0026-895X/05/6704-983–991$20.00
MOLECULAR PHARMACOLOGY
Vol. 67, No. 4
8268/1197151
Mol Pharmacol 67:983–991, 2005
Printed in U.S.A.

ACCELERATED COMMUNICATION

The Natural Mutation Encoding a C Terminus-Truncated 5-Hydroxytryptamine$_{2B}$ Receptor Is a Gain of Proliferative Functions[S]

Maud Deraet, Philippe Manivet, Agnes Janoshazi, Jacques Callebert, Silke Guenther, Ludovic Drouet, Jean-Marie Launay, and Luc Maroteaux

Institut de Génétique et de Biologie Moléculaire et Cellulaire, Centre National de la Recherche Scientifique, Institut National de la Santé et de la Recherche Médicale, Université L. Pasteur de Strasbourg, Illkirch, France (M.D., A.J., S.G., L.M.); and IFR6, Services de Biochimie et d'Angio-Hématologie, Hôpital Lariboisière, Paris, France (P.M., J.C., L.D., J.-M.L.)

Received October 14, 2004; accepted December 28, 2004

ABSTRACT

Although potentially implicated in several physiological functions, few functional mutations have been identified in the human 5-hydroxytryptamine (HT)$_{2B}$ receptor gene. A heterozygous mutation R393X in the 5-HT$_{2B}$ receptor was recently identified in a patient diagnosed with pulmonary hypertension after intake of the anorexigenic dexfenfluramine. Although reported to generate a lack of function, this C terminus-truncated 5-HT$_{2B}$ receptor should somehow affect transduction pathways relevant to pulmonary hypertension. In our study, we investigated putative modifications in transduction of the R393X-mutated 5-HT$_{2B}$ receptor. In stably transfected cells, we confirmed the loss of inositol 1,4,5-trisphosphate stimulation caused by the $G_{\alpha q}$ uncoupling, despite conserved ligand affinity between the normal and mutated receptors. We also observed a partial loss of nitric-oxide synthase stimulation. However, the truncated R393X receptor presented 1) a strong gain of efficacy in cell proliferation as assessed by mitogen-activated protein kinase activity and thymidine incorporation, 2) a preferential coupling to $G_{\alpha 13}$ as shown by blocking antiserum, and 3) an apparent lack of internalization upon agonist stimulation as observed by confocal microscopy. This work demonstrates that, in the 5-HT$_{2B}$ receptor, the C terminus, including the palmitoylation and phosphorylation sites, is absolutely required for proper transduction and internalization. For the first time, we show that the lack of C terminus can generate a switch of coupling to $G_{\alpha 13}$, a reduced NO synthase activation, and an increase in cell proliferation. All these modifications are relevant in pathophysiological vasoconstriction.

Knowledge of 5-HT$_{2B}$ receptor genetic variants is of the utmost interest in view of the involvement of these receptors in various pathologies (Roth and Shapiro, 2001). Few studies have investigated mutations in the 5-HT$_{2B}$ receptor gene (MIM 601122). It was identified as a candidate gene in obsessive-compulsive disorder: one single nucleotide polymorphism was described in intron 1, but no evidence for functional mutation was found in the coding regions of 5-HT$_{2B}$ receptor (Kim et al., 2000). More recently, by investigating the 5-HT$_{2B}$ receptor gene in patients who developed pulmonary hypertension after intake of fenfluramines, a heterozygous mutation was found in one female who 5 years before followed a 9-month anorexigenic treatment (Blanpain et al., 2003). The reported mutation consists of a C/T transition

This work has been supported by funds from the Centre National de la Recherche Scientifique, the Institut National de la Santé et de la Recherche Médicale, the Hôpital Universitaire de Strasbourg, the Université Louis Pasteur, and by grants from the Fondation de France, the Fondation pour la Recherche Médicale, the Association pour la Recherche contre le Cancer, the French ministry of research (Actions Concertées Incitatives), and the European Community.

[S] The online version of this article (available at http://molpharm.aspetjournals.org) contains supplemental material.

Article, publication date, and citation information can be found at http://molpharm.aspetjournals.org.
doi:10.1124/mol.104.008268.

ABBREVIATIONS: 5-HT, 5-hydroxytryptamine (serotonin); NOS, nitric-oxide synthase; IP_3, inositol 1,4,5-trisphosphate; PDZ, PSD-95, Dlg, ZO-1; GPCR, G protein-coupled receptor; MAPK, mitogen-activated protein kinase; [^{125}I]DOI, (6)-[^{125}I]1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane HCl; norDF, metabolite of dexfenfluramine; pEYFP, phosphorylated enhanced yellow fluorescent protein; HHV8, human herpes virus 8; NF-κB, nuclear factor-κB.

resulting in the substitution of a stop codon instead of Arg393 of the predicted protein sequence (R393X). This mutation truncates the complete carboxy-terminal tail of the receptor, removing 1) putative palmitoylation and phosphorylation sites essential for the internalization process (Parker et al., 2003), and 2) the PSD-95, Dlg, ZO-1 (PDZ) binding motif involved in the coupling of nitric-oxide synthase (NOS) (Manivet et al., 2000) or other scaffold proteins (Becamel et al., 2004).

The 5-HT_{2B} receptor transduction pathways may greatly depend on cell types, but cell transfection seems to be an indispensable tool to solve the complexity of 5-HT_{2B} receptor-coupling mechanisms. Blanpain et al. (2003) described, by transient transfection, that despite normal expression at the plasma membrane and normal agonist binding, this R393X mutation causes loss of 5-HT_{2B} receptor function as measured by IP_3 production and calcium-dependent aequorin luminescence. In this study, the mutated receptor displayed no constitutive activity compared with the wild-type receptor, and it did not produce dominant negative effects over the IP_3/Ca^{2+} pathway in cotransfection experiments.

Noteworthy, coupling of 5-HT_{2B} receptors to IP_3 stimulation has never been observed in vivo despite its contractile effects in rat stomach (Cox and Cohen, 1995) or vasculature (Ellis et al., 1995), or its proliferative effect in carcinoid tumor cells (Launay et al., 1996). Moreover, 5-HT_{2B} receptor can trigger IP_3-independent Ca^{2+} release from ryanodine-sensitive intracellular stocks in lung endothelial cells (Ullmer et al., 1996). Most mutations in G protein-coupled receptor (GPCR) leading to loss of function are autosomal recessive, whereas most disease caused by GPCR as gain of function mutations are inherited in an autosomal dominant manner (Spiegel and Weinstein, 2004). If the heterozygous R393X 5-HT_{2B} receptor mutation is related to the pulmonary hypertension, it must trigger transduction alterations relevant to this pathology.

