Molecular Basis of Receptor/G Protein Coupling Selectivity Studied by Coexpression of Wild Type and Mutant m2 Muscarinic Receptors with Mutant $G\alpha_q$ Subunits†

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ABSTRACT: The molecular basis of receptor/G protein coupling selectivity was studied by using the m2 muscarinic receptor, a prototypical $G_{i/o}$-coupled receptor as a model system. We could recently show that the m2 receptor can efficiently interact with mutant G protein α_q subunits in which the last five amino acids were replaced with α_{i2} or α_o sequence [Liu, J., Conklin, B. R., Blin, N., Yun, J., & Wess, J. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 11642−11646]. Additional mutagenesis studies led to the identification of a four-amino-acid motif on the m2 receptor (Val_{385}, Thr_{386}, Ile_{389}, and Leu_{390}) that is predicted to functionally interact with the C-terminal portion of $\alpha_{i/o}$ subunits. To further investigate the structural requirements for this interaction to occur, these four m2 receptor residues were replaced, either individually or in combination, with the corresponding residues present in the $G_{q/11}$-coupled muscarinic receptors (m1, m3, and m5). The ability of the resulting mutant m2 receptors to interact with a mutant α_q subunit (qo5) in which the last five amino acids were replaced with α_o sequence was investigated in co-transfected COS-7 cells [studied biochemical response: stimulation of phosphatidyl inositol (PI) hydrolysis]. Our data suggest that the presence of three of the four targeted m2 receptor residues (Val_{385}, Thr_{386}, and Ile_{389}) is essential for efficient recognition of C-terminal $\alpha_{i/o}$ sequences. To study which specific amino acids within the C-terminal segment of $\alpha_{i/o}$ subunits are critical for this interaction to occur, the wild type m2 receptor was co-expressed with a series of mutant α_q subunits containing single or multiple $\alpha_q \rightarrow \alpha_{i1,2}$ point mutations at their C-terminus. Remarkably, the wild type m2 receptor, while unable to efficiently stimulate wild type α_q, gained the ability to productively interact with three α_q single-point mutants, providing the first example that the receptor coupling selectivity of G protein α subunits can be switched by single amino acid substitutions. Given the high degree of structural homology among different G protein-coupled receptors and among different classes of G protein α subunits, our results should be of broad general relevance.

G protein-coupled receptors are integral plasma membrane proteins that transmit extracellular signals into the cell interior. Upon binding of extracellular ligands, these receptors are predicted to undergo conformational changes that allow the intracellular receptor surface to interact with specific classes of heterotrimeric G proteins (Dohlman *et al*., 1991; Savarese & Fraser, 1992; Hedin *et al*., 1993; Strader *et al*., 1994). Characteristically, each member of this receptor superfamily can recognize and activate only a limited set of the many structurally closely related G proteins expressed within a cell (Dohlman *et al*., 1991; Savarese & Fraser, 1992; Hedin *et al*., 1993). To understand how this selectivity is achieved at a molecular level has become the focus of an ever increasing number of laboratories.

For many years, we have used different members of the muscarinic acetylcholine receptor family (m1−m5) as model systems to analyze the molecular mechanisms underlying receptor/G protein coupling selectivity (Wess, 1996). Whereas the m2 and m4 receptor subtypes are selectively linked to G proteins of the $G_{i/o}$ class (primary biochemical response: inhibition of adenylyl cyclase), the m1, m3, and m5 receptors are preferentially coupled to G proteins of the $G_{q/11}$ family [primary biochemical response: stimulation of phosphatidylinositol (PI)[1] hydrolysis via activation of phospholipase Cβ (PLCβ)] (Peralta *et al*., 1988; Parker *et al*., 1991; Berstein *et al*., 1992; Offermanns *et al*., 1994).

Systematic site-directed mutagenesis studies have shown that the G protein coupling preference of a given muscarinic receptor subtype is determined by a limited number of amino acids located on different intracellular receptor domains including the i2 loop and the N- and C-terminal portions of the i3 domain (Blin *et al*., 1995; Wess, 1996). These findings

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[1] Abbreviations: i1-i3, the three intracellular loops in G protein-coupled receptors; IP_1, inositol monophosphate; NMS, *N*-methylscopolamine; PCR, polymerase chain reaction; PI, phosphatidylinositol; PLC, phospholipase C; TM VI, the sixth transmembrane domain in G protein-coupled receptors.

