dependent protein kinases (CaMKs) [69]. Sustained activation of MAP kinases has been observed during early HCMV infection, resulting in CREB-mediated induction of IE genes [46, 70]. Transient expression of pUS28 in COS-7 cells caused constitutive activation of CREB. Again, the constitutive activity of CREB induced by pUS28 in COS-7 cells was not altered by CC chemokines, while being partially inhibited by CX_3CL1 [66]. Unlike the induction of NF-κB activity by pUS28 mentioned above, the activation of CREB by pUS28 was blocked by specific p38 MAP kinase inhibitors, suggesting that the p38 MAP kinase pathway is involved in mediating the effect of pUS28 on the activity of CREB [66].

SRF activates the transcription of a variety of genes, including genes expressed in brain and muscle cells, as well as IE genes of HCMV [46, 71], by interacting with a specific recognition site referred to as the serum response element (SRE). The activity of SRF is regulated by extracellular mediators such as those present in serum (e.g., growth factors), and by phorbol esters [71]. Activation of SRF is mediated by Rho family GTPases and changes in actin polymerization. Thus, SRF links cytoskeletal reorganization to induction of gene transcription [72, 73]. Transient expression of pUS28 in COS-7 cells caused constitutive induction of SRF-mediated gene transcription. This effect is apparently mediated by $G\alpha_q$, as it was largely enhanced by coexpression of $G\alpha_q$ but is inhibited by the $G\alpha_q$-GTPase-activating protein RGS2 (Moepps et al., unpublished; see Chapter 8). Rho family GTPases were involved in this effect, since coexpression of RhoA and pUS28 produced synergistic stimulatory effects on SRF-mediated transcriptional activity. Furthermore, coexpression of pUS28 together with *Clostridium botulinum* C3 ADP-ribosyltransferase, which specifically ADP-ribosylates RhoA, -B, and -C and thereby interferes with RhoGEF-mediated (RhoGEF = Rho guanine nucleotide exchange factor) activation of these GTPases [74], abolished the stimulatory effect of pUS28 on SRF-mediated transcriptional activity (Moepps et al., unpublished).

Although it seems clear that expression of pUS28 causes constitutive activation of NF-κB, CREB, and SRF in pUS28-cDNA-transfected cells, the occurrence of these effects in HCMV-infected cells and the functional relevance of these changes to the course of HCMV infection and latency is poorly understood. Thus, expression of mRNA encoding pUS28 was detected at early times [i.e., 8–24 h post infection (p.i.)] and was maximal in the early-late phase of infection (e.g., at 48 h p.i.), making it unlikely that expression of pUS28 contributes to enhanced expression of viral immediate early genes at “immediate early“ time points (i.e., 1–4 h p.i.) [54, 64]. However, pUS28 has also been postulated to be part of the viral envelope and to be incorporated into the host cell membrane even without transcription of the viral genome [75]. Thus, pUS28 may already be present in the host cell membrane during the course of viral entry and during “immediate early“ times p.i. to activate the cellular transcription factors NF-κB, CREB, and SRF. It also seems likely that pUS28 affects the activity of cellular transcription factors during early and/or late times p.i. The activity of NF-κB observed during these phases might explain the modulation of the expression

of genes encoding cellular chemokines (e.g., CXCL8) [76, 77]. Enhanced activity of CREB has been described in early and/or late phases of HCMV infection [78]. Whether these changes are induced by pUS28 remains an intriguing question to be clarified by future experimentation. A summary of signaling pathways regulated by pUS28 is given in Figure 15.2.

15.2.5
Regulation of Constitutively Active pUS28

Phosphorylation by members of a family of serine-threonine protein kinases termed G protein-coupled receptor kinases (GRKs) together with the recruitment of arrestins are crucial steps in agonist-induced desensitization and internalization of G protein-coupled receptors [79–81]. Desensitization is characterized by phosphorylation of serine-threonine residues present in the carboxyl-terminal portions of the receptors by GRKs, followed by recruitment and binding of arrestins to these portions. Binding of arrestins to the receptors prevents their interaction with G proteins and initiates