Previous studies established that, in addition to its role in phosphoinositide hydrolysis, the 5-HT_{2B} receptor can transactivate growth factor receptors that control mitogen-activated protein kinase (MAPK) signaling and cell cycle progression (Launay et al., 1996; Nebigil et al., 2000). The 5-HT_{2B} receptor protein is overexpressed in lung arteries of pulmonary hypertension patients as well as in mice chronically submitted to hypoxia, a model that shares several features with pulmonary hypertension (Launay et al., 2002). The 5-HT_{2B} receptor is required for smooth muscle cell proliferation and structural remodeling of arteries, consistent with the ability of 5-HT_{2B} receptor to initiate cell cycle progression in mouse fibroblasts (Nebigil et al., 2000). The 5-HT_{2B} receptor can also elicit a reversible endothelium-dependent relaxation of pulmonary arteries, associated with an increase in cyclic GMP (Glusa and Pertz, 2000), through coupling to endothelial NOS. The 5-HT_{2B} receptor-dependent NOS activation requires the PDZ motif at the C terminus (Manivet et al., 2000). Because the R393X mutation abolishes IP_3 release and removes the C terminus PDZ motif, Blanpain et al. (2003) suggested that the mutated receptor had lost its G_q coupling and probably its ability to stimulate NOS. Besides endothelium-dependent vasodilatation, the 5-HT_{2B} receptor could participate in pulmonary arterial smooth muscle cells proliferation or remodeling (Launay et al., 2002). If this 5-HT_{2B} receptor-myoproliferative effect is relevant to smooth muscle cells in pulmonary hypertension patients, we hypothesize that it should be affected by the mutated R393X allele. In this study, we therefore investigated a putative gain of function of the R393X 5-HT_{2B} receptor and showed that despite its loss of IP_3 stimulation as a result of $G_{\alpha q}$ uncoupling and its loss of endothelial NOS stimulation because of the absence of the PDZ binding domain, this receptor retains some NO coupling and presents higher efficacy for cell proliferation via a nearly exclusive $G_{\alpha 13}$ coupling. In addition to the switch in coupling pathways, R393X 5-HT_{2B} receptor showed no apparent internalization upon agonist stimulation.

Materials and Methods

Reagents. Neurochemicals were provided by Roche (Basel, Switzerland), Eli Lilly (Indianapolis, IN), and GlaxoSmithKline (Welwyn Garden City, Hertfordshire, UK) companies (Table 1 for chemical makeup of compound numbers). All other chemicals were reagent grade and purchased from commercial sources. The radioactive compound (6)-[^{125}I]1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane HCl ([^{125}I]DOI; 81.4 TBq/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Rabbit antisera against rodent G_s (C-18), $G_{q/11}$ (C-19), and G_{13} (A-20) were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).

Mutagenesis. The different constructs of the human wild-type 5-HT_{2B} and truncated 5-HT_{2B} receptor cDNA were performed by polymerase chain reaction. The mutated reading frames were subcloned into enhanced green fluorescent protein variants, pEYFP fused at the N terminus to generate wild-type 5-HT_{2B} receptor-pEYFP and R393X 5-HT_{2B} receptor-pEYFP. All the constructs were verified by sequencing before transfection.

Cell Culture. LMTK$^-$ cell lines were maintained in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum. For transfection experiments, cells were seeded at a density of 1.5×10^6 cells/10-mm dish and cultured for 24 h. Transient transfection was then performed using Jet Pei according the manufacturer's recommendations. The cells were then cultured in the same medium for 48 h. The stable expressing human 5-HT_{2B} receptor and R393X 5-HT_{2B} receptor were grown with 0.8 mg/ml G418 (Geneticin).

Confocal Imaging. LMTK$^-$ cells expressing wild-type 5-HT_{2B} receptor-pEYFP or R393X 5-HT_{2B} receptor-pEYFP were grown in a glass-bottomed dish and observed in serum-free medium at room temperature. To visualize the receptor internalization movement after agonist treatment, time-lapse series were taken every 5 min over a 30-min period. To calculate the internalization kinetics of the receptors, we selected more than five regions of interest on the membrane per cell and followed the relative intensity changes of these regions over time, with three individual cells per experiment. Our data represent more than four independent experiments. The kinetic curves were corrected for the bleaching by using the intensity of the whole cell as a normalization factor.

Radioligand Binding Experiments. Radioligand binding experiments were performed as detailed previously (Tournois et al., 1998). Stable LMTK$^-$ clones expressed 178 and 128 fmol of 5-HT_{2B} receptor and R393X 5-HT_{2B} receptor/mg protein, respectively, as measured by [^{125}I]DOI binding.

Determination of Intracellular Inositol 1,4,5-Trisphosphate Levels. Cells were washed twice with fresh serum-free medium, incubated for 5 min at 37°C with agonists, and scraped with a rubber policeman. Intracellular IP_3 levels were then measured by radioreceptor assay as detailed previously (Tournois et al., 1998). The E_{max} value for serotonin of the wild-type receptor was 120 pmol/mg protein.

NOS Assay. Cells were incubated with agonists for 10 min. Cell lysate supernatant proteins were assayed for NOS activity by

TABLE 1

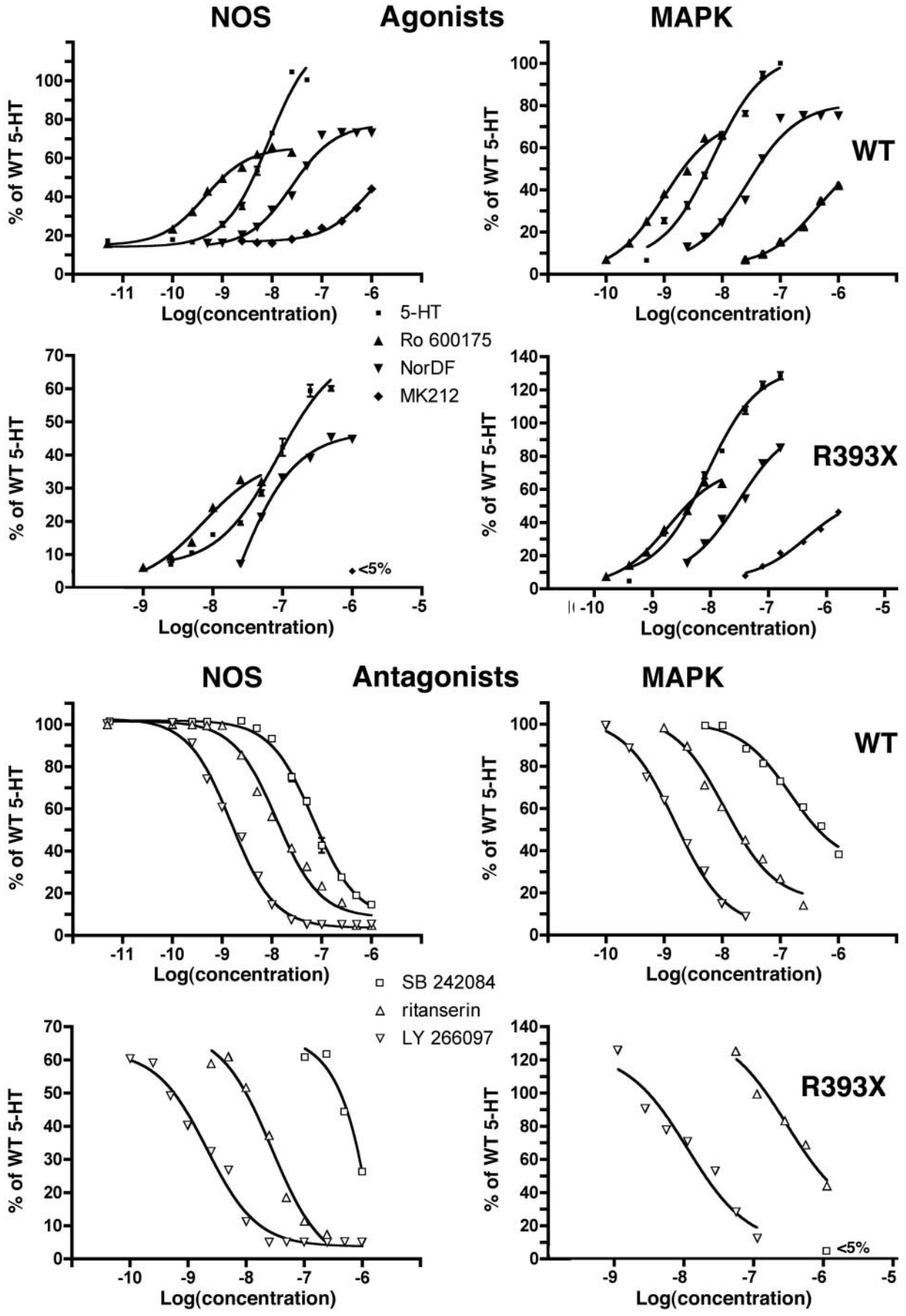
Pharmacology of wild-type and R393X mutated 5-HT$_{2B}$ receptors