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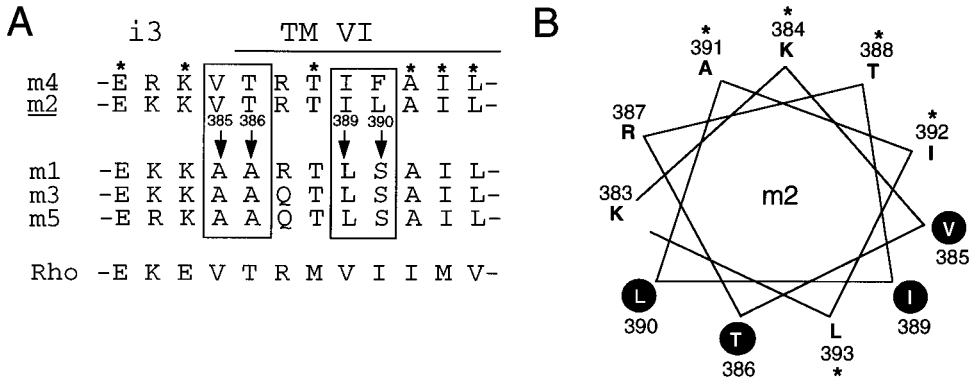

FIGURE 1: (A) Comparison of m1−m5 muscarinic receptor sequences at the i3 loop/TM VI junction. Mutant m2 receptors were created by replacing the highlighted m2 receptor residues (arrows) with the corresponding m1 (= m3, m5) receptor residues, either individually or in combination. Numbers refer to amino acid positions in the human m2 muscarinic receptor (Bonner *et al*., 1987). Asterisks indicate positions where all muscarinic receptor subtypes have identical residues. For comparison (see Discussion), the corresponding rhodopsin (Rho) sequence is also shown. The single-letter amino acid code is used. (B) Helical wheel representation of the m2 receptor sequence at the i3 loop/TM VI junction, as viewed from the cytoplasm. The residues predicted to form the functionally important VTIL motif (Liu *et al*., 1995, 1996) are highlighted.

are consistent with results from many different laboratories studying other classes of G protein-coupled receptors by molecular genetic or biochemical approaches (Dohlman *et al*., 1991; Savarese & Fraser, 1992; Hedin *et al*., 1993; Gudermann *et al*., 1996).

To gain deeper insight into the molecular basis governing receptor/G protein coupling selectivity and receptor-mediated G protein activation, the sites on the G protein(s) that are contacted by the receptor regions known to be critical for coupling selectivity need to be identified. Unfortunately, high-resolution structural data of receptor/G protein complexes are not available at present. However, molecular genetic and biochemical strategies have been employed to map functionally important G protein regions predicted to be located at the receptor/G protein interface (Conklin & Bourne, 1993; Rens-Domiano & Hamm, 1995; Neer, 1995).

Using the m2 muscarinic receptor [m2(wt)], a prototypical $G_{i/o}$-coupled receptor, as a model system, we (Liu *et al*., 1995) have recently identified the receptor region that can functionally interact with the C-terminus of G protein α subunits, a G protein site that has been implicated most consistently in receptor/G protein coupling (Conklin & Bourne, 1993; Spiegel, 1994; Rens-Domiano & Hamm, 1995). We could demonstrate that m2(wt), similar to other $G_{i/o}$-coupled receptors (Conklin *et al*., 1993), does not efficiently interact with wild type α_q [q(wt)], but can productively couple to mutant α_q subunits in which the last five amino acids of q(wt) are replaced with the corresponding α_i or α_o sequences (resulting in qi5 and qo5, respectively) (Liu *et al*., 1995).