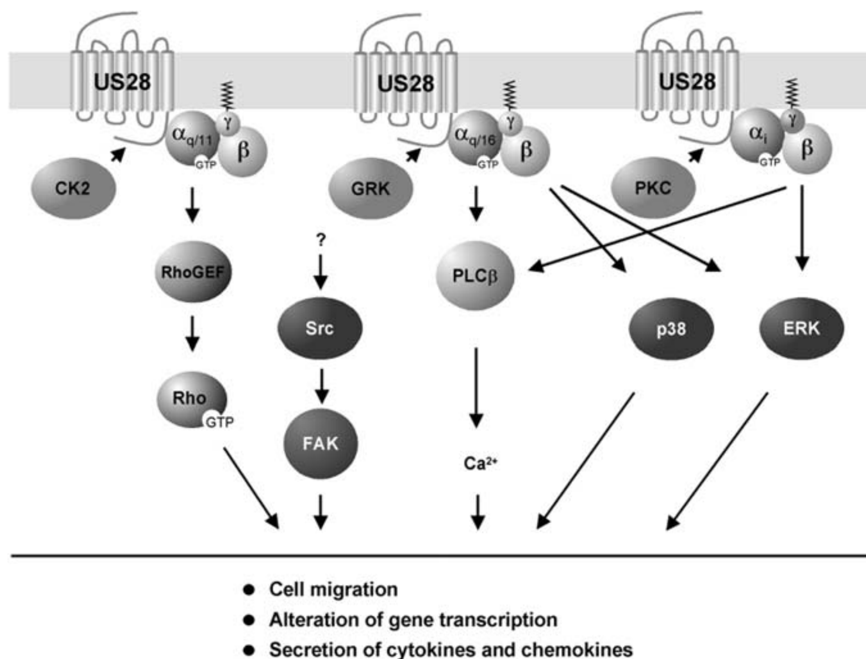

Fig. 15.2 Model of signaling pathways activated by HCMV-encoded pUS28. pUS28 constitutively activates PLCβ through $G\alpha_q$ and/or $G\alpha_{11}$. ERK1/2 and p38 may be activated by Gβγ. The activity of pUS28 is regulated through phosphorylation by members of the GRK, PKC, and/or CK2 families. In addition, pUS28 constitutively activates the transcription factors NF-κB, CREB, and SRF. SRF-stimulated gene transcription by pUS28 is mediated by RhoA-family members. pUS28 may also couple to G_i proteins in a ligand-dependent manner, which may in turn result in activation of FAK through Src.

receptor internalization. In pUS28-cDNA-transfected cells, pUS28 was phosphorylated and internalized by the cellular endocytic pathway [82–85]. However, unlike its cellular chemokine receptor counterparts, pUS28 was constitutively phosphorylated and internalized in a ligand-independent manner, and neither of these functions was further enhanced upon addition of CCL5 and CCL3 [82]. Thus, upon expression of an epitope-tagged pUS28 in HEK293A cells, only a small fraction was present on the cell surface, while the majority of the protein was located in the perinuclear endosomes [83]. Coexpression of GRK2, GRK5, or GRK6 together with pUS28 increased pUS28 phosphorylation and reduced constitutive signaling of pUS28 to downstream effectors such as PLCβ and SRF in COS-7 cells [82, 84, Moepps et al., unpublished]. In accordance with these findings, cells transfected with carboxyl-terminal deletion mutants of pUS28 showed further increases in constitutively enhanced inositol phosphate formation and NF-κB and CREB activity, which is probably due to the fact that these mutants are not phosphorylated by GRKs and are not internalized [84, 85]. Interestingly, a carboxyl-terminal deletion mutant of pUS28 was impaired in its ability to constitutively activate the p38 MAP kinase pathway, implying that some of the intracellular signaling mechanisms of pUS28 are positively influenced by GRKs and/or arrestins [84]. Recently, the involvement of arrestins in pUS28 internalization has been questioned. Thus, expression of pUS28 in arrestin-deficient cells resulted in pUS28 internalization with a rate similar to that reported for arrestin-expressing cells [86]. Internalization of pUS28 in these cells was blocked by inhibition of clathrin-mediated endocytosis, indicating that pUS28 is internalized in a clathrin-dependent but arrestin-independent manner [86]. In addition to GRKs, other serine/threonine protein kinases, such as PKC and casein kinase 2 (CK2) might also regulate pUS28 through phosphorylation [82].

The fact that CCL2 and CCL5 are depleted from the environments of HCMV-infected cells and that pUS28 is rapidly internalized and recycled to the cell surface suggests that pUS28 acts as a cellularly expressed chemokine scavenger [37, 54]. Thus, internalization of chemokines may be another strategy of the virus to prevent recruitment of host immune cells and thus to manipulate the host immune system.