Ten concentrations of each competing drug were used. Reported values are the means of four independent experiments performed in triplicates and are expressed in nanomolar or percentage of wild type (E_{max}) ± S.E.M. IP_3 levels were determined 5 min, NOS activities 10 min, and MAPK activities 20 min after agonists addition.

Agonist	HT_{2B} WT							R393X						
	Binding K_i	IP_3		NOS Activity		MAPK Activity		Binding K_i	IP_3**		NOS Activity		MAPK Activity	
		EC_{50}	E_{max}	EC_{50}	E_{max}	EC_{50}	E_{max}		EC_{50}	E_{max}	EC_{50}	E_{max}	EC_{50}	E_{max}
	nM	*nM*	%	*nM*	%	*nM*	%	*nM*	*nM*	%	*nM*	%	*nM*	%
MK 212	151 ± 12	468 ± 31	62 ± 8	178 ± 27	42 ± 5	151 ± 36	41 ± 11	89 ± 5*	>1000	N.D.	>1000	N.D.**	120 ± 22	47 ± 8
Ro 600332	66 ± 7	24 ± 2	91 ± 7	66 ± 6	39 ± 4	76 ± 6	34 ± 9	107 ± 5*	>1000	N.D.	240 ± 26**	21 ± 5*	15 ± 1**	67 ± 10*
DOI	14 ± 4	7.9 ± 0.7	68 ± 8	16 ± 3	43 ± 6	17 ± 5	43 ± 8	22 ± 6	>1000	N.D.	135 ± 16**	14 ± 4*	7.6 ± 0.8*	52 ± 11
BW 723C86	12 ± 2	5.1 ± 0.8	92 ± 9	23 ± 4	56 ± 7	25 ± 7	52 ± 11	14 ± 3	>1000	N.D.	98 ± 7**	23 ± 7*	3.5 ± 0.8**	61 ± 7
Ro 600175	0.71 ± 0.10	0.69 ± 0.07	84 ± 7	0.66 ± 0.06	64 ± 8	0.71 ± 0.18	65 ± 8	0.5 ± 0.2	>1000	N.D.	5.9 ± 0.8**	32 ± 6*	0.63 ± 0.02	64 ± 12
norDF	13 ± 3	18 ± 2	91 ± 1	22 ± 4	73 ± 9	22 ± 4	73 ± 3	17 ± 1	>1000	N.D.	87 ± 6**	45 ± 8*	12 ± 3*	86 ± 5*
5-HT	3.1 ± 0.9	4.6 ± 0.7	100 ± 5	5.6 ± 0.4	100 ± 6	6.0 ± 0.8	100 ± 7	4.9 ± 0.8	>1000	N.D.	56 ± 7**	60 ± 9*	3.1 ± 0.5*	127 ± 4*
Antagonist (vs. 5-HT)		K_B		K_B		K_B			K_B		K_B		K_B	
		nM		*nM*		*nM*			*nM*		*nM*		*nM*	
Lisuride	1.1 ± 0.4	1.2 ± 0.3		1.3 ± 0.6		2.1 ± 0.9		1.3 ± 0.4	N.D.		7.4 ± 0.3*		13 ± 3*	
Ro 600869	8.9 ± 1.2	63 ± 6		8.5 ± 0.8		7.1 ± 0.9		11.2 ± 1.1	N.D.		17.4 ± 0.7*		9.3 ± 0.8	
RS 102221	93 ± 5	436 ± 15		78 ± 5		89 ± 9		174 ± 8*	N.D.		302 ± 26**		>1000**	
SB 242084	85 ± 7	37 ± 5		36 ± 3		49 ± 8		53 ± 4*	N.D.		123 ± 16**		158 ± 20**	
Ketanserine	54 ± 9	209 ± 29		151 ± 12		93 ± 12		35 ± 6	N.D.		115 ± 19		141 ± 15*	
Mesulergine	2.1 ± 0.4	2.8 ± 0.5		10.5 ± 0.7		4.5 ± 0.8		2.9 ± 0.4	N.D.		8.5 ± 0.7		15 ± 1**	
SB 206553	12 ± 1	5.6 ± 0.3		7.4 ± 1.3		21 ± 3		7.9 ± 0.3	N.D.		29 ± 5**		102 ± 27**	
Mianserin	15 ± 1	5.9 ± 0.8		7.1 ± 0.9		33 ± 5		7.6 ± 0.6*	N.D.		25 ± 2**		229 ± 24**	
Ritanserin	5.5 ± 0.9	1.4 ± 0.4		8.7 ± 1.0		6.9 ± 0.8		7.1 ± 0.9	N.D.		12 ± 1		138 ± 18**	
SB 215505	1.2 ± 0.3	9.0 ± 0.1		2.2 ± 0.4		1.8 ± 0.5		1.4 ± 0.1	N.D.		6.0 ± 0.8*		13 ± 2**	
SB 204741	74 ± 8	44 ± 6		67 ± 6		60 ± 8		71 ± 4	N.D.		148 ± 13**		759 ± 61**	
LY 266097	0.24 ± 0.08	0.18 ± 0.06		0.65 ± 0.06		0.71 ± 0.07		0.33 ± 0.11	N.D.		1.07 ± 0.15*		2.3 ± 0.6*	
RS 127445	0.33 ± 0.11	0.49 ± 0.10		0.43 ± 0.07		0.45 ± 0.08		0.30 ± 0.09	N.D.		0.52 ± 0.09		0.49 ± 0.31	
MDL 100907	91 ± 8	67 ± 7		245 ± 16		155 ± 19		81 ± 3	N.D.		166 ± 17*		181 ± 16	

* $P < 0.05$; ** $P < 0.01$ versus wild type by Student's t test.