Moreover, by analyzing a large number of mutant m2 receptors, we could show that the ability of m2(wt) to interact with such hybrid α_q subunits can be specifically disrupted by replacing a set of four amino acids (VTIL: Val_{385}, Thr_{386}, Ile_{389}, and Leu_{390}), predicted to be located at the i3 loop/TM VI junction, with the corresponding residues (AALS) conserved among the three $G_{q/11}$-coupled muscarinic receptors (Liu *et al*., 1995). In addition, gain-of-function studies showed that substitution of the VTIL motif into mutant m3 muscarinic receptors [which were unable to couple to q(wt)] could confer onto these receptors the ability to efficiently couple to mutant α_q subunits such as qo5 or qi5 (Liu *et al*., 1995). The most straightforward explanation for these findings is that the VTIL motif (which is predicted to form the hydrophobic face of an α-helical, amphiphilic receptor region; Figure 1B) can contact the C-terminus of $\alpha_{i/o}$ subunits and that this interaction is intimately involved in determining coupling selectivity and triggering G protein activation.

On the basis of these results, the present study was designed to gain deeper insight into the structural requirements necessary for this interaction to occur. First, we wanted to examine which specific residues within the VTIL motif are most critical for proper recognition of the C-terminus of $\alpha_{i/o}$ subunits. Towards this goal, single or multiple point mutations were introduced into m2(wt) replacing these amino acids with the residues present at the corresponding positions in the $G_{q/11}$-coupled muscarinic receptors (AALS; Figure 1A). The ability of the resultant mutant receptors to functionally interact with a mutant α_q subunit (qo5) in which the last five amino acids of α_q were replaced with the corresponding α_o sequence was determined in co-transfected COS-7 cells. In an analogous fashion, to study the relative importance of residues at the C-terminus of $\alpha_{i/o}$ for receptor/G protein coupling selectivity, coexpression experiments were carried out with the wild type m2 muscarinic receptor and mutant α_q subunits in which the C-terminal five amino acids of q(wt) were systematically modified by substitution with the corresponding $\alpha_{i1,2}$ residues.

Our data indicate that the structural integrity of Val_{385}, Thr_{386}, and Ile_{389} is essential for efficient recognition of the C-terminus of $\alpha_{i/o}$ subunits by m2(wt). Moreover, we made the surprising observation that the receptor coupling selectivity of α_q can be changed by single amino acid substitutions at the C-terminus.

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis

To introduce mutations into the human m2 muscarinic receptor gene (at a region corresponding to the i3 loop/TM VI junction), the mammalian expression plasmid, Hm2pcD (Bonner *et al*., 1987), was used. A 118-bp *Sma*I-*Dra*III restriction fragment was cut out from Hm2pcD and replaced, in a two-piece ligation, with a PCR fragment containing the desired mutations (Higuchi, 1989). The construction of expression plasmids encoding the mutant m3 muscarinic

Table 1: Radioligand Binding and Functional Properties of Mutant m2 Muscarinic Receptors Coexpressed with the Mutant G Protein α_q Subunit, qo5[a]

		[^{3}H]NMS binding		carbachol binding	PI hydrolysis	
receptor	co-expressed G protein α subunit	K_D (pM)	B_{max} (fmol/mg)	IC_{50} (μM)	EC_{50} carbachol (μM)	maximum increase in IP_1 above basal (fold)
m2(wt)	qo5	232 ± 34	1032 ± 240	10.6 ± 1.1	0.39 ± 0.07	4.7 ± 0.4
m2 (wt)	q(wt)				nd[b]	2.5 ± 0.1
m2(wt)	− (vector DNA [pcDNAI])				nd[b]	1.8 ± 0.2
m2(VTIL→AALS)	qo5	642 ± 23	1166 ± 204	32.6 ± 1.2	nd[b]	2.0 ± 0.2
m2(V→A)	qo5	428 ± 5	1316 ± 214	18.7 ± 3.2	6.3 ± 2.5	3.4 ± 0.3
m2(T→A)	qo5	425 ± 6	1098 ± 194	5.7 ± 0.9	0.45 ± 0.15	3.4 ± 0.2
m2(I→L)	qo5	535 ± 35	1293 ± 223	66.1 ± 4.4	29.1 ± 17.8	4.1 ± 0.6
m2(L→S)	qo5	704 ± 16	1260 ± 189	10.9 ± 2.2	0.37 ± 0.14	4.9 ± 0.3
m2(VT→AA)	qo5	422 ± 9	1293 ± 200	3.8 ± 0.2	nd[b]	1.3 ± 0.1
m2(IL→LS)	qo5	471 ± 49	1076 ± 160	47.8 ± 0.5	8.9 ± 3.6	4.9 ± 0.7
m2(VI→AL)	qo5	403 ± 27	1135 ± 198	589 ± 105	nd[b]	2.1 ± 0.2
m2(TL→AS)	qo5	658 ± 43	997 ± 148	2.9 ± 0.3	0.03 ± 0.01	4.2 ± 0.3