15.2.6 Cellular Functions of pUS28

The finding that pUS28 constitutively regulates several intracellular signaling pathways by activating members of the $G\alpha_q$ family in a ligand-independent fashion is not easily consistent with earlier findings showing: i) that CC chemokines, including CCL2 and CCL5, increase the concentration of cytosolic free Ca^{2+} both in pUS28 cDNA-transfected and in HCMV-infected cells [53, 62], and ii) that this effect was blocked by PTX in HCMV-infected cells [53]. The finding is also not in accord with the previous observation that migration of HCMV-infected smooth muscle cells (SMCs) was critically dependent on the presence of the chemokines CCL2 and CCL5 in the extracellular medium [87]. SMC migration may be mediated by the interaction of CCL5-activated pUS28 with Src, followed by activation of focal adhesion kinase (FAK)

and reorganization of the actin cytoskeleton [88]. Several possible explanations to explain these discrepancies have been put forward. One is that different cell types have been used to study the cellular functions of pUS28, and these cells could differ in their relative abundances of pUS28 and intracellular signal transduction components [63, 87, 89]. For example, the transient expression of pUS28 in HEK-293 cells, but not in COS-7 cells, activated MAP kinase [53, 89]. Cell type specificity has also been observed for pUS28-stimulated migration of HCMV-infected SMCs from different types of blood vessels. Specifically, this migratory response was observed for SMCs of coronary and pulmonary arteries and the aorta, but not for SMCs of saphenous veins or for human dermal fibroblasts and human umbilical vein endothelial cells (HUVECs) [87].

Transient expression of pUS28 in COS-7 cells together with the CC chemokine receptor CCR1 resulted in an enhanced cellular response to stimulation of CCR1 by its ligand CCL5 [90]. Importantly, this response was blocked by pretreatment of the cells with PTX. These results suggest that constitutively active pUS28 may facilitate agonist-induced signaling of cellular G_i-coupled chemokine receptors [90]. Whether the dependence of cellular functions of HCMV-infected cells on chemokines observed in some cells [53, 88] is based on such a mechanism needs further analysis.

While much has been learned about the functions of pUS28 in transfected cells, one needs to be cognizant of the fact that the genome of HCMV encodes more than 200 unique proteins, many of which are of unknown function [91]. Furthermore, HCMV infection of human cells results in altered expression of more than 1400 cellular mRNAs [92]. Both coexpression of virally encoded proteins and altered expression of cellular proteins could conceivably influence the function of pUS28 during natural infection, so it is of great interest to determine the transmembrane signaling functions of pUS28 in HCMV-infected rather than transfected cells [64]. To study the specific functional role of pUS28 in infected cells, recombinant viruses with deleted ORF28 genes have been generated [54, 62, 64, 87]. Infection of human fibroblasts with these deletion mutants revealed that pUS28 apparently plays no major role as a regulator of viral replication, at least in cultured fibroblasts. However, the observation that CC-chemokine-stimulated migration of certain HCMV-infected SMCs is critically dependent on pUS28 [87] indicates that pUS28 might be involved in regulating viral dissemination. This notion is supported by the finding that the gene encoding pM33, which may be the functional equivalent of pUS28 in cells infected by murine CMV, is essential for the targeting of murine CMV to the salivary glands and for the replication of the virus in this organ *in vivo* [93, 94]. Like pUS28, mouse pM33 constitutively activates PLCβ, CREB, and NF-κB [66]. Whether pUS28 fulfils a similar function in targeting human HCMV to specific host tissues and in regulating replication in these tissues is unknown.