N.D., not detectable; BW 723C86, 1-[5-(2-thienylmethoxy)-1*H*-3-indolyl]propan-2-amine HCl; LY 266097, 1-(2-chloro-3,4-dimethoxybenzyl)-6-methyl-1,2,3,4-tetrahydro-9*H*-pyrido[3,4-*b*]indole hydrochloride; MDL 100907, [(+)-α-(2,3-dimethoxyphenyl)-1-[2-(4-fluorophenylethyl)]-4-piperidinemethanol; MK 212, 6-chloro-2-[1-piperazinyl]-pyrazine; Ro 600175, (*S*)-2-(6-chloro-5-fluoroindol-1-yl)-1-methlethylamine); Ro 600332, 1(*S*)-methyl-2-(4,4,7-trimethyl-1,4-dihydroindeno[1,2-*b*]pyrrol-1-yl)ethylamine fumarate; Ro 600869, *cis*-7-ethyl-6-hydroxy-8-methoxy-2-methyl- 1,2,3,3*a*,4,9*b*-hexahydro-5*H*-benzo[*e*]isoindol-5-one; RS 102221, 8-[5-(2,4-dimethoxy-5-(4-trifluorophenylsulfonamido)phenyl-5-oxopentyl]-1,3,8-triazo-spiro[4.5]decane-2,4-dione hydrochloride; RS 127445, 2-amino-4-(4-fluoronaphth-1-yl)-6-isopropylpyrimidine; SB 204741, *N*-(1-methyl-5-indolyl)-*N*′-(3-methyl-5-isothiazolyl) urea; SB 206553, 5-methyl-1-(3-pyridylcarbamoyl)-1,2,3,5-tetrahydropyrrolo[2,3-*f*]indole; SB 215505, 6-chloro-5-methyl-1-(5-quinolylcarbamoyl) indoline; SB 242084, 6-chloro-5-methyl-1-[2-(2-methylpyridyl-3-oxy)-pyrid-5-yl carbonyl] indoline.


NOS
Agonists
MAPK
% of WT 5-HT
Log(concentration)
WT
R393X
5-HT
Ro 600175
NorDF
MK212
<5%
NOS
Antagonists
MAPK
SB 242084
ritanserin
LY 266097
<5%

monitoring the conversion of ^{3}H-labeled L-arginine to ^{3}H-labeled L-citrulline as described previously (Manivet et al., 2000). The E_{max} value for serotonin of the wild-type receptor was 16 pmol/mg protein/min.

Assay of MAPK Activity. Cells were incubated with agonists for 20 min. Cell lysate supernatant proteins were assayed for kinase activity in reaction buffer containing 1 μCi of [γ-^{32}P]ATP, 20 μM ATP, and 1.5 mg/ml myelin basic protein (Sigma-Aldrich, St. Louis, MO) for 20 min at 30°C, essentially as described previously (Launay et al., 1996). The E_{max} value for serotonin of the wild-type receptor was 1.15 pmol/mg of protein/10 min.

Thymidine Incorporation. Cells were seeded on 24-well plates at a density of 10^3 cells per well, grown overnight, and serum-starved for 24 h. Quiescent cells were treated with serotonin at different concentrations for 18 h, and 0.5 μCi (1 Ci = 37 GBq) of [^{3}H]thymidine was added to the culture during the last 4 h of incubation. The free radioactive thymidine was washed away in 5% trichloroacetic acid, and the incorporated radioactive thymidine was quantified by scintillation counting as described previously (Nebigil et al., 2000).

Cell Permeabilization. The cells were washed twice with phosphate-buffered saline containing 0.1% bovine serum albumin and exposed to 1 hemolytic unit of alveolysin/10^6 cells at 37°C under agitation as described previously (Manivet et al., 2000).

Statistics. Binding data were analyzed using the iterative nonlinear fitting software GraphPad Prism 2.0 (GraphPad Software Inc., San Diego, CA). This allowed to calculate inhibition constants (K_i) according to the Cheng-Prusoff equation (Cheng and Prusoff, 1973). All values represent average of independent experiments ± S.E.M. (n is number of experiments as indicated in the text). Comparisons between groups were performed using Student's unpaired t test or analysis of variance and a Fisher test. Significance was set at $P < 0.05$.

Results

Because it was not possible to study the mutated 5-HT$_{2B}$ receptor R393X in patients' native tissues, we used stable recombinant expression systems. The two human 5-HT$_{2B}$ receptors cDNA, wild type and R393X, were stably transfected into LMTK$^-$ cells, thus ensuring they were studied in an identical cell background. Under the same experimental paradigm, parental nontransfected cells did not exhibit a concentration-dependent rise in second messengers after agonist stimulation. Single clones were chosen for each receptor according their relatively low expression to be as close as possible to physiological levels. Moreover, cells were used at nearly similar passages to ensure stable expression levels. Classic pharmacological binding studies clearly confirmed that the R393X mutation had no significant effect on the binding profile of either agonists or antagonists (Table 1). We also confirmed that the IP$_3$ coupling was lost by this mutated receptor (Table 1). However, because NO coupling and cell proliferation may be more physiologically relevant signaling pathways for this receptor, we asked whether the R393X mutation had differential effect on these two pathways.

Pharmacological values obtained for R393X 5-HT$_{2B}$ receptor versus wild-type receptor clearly demonstrate that EC$_{50}$ values for NO coupling were increased for all agonists (Fig. 1; Table 1; supplemental figure). For some ligands such as serotonin, the EC$_{50}$ value was increased by a factor 10. The maximal levels of NOS activation (E_{max}) were all reduced. The E_{max} value for serotonin was reduced by 40%, with serotonin behaving as a partial agonist. The E_{max} value for DOI changed from more than 40% of that of serotonin for wild-type receptor to 14% for the mutant receptor, with DOI becoming a nearly silent agonist (Table 1). Most K_B values for antagonists were increased (Table 1; supplemental figure). These results indicate a partial loss of NO coupling by the R393X receptor.

In contrast, most EC$_{50}$ values for the coupling to MAPK activation were decreased. BW 723C86 (a selective 5-HT$_{2B}$ receptor agonist) showed a 7-fold decreased EC$_{50}$ value. It is interesting that the E_{max} value of R393X 5-HT$_{2B}$ receptor for serotonin was increased by 27% over the wild-type value, and the E_{max} value for the active metabolite of dexfenfluramine, norDF, changed from 73 to 86% of that of serotonin for the wild-type receptor (Fig. 1; Table 1). For antagonists, most K_B values toward MAPK activation were increased, by 20-fold for ritanserin, for example (Fig. 1; supplemental figure). These results indicate a clear gain of efficacy in MAPK stimulation by the R393X receptor.

Because previous studies have indicated that 5-HT$_{2B}$ receptor-dependent MAPK activation can led to cell proliferation and/or survival, we performed a proliferation assay on both cell lines expressing either wild-type or R393X 5-HT$_{2B}$ receptors. According to these studies, agonist activation of 5-HT$_{2B}$ receptors triggered cell proliferation as assessed by tritiated thymidine incorporation (Fig. 2). In agreement to the pharmacological study on MAPK activation, the cell proliferation triggered by stimulation of the R393X-mutated 5-HT$_{2B}$ receptor was strikingly increased (3-fold at 1 μM serotonin), and the stimulation by 100 nM serotonin of this mutated receptor was similar to that of the wild-type receptor by 1 μM serotonin (Fig. 3A). Basal thymidine incorporation was also elevated in R393X 5-HT$_{2B}$ receptor (1.7 ± 0.3-fold basal wild type; $n = 8$). These results indicate a clear gain of efficacy in thymidine incorporation and thus of cell proliferation for R393X 5-HT$_{2B}$ receptor.

We further investigated this gain of cell proliferation using blocking antibodies to various G proteins on alveolysin-permeabilized living cells. We observed that the thymidine incorporation was controlled by both G$_q$ and G$_{13}$ in the wild-type receptor-expressing cells. Indeed, anti-G$_s$ antiserum was inactive in the same assay. By contrast, the thymidine incorporation seemed almost entirely dependent on G$_{13}$, but not anymore on G$_q$ (or G$_s$) in R393X 5-HT$_{2B}$ receptor-expressing cells (Fig. 3B). These results suggest that the gain of efficacy to cell proliferation by the R393X 5-HT$_{2B}$ receptor is subsequent to a change in G protein coupling to G$_{13}$.

Finally, a previous report indicated that upon agonist stimulation, 5-HT$_{2B}$ receptor is quickly desensitized (Porter et al., 2001), a phenomenon that likely relies, at least in part, on phosphorylation of residues in the C terminus of GPCRs. We

Fig. 1. Comparison of the effects of selected ligands on the coupling of the 5-HT$_{2B}$ receptor to the wild-type (WT) and the R393X receptor. The dose-response effect of agonists (four top panels) on NOS activity has been directly followed by NOS activity assay (four left panels). In the case of MAPK, direct kinase assay was used to assess MAPK activity (four right panels). The antagonist effect (bottom four panels) to the receptor was assessed through competition 5-HT effect. Ten different concentrations of each drug were used. Reported values are the means of four independent experiments performed in triplicates and are expressed as percentage of 5-HT effects on wild-type receptor. Deduced absolute values ± S.E.M. for EC$_{50}$, E_{max}, and K_B and statistical significance are shown in Table 1.

therefore compared the wild-type and the R393X 5-HT_{2B} receptor-pEYFP for internalization upon stimulation by 1 μM serotonin by confocal time-lapse videomicroscopy. Stimulation of the 5-HT_{2B} receptor wild-type-expressing cells led to a rapid internalization indicated by the quick disappearance of receptors from the plasma membrane with a half-life of 4. 7 $\pm$ 0.9 min (n = 7). For R393X 5-HT_{2B} receptor-expressing cells, no significant internalization was detected with half-life longer than 60 min (Fig. 4; supplemental movies). These results indicate that the absence the C terminus-truncated receptor is either partially retained intracellularly or constitutively internalized.

Discussion

Using stably transfected cell lines expressing human cDNAs encoding wild-type and R393X 5-HT_{2B} receptors, we first confirmed the initial Blanpain et al. (2003) results that the R393X 5-HT_{2B} receptor has kept the same binding affinity for all tested compounds but lost its coupling to G_q and to IP_3. We then validated their hypothesis that endothelial NOS coupling is lost in the R393X 5-HT_{2B} receptor. Both of these findings are loss of function.

Moreover, we demonstrate here, for the first time, that the lack of C-terminal tail containing the palmitoylation site in the R393X 5-HT_{2B} receptor is associated with a loss of rapid internalization as shown by confocal analysis of yellow fluorescent protein-tagged receptors. In addition, the R393X 5-HT_{2B} receptor retains its capacity to stimulate NOS activity despite lower efficacy. Furthermore, the R393X 5-HT_{2B} receptor has acquired a striking increase in proliferative capacity, as indicated by two independent criteria, an increase in MAPK E_{max} value and in thymidine incorporation levels. These latter data seem to be the consequence of a switch from a dual $G_{\alpha q}/G_{\alpha 13}$ coupling in wild-type receptor to a nearly exclusive coupling to $G_{\alpha 13}$ in the R393X 5-HT_{2B} receptor as shown by blocking antibodies. This switch to $G_{\alpha 13}$ coupling and cell proliferation is clear gain of functions for this mutant receptor in transduction pathways, which is relevant for vascular proliferation and remodeling.

In mouse $LMTK^-$ fibroblasts stably transfected with the full-length cDNA encoding the 5-HT_{2B} receptor, a serotonin-dependent NO coupling mediated by endothelial and inducible NOS has been observed. The endothelial NOS stimulation is PDZ-dependent, whereas the inducible NOS is controlled by G_{13} (Manivet et al., 2000). In transfected cells expressing a partial C terminus-truncated form of the 5-HT_{2B} receptor (K403X), DOI-dependent IP_3 coupling was retained (Manivet et al., 2000). Combined with our data, this clearly indicates that the Cys397 is required for the formation of the VIII α helix and identifies this helix as responsible for the G_q association, because it is destroyed by the absence of the palmitoylation site but retained in the K403X 5-HT_{2B} receptor, as documented previously by structural studies of the rhodopsin/transducin interactions (Sakmar et al., 2002).

A nearly complete lack of apparent internalization was observed in R393X 5-HT_{2B} receptor by time-lapse video confocal microscopy and thus of desensitization that is especially relevant to chronic diseases. This result indicates that the truncated receptor is either partially retained intracellularly or constitutively internalized. Multi-PDZ domain-containing