15.3 The Human Kaposi's Sarcoma Virus-encoded Chemokine Receptor KSHV-GPCR

15.3.1 Characteristics of Human Kaposi's Sarcoma Virus Infection

Human herpes virus 8 (HHV8), also referred to as Kaposi's sarcoma herpes virus (KSHV) is likely to be involved in the pathogenesis of: i) all forms of Kaposi's sarcoma (classic, iatrogenic, and epidemic), ii) primary effusion lymphomas (a subset of malignant lymphomas), and iii) multicentric Castleman's disease (an angiolymphoproliferative disorder) [95–97]. KSHV belongs to the mammalian γ-herpes virus family that also includes human Epstein Barr virus (EBV), nonhuman herpes virus saimiri (HVS), equine herpes virus type 2 (EHV-2), and murine γ-herpes virus 68 (MHV68) [95]. These viruses are characterized by their narrow host ranges and, except for EBV, by the presence of genes encoding viral chemokines and chemokine receptors in their genomes [3]. For example, the viral chemokine receptor KSHV-GPCR is encoded by ORF74, which has been identified in all γ-herpes virus genomes [98–101]. Kaposi's sarcoma (KS) is a multifocal neovascular tumor, characterized by proliferating spindle-shaped tumor cells thought to be of endothelial origin, angiogenesis, edema, and a variable inflammatory cell infiltrate [95–97]. In cell culture, exposure of endothelial cells to KSHV caused cell transformation [102]. Although only a subset of the exposed cells was infected by KSHV (1–6 %), all cells survived upon long-term cultivation. The survival of uninfected cells may be due to the paracrine induction of vascular endothelial growth factor (VEGF) receptor 2 expression. This receptor is thought to play an important role as a regulator of endothelial cell survival [102, 103]. Interestingly, only a small percentage of the microvascular endothelial cells and the spindle-shaped cells ($< 10\%$) were infected by KSHV in early KS lesions [104]. Thus, KS tumorigenesis may also rely on paracrine effects [95, 97].

Sequencing of the KSHV genome has identified several genes with potential transforming or angiogenic properties, including ORF74 [104]. Importantly, transcripts of ORF74 were found in KS lesions [98, 104]. Furthermore, transient expression of KSHV-GPCR in NIH/3T3 fibroblasts and endothelial cells resulted in cellular transformation and acquisition of spindle cell morphology, respectively [98, 105–107]. Furthermore, multifocal KS-like lesions were observed in KSHV-GPCR transgenic mice. These findings provide strong support for the notion that KSHV-GPCR expression contributes to the pathogenesis of KS [108]. Interestingly, NIH/3T3 cells stably expressing KSHV-GPCR, as well as tumors arising from these cells when injected into nude mice, secrete high levels of VEGF [109].

15.3.2 Functional Characteristics of KSHV-GPCR

KSHV-GPCR displays homology to the human CXC chemokine receptors CXCR1 and CXCR2 [98, 110]. Unlike these receptors, KSHV-GPCR is promiscuous in its interac-

tion with chemokines, displaying unselective binding of the CC chemokines CCL1, CCL2, CCL4, and CCL5, as well as of the CXC chemokines CXCL1, CXCL4, CXCL7, and CXCL8 [98, 111]. KSHV-GPCR expression, similarly to that of pUS28, mediates constitutive activation of PLCβ and several transcription factors, such as NF-κB and CREB [98, 112–115]. Constitutive activation of the PLCβ pathway by KSHV-GPCR is likely to be mediated by PTX-insensitive G_q proteins [98, 116]. Like pUS28, KSHV-GPCR is constitutively phosphorylated and internalized in a ligand-independent manner [117]. Constitutive KSHV-GPCR activity was reduced by coexpression of GRK4, GRK5, or GRK6, or by stimulation of PKC [117]. Moreover, KSHV-GPCR-stimulated proliferation of mouse NIH/3T3 cells was inhibited by coexpression of GRK5 [117].

15.3.3 Signaling Pathways Regulated by KSHV-GPCR

Expression of KSHV-GPCR causes constitutive activation of several signal transduction pathways involving MAP kinases, including ERK1/2, p38, and c-Jun amino-terminal kinase (JNK), as well as PI3-K and the protein kinase Akt, also referred to as protein kinase B [89, 107, 113, 115, 118]. Activation of Akt by KSHV-GPCR is probably caused by the release of Gβγ subunits from activated PTX-sensitive G_i proteins, and is likely to require the activation of PI3-K [89, 113]. Inhibition of PI3-K and Akt in KSHV-GPCR-expressing cells resulted in an increase in apoptotic cells, suggesting that activation of this pathway plays a key role in promoting cell survival in infected cells [113]. Activation of ERK1/2 by KSHV-GPCR involves both PTX-sensitive and PTX-insensitive G proteins [89]. Release of Gβγ from the former promotes activation of PI3-K, which is coupled to stimulation of the Ras-Raf-MEK-ERK1/2 pathway [89]. Interaction of KSHV-GPCR with PTX-insensitive G_q proteins may activate PKC through PLCβ, which has been suggested to cause activation of the same pathway by phosphorylation of Raf [89]. Activation of the JNK kinases by KSHV-GPCR appears to be mediated by related adhesion focal tyrosine kinase (RAFTK), also referred to as proline-rich kinase 2 (Pyk2), which in turn enhances the activity of the Src family member Lyn [119]. The nature of the G proteins mediating activation of RAFTK is currently unknown. Similarly, little is known about the pathways leading to activation of p38 by KSHV-GPCR.

15.3.4 Regulation of Transcriptional Activity by KHSV-GPCR

The activity of several cellular transcription factors, including NF-κB, hypoxia-inducible factor 1α (HIF-1α), CREB, activator protein-1 (AP-1), and nuclear factor of activated T cells (NFAT) is modified upon KSHV-GPCR expression [113, 114, 118, 120]. Specifically, an increase in NF-κB and/or AP-1 activity was observed in COS-7, HEK293, THP-1, and Jurkat T cells, as well as in KS-derived KSHV-negative endothelial cell line (KSIMM) [121] and primary human endothelial cells. In the latter cells, the increase in NF-κB activity was linked to an enhanced secretion of several proinflammatory cyto-

kines and chemokines, such as IL-6, CXCL8, and CCL5, and to increased expression of several adhesion molecules, including vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (I-CAM-1), and E-selectin [107, 120]. In COS-7 cells, induction of NF-κB activity by KSHV-GPCR is mediated by Akt [113]. Induction of NF-κB may also involve activation of RhoA by $G\alpha_{13}$ and a Rho-GEF (e.g., p115RhoGEF), as described for HeLa cells [122]. Activation of ERK1/2 and p38 by KSHV-GPCR caused an increase in HIF-1α activity, which may in turn result in enhanced secretion of the angioproliferative and antiapoptotic mediator VEGF [118]. Thus, inhibitors of signaling pathways involving ERK1/2 and p38 reduced the secretion of VEGF by NIH/3T3 cells stably expressing KSHV-GPCR [118]. A summary of signaling pathways activated by KSHV-GPCR is given in Figure 15.3.

Very recently, activation of the transcription factors NF-κB, AP-1, CREB, and NFAT by KSHV-GPCR has been reported in primary effusion lymphoma cells [114, 123]. Interestingly, unlike in COS-7 cells, PI3-K/Akt does not mediate NF-κB activity in these cells, indicating that KSHV-GPCR might activate transcription factors in a