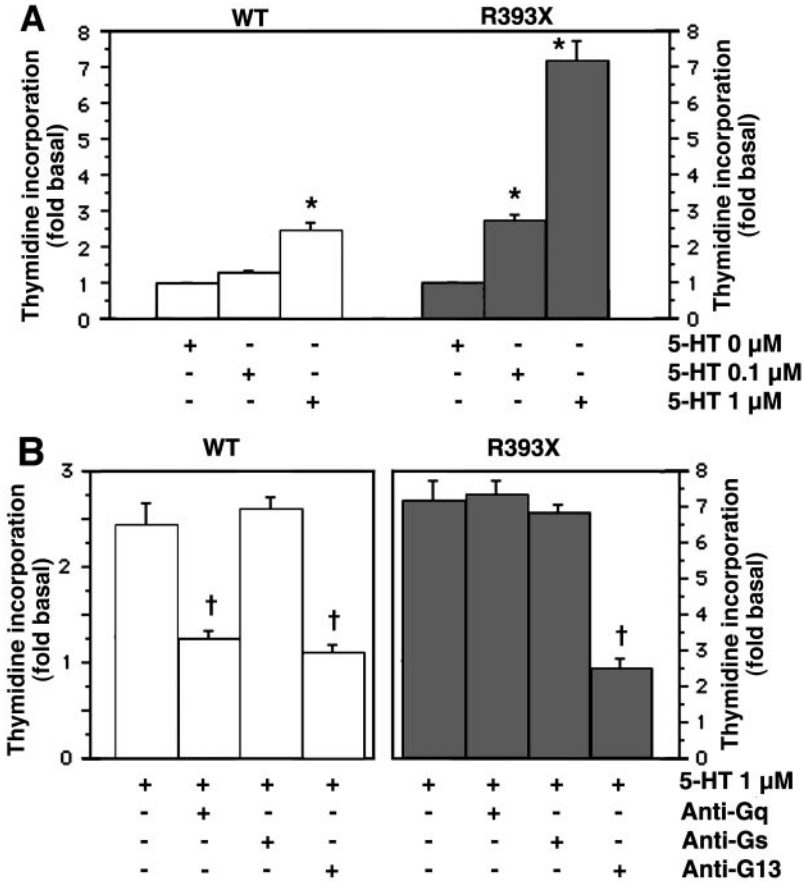


Fig. 2. Gα_{13} is involved in the 5-HT_{2B} receptor-mediated thymidine incorporation. The thymidine incorporation evaluated 24 h after stimulation and represented as fold basal incorporation is significantly elevated by 1 μM serotonin in wild-type receptor and by 100 nM and 1 μM in mutant receptor (A). Basal thymidine incorporation was 5516 $\pm$ 76 dpm/mg of protein for wild-type and 9345 $\pm$ 105 dpm/mg protein for the mutant receptor (n = 8). Exposing permeabilized cells to $G_{\alpha q}$ and $G_{\alpha 13}$ antibodies significantly interferes with the wild-type 5-HT_{2B} receptor-mediated thymidine incorporation but not with antibodies against $G_{\alpha s}$ (B). Antibodies against $G_{\alpha 13}$ nearly completely abolished serotonin-induced thymidine incorporation, but antibodies against $G_{\alpha s}$ or $G_{\alpha q}$ had no effect on the R393X 5-HT_{2B} receptor response. *, $P < 0.05$ versus unstimulated cells; †, $P < 0.05$ versus 1 μM serotonin stimulated cells; white columns, wild-type (WT) control; gray columns, R393X 5-HT_{2B} receptor.

protein 1, a putative scaffolding protein with 13 PDZ domains, was identified as a 5-HT$_{2C}$R-interacting protein (Ullmer et al., 1998). 5-HT$_{2C}$R interaction with multi-PDZ domain-containing protein 1 is dynamically regulated by phosphorylation at Ser458 (Parker et al., 2003). Likewise, PSD-95 was shown to modulate 5-HT$_{2A}$R's activity, intracellular trafficking, and distribution (Xia et al., 2003), and agonist-mediated desensitization of 5-HT$_{2A}$Rs requires the presence of S421 in the C-terminal tail (Gray et al., 2003). In the R393X 5-HT$_{2B}$ receptor, the truncation of most of the carboxy-terminal tail of the receptor removes putative phosphorylation sites and thus PDZ-dependent intracellular trafficking motifs, likely explaining the absence of internalization.

Besides the phosphorylation by second messenger-activated kinases, GPCRs can be also phosphorylated by GPCR kinases, which lead to recruitment of the arrestin proteins. Recent discoveries indicate that the β-arrestins play widespread roles as scaffolds and/or adapter molecules that organize a variety of complex signaling pathways emanating from GPCRs (Miller and Lefkowitz, 2001). G protein-independent, β-arrestin-dependent activation of MAPK has been described previously (Azzi et al., 2003). It was found that inverse agonists for G_s-activated adenylate cyclase could be positive partial agonists for β-arrestin-dependent MAPK activation. These authors suggested that multiple active conformations of a receptor may activate different types of effectors such as G proteins and β-arrestin (Lefkowitz and Whalen, 2004). Although binding affinities are not affected by the absence of C terminus in R393X 5-HT$_{2B}$ receptors, the efficacy of compounds is differentially affected depending on the

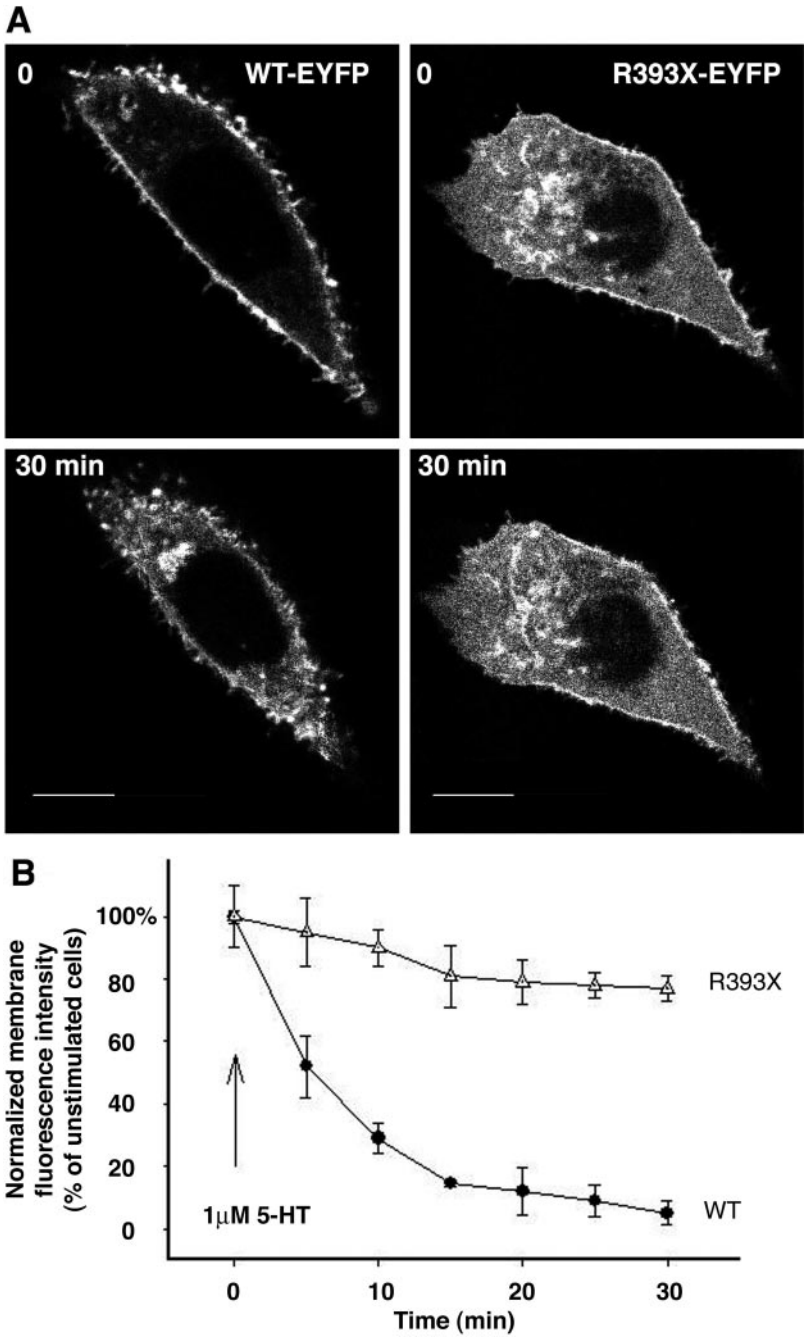


Fig. 3. Internalization of 5-HT$_{2B}$ receptor wild-type and R393X 5-HT$_{2B}$ receptor mutant. Series of single confocal plane images were taken from living LMTK$^-$ cells expressing 5-HT$_{2B}$ receptor-pEYFP wild-type (WT) or R393X 5-HT$_{2B}$ receptor-pEYFP by time-lapse video after stimulation. A, distribution of 5-HT$_{2B}$ receptor expressed at 0 and 30 min of serotonin stimulation. Corresponding sets of images are shown as supplemental videos. These images are representative of three cells observed in each of at least four independent experiments. Scale bars, 2 μm. B, internalization kinetics for wild-type 5-HT$_{2B}$ receptor-pEYFP and R393X 5-HT$_{2B}$ receptor-pEYFP after stimulation by 1 μM serotonin assessed by fluorescence intensity from more than five different regions of interest per cell at the membrane and expressed as percentage of unstimulated cells ± S.E.M.

transduction pathways. The partial agonist DOI becomes a silent agonist toward NOS activity in the mutant receptor, whereas the partial agonist norDF becomes a full agonist toward MAPK activation. These results clearly reinforce the notion that active conformation of GPCRs differs between different couplings and thus agonist efficacy must be dependent on cellular G protein plethora.

Introduction of anti-G_{13} antibodies in permeabilized cells systematically blocked the 5-HT_{2B} receptor-mediated inducible NOS activation (Manivet et al., 2000). Our present study also provides evidence that the α subunit of the G_{13} protein plays a critical role in the mechanisms of 5-HT_{2B} receptor/NOS coupling. G_{13} has been reported to activate inducible NOS through a mechanism distinct from other G_{α} chains (Kitamura et al., 1996). The Src family kinase (other effectors of HT_{2B}Rs) (Nebigil et al., 2000) acts upstream of Rho activation for the $G_{12/13}$-induced c-Jun NH_2-terminal kinase activation (Nagao et al., 1999). The small G protein RhoA and its effector Rho kinase play a major role in the sustained rise in tension induced by vasoconstrictors. Rho/Rho-kinase activation is important in the effects of both acute and chronic hypoxia on the pulmonary circulation, possibly by contributing to both vasoconstriction and vascular remodeling (Fagan et al., 2004). The gain in G_{13} coupling of the R393X 5-HT_{2B} receptor seems thus relevant to pathological vasoconstriction and remodeling.

Recent work on endothelin B receptor, another G_q-coupled receptor implicated in pulmonary vasoconstriction, showed that although the three intracellular loops of this receptor are involved in coupling to G proteins, residues in the first intracellular loop are specifically required for activation of G_{13} (Liu and Wu, 2003). It is interesting that these authors have also reported that the deletion of endothelin B receptor C terminus up to residue Cys405, a myristoylation site, increased response of serum response element-mediated transcription to endothelin 1 in both wild-type and $G_{q/11}$-mutated fibroblasts. Their conclusion that G_{13} transduces signals from GPCRs to stimulate growth-promoting pathways via the serum response element seems similar to the coupling of the R393X 5-HT_{2B} receptor to cell proliferation.

A recent report showed that lung tissue samples from several patients with pulmonary hypertension present evidence of infection with Kaposi's sarcoma herpes virus (human herpes virus 8; HHV8), suggesting a pathogenetic role in primary pulmonary hypertension (Cool et al., 2003). The HHV8 open reading frame 74 encodes a constitutively active GPCR for chemokines that leads to cell transformation and vascular overgrowth by constitutive activation of nuclear factor-κB (NF-κB) and involves activation of G_{13} and RhoA (Shepard et al., 2001). Mouse lung endothelial cell model in which HHV8-GPCR is stably expressed stimulates phosphatidylinositol 3-kinase and activates NF-κB (Couty et al., 2001). Because we previously reported that 5-HT_{2B} receptor via phosphatidylinositol-3 kinase/Akt can activate NF-κB (Nebigil et al., 2003), it is tempting to speculate that HHV8-GPCR and 5-HT_{2B} receptor use common G_{13}-dependent pathways leading to vascular remodeling.

Disruption of the association between PDZ proteins and their targets contributes to the pathogenesis of a number of human diseases, most likely because of the failure of PDZ proteins to appropriately target and modulate the actions of the associated proteins. Our results not only confirm the importance of C-terminal tail of GPCR for both desensitization and coupling but also highlight its importance for G protein repertoire selectivity.

Acknowledgments

We thank P. Hickel for excellent technical assistance, Dr. S. Brooks for English corrections, and Drs. F. Mentré and F. Godmard for statistical expertise.

References

Azzi M, Charest PG, Angers S, Rousseau G, Kohout T, Bouvier M, and Pineyro G (2003) beta-Arrestin-mediated activation of MAPK by inverse agonists reveals distinct active conformations for G protein-coupled receptors. *Proc Natl Acad Sci USA* **100:**11406–11411.

Becamel C, Gavarini S, Chanrion B, Alonso G, Galeotti N, Dumuis A, Bockaert J, and Marin P (2004) The serotonin 5-HT2A and 5-HT2C receptors interact with specific sets of PDZ proteins. *J Biol Chem* **279:**20257–20266.

Blanpain C, Le Poul E, Parma J, Knoop C, Detheux M, Parmentier M, Vassart G, and Abramowicz MJ (2003) Serotonin 5-HT_{2B} receptor loss of function mutation in a patient with fenfluramine-associated primary pulmonary hypertension. *Cardiovasc Res* **60:**518–528.

Cheng Y and Prusoff WH (1973) Relationship between the inhibition constant (K1) and the concentration of inhibitor which causes 50 per cent inhibition (I50) of an enzymatic reaction. *Biochem Pharmacol* **22:**3099–3108.

Cool CD, Rai PR, Yeager ME, Hernandez-Saavedra D, Serls AE, Bull TM, Geraci MW, Brown KK, Routes JM, Tuder RM, et al. (2003) Expression of human herpesvirus 8 in primary pulmonary hypertension. *N Engl J Med* **349:**1113–1122.