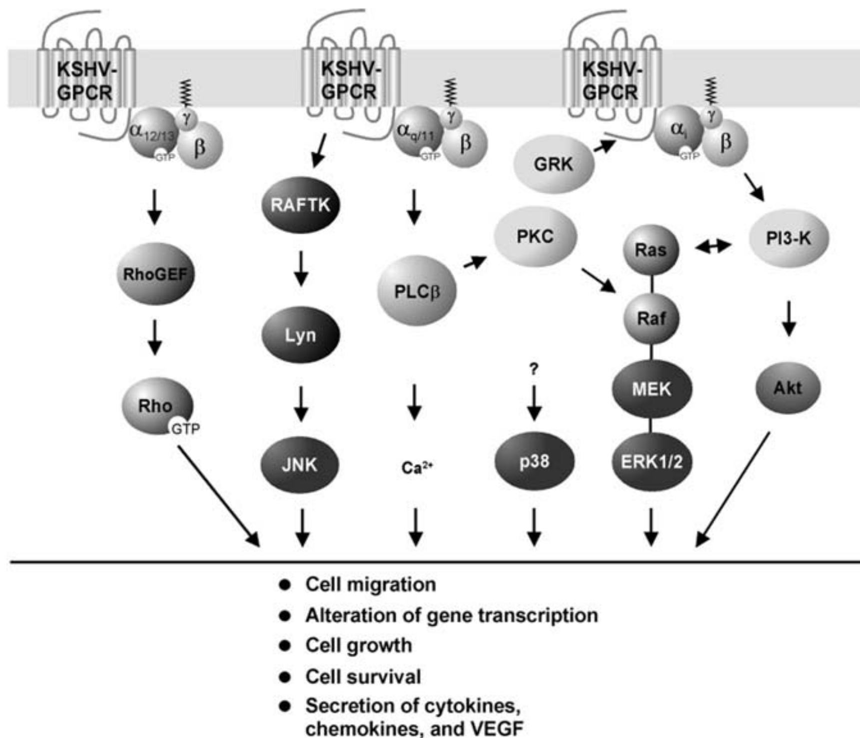

Fig. 15.3 Model of signaling pathways activated by KSHV-encoded KSHV-GPCR. KSHV-GPCR constitutively activates PLCβ through $G\alpha_q$ and $G\alpha_{11}$. Activation of Akt is likely to be caused by the release of Gβγ subunits from G_i proteins and to require activation of PI3-K. Activation of ERK1/2 probably involves both $G_{i/o}$ and $G_{q/11}$ proteins and activation of the Ras-Raf-MEK-ERK1/2 cascade. Activation of JNK/SAPK by KSHV-GPCR is mediated by Lyn activation through RAFTK/Pyk2. Activity of KSHV-GPCR is regulated through phosphorylation by members of the GRK and/or PKC families. KSHV-GPCR also couples to $G_{12/13}$ proteins to stimulate RhoA through activation of a RhoGEF.

cell type-specific manner [123]. This cell type-specific regulation may explain the differences observed in the patterns of the factors secreted by KSHV-GPCR-expressing cells. Thus, Jurkat T cells specifically secrete the cytokines IL-2 and IL-4, while the B cell line BJAB predominantly release the CC chemokine MIP-1α into the medium [120, 124]. Secretion of proinflammatory cytokines and chemokines, as well as angiogenic growth factors such as VEGF, by KSHV-GPCR-infected cells may contribute significantly to the development of KS lesions by both paracrine and autocrine mechanisms and may be involved in cellular transformation and tumor vascularization [109, 125].

15.3.5
Regulation of KSHV Activity by Chemokines

In marked contrast to the activity of pUS28, that of KSHV-GPCR is modulated by chemokines, which act at KSHV-GPCR as agonists, inverse agonists, or antagonists [89, 111, 119, 126–128]. For example, binding of CXCL1 to KSHV-GPCR further stimulates PLCβ, while binding of CXCL10 has the opposite effect [112]. Activation of ERK1/2 and Akt is also significantly reduced by CXCL10 [89, 113]. The activities of the transcription factors NF-κB, AP-1, and CREB are also subject to regulation by chemokines [114, 120]. Interestingly, the chemokines modulating KSHV-GPCR signaling belong to the subfamily of CXC chemokines known to regulate angiogenesis [16, 19, 22]. Thus, CXCL1 has angiogenic and CXCL10 angiostatic properties [16, 19, 22]. Surprisingly, the activation of PLCβ by KSHV-GPCR was stringently dependent on the presence of KSHV-GPCR ligands in endothelial cells with stable expression of KSHV-GPCR [125]. This finding shows that the constitutive activity of KSHV-GPCR is observed in some but not all cell types. The molecular basis for the cell type specificity of the constitutive activity of KSHV-GPCR is unknown.

15.3.6
Structure–Function Relationships of KHSV-GPCR

The identification of the structural elements that determine constitutive activity of KSHV-GPCR may allow the development of strategies through which to interfere specifically with the functions of KSHV-GPCR in herpes virus-infected cells (with its transforming activity and its stimulatory effect on secretion of VEGF, for example). Deletion of the amino-terminal portion of KSHV-GPCR caused a loss of chemokine binding without influencing the constitutive activity of the receptor [129, 130]. In marked contrast, deletion of the five C-terminal amino acids strongly reduced the transforming and NF-κB-inducing activities without affecting ligand binding [120]. Interestingly, the latter truncation only marginally affected the stimulation of chemokine and VEGF production by KSHV-GPCR [120]. This could be interpreted as evidence for multiple GPCR conformations that differentially regulate various downstream effector pathways (see also Chapter 9). Other determinants of constitutive signaling of KSHV-GPCR were identified in the second transmembrane domain (TM II)

and at the boundaries between TM II and TM III and the corresponding intracellular segments [130, 131]. Thus, the substitutions $Lys^{91}Asp$ and $Lys^{94}Asp$ significantly lowered basal activity while preserving chemokine responsiveness [130]. The substitutions $Asp^{83}Ala$ and $Val^{142}Asp$ increased basal KSHV-GPCR activity [131]. Other residues involved in mediating the stimulatory effects of chemokine agonists on KSHV-GPCR activity were identified at the extracellular transmembrane helix boundaries of TM V and TM VI. As an example, the substitutions $Arg^{208}His$ and $Arg^{212}His$ abolished the response to CXCL1, while binding of the chemokine and basal constitutive activity were preserved [130].