Couty JP, Geras-Raaka E, Weksler BB, and Gershengorn MC (2001) Kaposi's sarcoma-associated herpesvirus G protein-coupled receptor signals through multiple pathways in endothelial cells. *J Biol Chem* **276:**33805–33811.

Cox DA and Cohen ML (1995) 5-Hydroxytryptamine $_{2B}$ receptor signaling in rat stomach fundus: role of voltage-dependent calcium channels, intracellular calcium release and protein kinase C. *J Pharmacol Exp Ther* **172:**143–150.

Ellis ES, Byrne C, Murphy OE, Tilford NS, and Baxter GS (1995) Mediation by 5-hydroxytryptamine$_{2B}$ receptors of endothelium-dependent relaxation in rat jugular vein. *Br J Pharmacol* **114:**400–404.

Fagan KA, Oka M, Bauer NR, Gebb SA, Ivy DD, Morris KG, and McMurtry IF (2004) Attenuation of acute hypoxic pulmonary vasoconstriction and hypoxic pulmonary hypertension in mice by inhibition of Rho-kinase. *Am J Physiol* **287:**L656–L664.

Glusa E and Pertz HH (2000) Further evidence that 5-HT-induced relaxation of pig pulmonary artery is mediated by endothelial 5-HT_{2B} receptors. *Br J Pharmacol* **130:**692–698.

Gray JA, Compton-Toth BA, and Roth BL (2003) Identification of two serine residues essential for agonist-induced 5-HT2A receptor desensitization. *Biochemistry* **42:**10853–10862.

Kim SJ, Veenstra-VanderWeele J, Hanna GL, Gonen D, Leventhal BL, and Cook EH Jr (2000) Mutation screening of human 5-HT_{2B} receptor gene in early-onset obsessive-compulsive disorder. *Mol Cell Probes* **14:**47–52.

Kitamura K, Singer WD, Star RA, Muallem S, and Miller RT (1996) Induction of inducible nitric-oxide synthase by the heterotrimeric G protein Gα13. *J Biol Chem* **271:**7412–7415.

Launay J-M, Birraux G, Bondoux D, Callebert J, Choi D-S, Loric S, and Maroteaux L (1996) Ras involvement in signal transduction by the serotonin 5-HT_{2B} receptor. *J Biol Chem* **271:**3141–3147.

Launay JM, Hervé P, Peoc'h K, Tournois C, Callebert J, Nebigil C, Etienne N, Drouet L, Humbert M, Simonneau G, et al. (2002) Function of the serotonin 5-hydroxytryptamine $_{2B}$ receptor in pulmonary hypertension. *Nat Med* **8:**1129–1135.

Lefkowitz RJ and Whalen EJ (2004) beta-arrestins: traffic cops of cell signaling. *Curr Opin Cell Biol* **16:**162–168.

Liu B and Wu D (2003) The first inner loop of endothelin receptor type B is necessary for specific coupling to Gα 13. *J Biol Chem* **278:**2384–2387.

Manivet P, Mouillet-Richard S, Callebert J, Nebigil CG, Maroteaux L, Hosoda S, Kellermann O, and Launay J-M (2000) PDZ-dependent activation of nitric-oxide synthases by the serotonin $_{2B}$ receptor. *J Biol Chem* **275:**9324–9331.

Miller WE and Lefkowitz RJ (2001) Expanding roles for beta-arrestins as scaffolds and adapters in GPCR signaling and trafficking. *Curr Opin Cell Biol* **13:**139–145.

Nagao M, Kaziro Y, and Itoh H (1999) The Src family tyrosine kinase is involved in Rho-dependent activation of c-Jun N-terminal kinase by Galpha12. *Oncogene* **18:**4425–4434.

Nebigil CG, Etienne N, Messaddeq N, and Maroteaux L (2003) Serotonin is a novel survival factor of cardiomyocytes: mitochondria as a target of 5-HT_{2B}-receptor signaling. *FASEB J* **17:**1373–1375.

Nebigil CG, Launay J-M, Hickel P, Tournois C, and Maroteaux L (2000) 5-Hydroxytryptamine $_{2B}$ receptor regulates cell-cycle progression: cross talk with tyrosine kinase pathways. *Proc Natl Acad Sci USA* **97:**2591–2596.

Parker LL, Backstrom JR, Sanders-Bush E, and Shieh BH (2003) Agonist-induced phosphorylation of the serotonin 5-HT2C receptor regulates its interaction with multiple PDZ protein 1. *J Biol Chem* **278:**21576–21583.

Porter RH, Malcolm CS, Allen NH, Lamb H, Revell DF, and Sheardown MJ (2001) Agonist-induced functional desensitization of recombinant human 5-HT2 receptors expressed in CHO-K1 cells. *Biochem Pharmacol* **62:**431–438.

Roth BL and Shapiro DA (2001) Insights into the structure and function of 5-HT(2) family serotonin receptors reveal novel strategies for therapeutic target development. *Expert Opin Ther Targets* **5:**685–695.

Sakmar TP, Menon ST, Marin EP, and Awad ES (2002) Rhodopsin: insights from recent structural studies. *Annu Rev Biophys Biomol Struct* **31:**443–484.

Shepard LW, Yang M, Xie P, Browning DD, Voyno-Yasenetskaya T, Kozasa T, and Ye RD (2001) Constitutive activation of NF-κB and secretion of interleukin-8

induced by the G protein-coupled receptor of Kaposi's sarcoma-associated herpesvirus involve Gα_{13} and RhoA. *J Biol Chem* **276:**45979–45987.
Spiegel AM and Weinstein LS (2004) Inherited diseases involving G proteins and G protein-coupled receptors. *Annu Rev Med* **55:**27–39.
Tournois C, Mutel V, Manivet P, Launay JM, and Kellermann O (1998) Cross-talk between 5-hydroxytryptamine receptors in a serotonergic cell line. Involvement of arachidonic acid metabolism. *J Biol Chem* **273:**17498–17503.
Ullmer C, Boddeke HGWM, Schmuck K, and Lübbert H (1996) 5-HT$_{2B}$ receptor-mediated calcium release from ryanodine-sensitive intracellular stores in human pulmonary artery endothelial cells. *Br J Pharmacol* **117:**1081–1088.
Ullmer C, Schmuck K, Figge A, and Lubbert H (1998) Cloning and characterization of MUPP1, a novel PDZ domain protein. *FEBS Lett* **424:**63–68.
Xia Z, Gray JA, Compton-Toth BA, and Roth BL (2003) A direct interaction of PSD-95 with 5-HT2A serotonin receptors regulates signal transduction and receptor trafficking. *J Biol Chem* **278:**21901–21908.

Address correspondence to: Dr. Luc Maroteaux, INSERM U616 Hospital Pitié-Salpetrière, Batiment Pédiatrie, 47 bd de l'Hôpital, 75651 Paris Cedex 13, France. E-mail: luc.maroteaux@chups.jussieu.fr