15.3.7
Cellular Functions of KHSV-GPCR *in vivo*

Similarly to pUS28, KSHV-GPCR may not only regulate specific intracellular signaling pathways on its own, but may also modulate the function of cellular GPCRs. On one hand, increased expression of CXCL8 in herpes virus-infected cells may result in activation of CXCR1 and/or CXCR2, which are expressed in KS lesions [132, 133]. On the other hand, induction of heterologous desensitization of other GPCRs and depletion of $InsP_3$-releasable ($InsP_3$ = inositol 1,4,5-triphosphate) Ca^{2+} stores has been reported for cells expressing KSHV-GPCR [134]. Thus, coexpression of KSHV-GPCR resulted in a marked inhibition of the agonist response of the thyrotropin-releasing hormone receptor (THR-R) in *Xenopus* oocytes [134]. This effect was partially reversed by CXCL10 [134], a chemokine that acts as an inverse agonist on KSHV-GPCR.

An intriguing question currently being addressed in several laboratories relates to which of the signaling functions of KSHV-GPCR observed in transfected cells are of biological importance *in vivo* (for the course of the herpes virus infection, for example, or the development of KS). To analyze the *in vivo* functions of KSHV-GPCR systematically and to determine its impact on KS formation, several transgenic mouse models have been developed and analyzed [108, 109, 135–137]. Transgenic mice expressing the KSHV-GPCR under the control of the CD2 promoter that specifically targets the expression of the viral protein to certain hematopoietic cells (e.g., T cells), or under the control of the simian virus 40 (SV40) early promoter-enhancer developed highly vascularized KS-like tumors [108, 135]. These findings provide strong evidence that KSHV-GPCR expression is causally linked to formation of KS [108, 109, 135]. Furthermore, expression of KSHV-GPCR mutants deficient either in constitutive activity or in chemokine binding in transgenic mice did not result in the development of KS-like tumors, demonstrating that both constitutive activity and modulation of signaling by chemokines are required for KSHV-GPCR to stimulate tumorigenesis [136]. Recently, cell-specific expression of several candidate KSHV oncogenes, including ORF74, ORF K9, encoding viral interferon factor 1 (vIRF-1), and ORF 16, encoding viral Bcl-2 (vBCL-2) in endothelial cells of transgenic mice, revealed that only expression of ORF74 induced angioproliferative tumors that resembled KS, while the expression of other two candidate viral genes did not [137]. The products of these genes are suspected to play important roles in spindle cell formation [137]. Similarly to KS, the an-

gioproliferative tumors induced by KSHV-GPCR expression in endothelial cells contained only a small number of cells expressing KSHV-GPCR, which is in agreement with the paracrine mechanism of KS tumor formation mentioned above [136]. From their results, the authors hypothesized that the KSHV-GPCR encoded by ORF74 not only initiates, but also promotes *in vivo* KS tumor development, and suggested that KHSV-GPCR is an attractive candidate for the development of pathogenesis-based therapies of this disease [137].

15.4 Conclusions

Viral strategies to evade the host immune system include the expression of viral proteins that either mimic or interfere with functions of host proteins playing important roles in the control of the host immune system. In host cells, chemokine receptors regulate the activity of a variety of intracellular effectors which control for example, chemotaxis, adhesion, and apoptosis of leukocytes. Viruses interfere with these functions by causing expression of virally encoded chemokine receptor homologues, with either ligand-dependent or ligand-independent constitutive activity. Heterologous expression of these receptor homologues may allow the virus to remain undetected by the host's immune system and to propagate and disseminate in the host. Interestingly, the functional properties of the receptor homologues have been adapted by viruses to fulfil various specific functions in infected cells, ranging from removal of chemokines from the extracellular environment to induction of a specific response to chemokines present in this environment or initiation of chemokine-independent changes in host cell signaling. Thus, analysis of signaling properties of viral chemokine receptor homologues and relating of these to functional changes of specific host cells should not only improve our understanding of viral immunopathogenesis, but might also help in the development of new therapeutic strategies to treat virally transmitted human diseases.

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