[a] COS-7 cells were co-transfected with expression plasmids coding for the wild type or mutant m2 muscarinic receptors and the mutant α_q subunit, qo5, in which the last five amino acids of q(wt) were replaced with the corresponding α_o sequence (Figure 4). The structure of the different mutant receptors is given in Figure 1. Radioligand binding studies and functional PI assays were carried out as described under Experimental Procedures. Data are given as means ± SE of at least three independent experiments, each carried out in duplicate. [b] Not determinable with sufficient accuracy.

receptors (rat), CR2 and CR15, has been described previously (Liu *et al*., 1995). In CR2, a sequence coding for the N-terminal portion of the i3 loop of the rat m3 muscarinic receptor (residues 252−387; Bonner *et al*., 1987) was replaced with the corresponding human m2 receptor sequence (residues 208−287). CR15 was derived from CR2 by introducing four additional point mutations at the i3 loop/TM VI junction (*m*3Ala$_{488}$Ala$_{489}$, Leu$_{492}$Ser$_{493}$ → *m2*Val$_{385}$-Thr$_{386}$, Ile$_{389}$Leu$_{390}$).

To introduce mutations into the C-terminal domain of wild type α_q, a pcDNAI-based expression plasmid (coding for murine α_q) containing an internal hemagglutinin (HA) epitope tag (DVPDYA) was used (Wedegaertner *et al*., 1993). The presence of the epitope tag which replaced α_q residues 125−130 did not affect the receptor and effector coupling properties of wild type α_q (Wedegaertner *et al*., 1993; Conklin *et al*., 1993). PCR-based mutagenesis was carried out essentially as described previously by Conklin *et al*. (1993). The pCIS-based expression plasmid coding for wild type α_{15} (mouse) has been described previously (Offermanns & Simon, 1995).

The identity of all mutant constructs and the correctness of all PCR-derived sequences were verified by dideoxy sequencing of the mutant plasmids (Sanger *et al*., 1977).

Transfection and Cell Culture

COS-7 cells were grown in Dulbecco's modified Eagle's medium, supplemented with 10% fetal calf serum, at 37 °C in a humidified 5% CO_2 incubator. For transfections, 1 × 10^6 cells were seeded into 100-mm dishes. About 24 h later, COS-7 cells were co-transfected with expression plasmids coding for the wild type or mutant m2 muscarinic receptors (4 μg of DNA/dish) and the different G protein constructs (1 μg of DNA/dish), by using a DEAE/dextran procedure (Cullen, 1987).

Radioligand Binding Assays

Radioligand binding assays were carried out with membrane homogenates prepared from transfected COS-7 cells essentially as described (Dörje *et al*., 1991). Incubation buffer consisted of 25 mM sodium phosphate (pH 7.4) containing 5 mM $MgCl_2$. In the [^{3}H]-*N*-methylscopolamine ([^{3}H]NMS; 79.5 Ci/mmol; Dupont-New England Nuclear) saturation binding experiments, six different concentrations of the radioligand were used (25−800 pM). In carbachol competition binding studies, ten different carbachol concentrations were used. The [^{3}H]NMS concentration employed in these experiments was 200 pM. Incubations were carried out for 3 h at 22 °C. Nonspecific binding was determined in the presence of 1 μM atropine. Protein concentrations were determined by the method of Bradford (1976).

Binding data were analyzed by nonlinear least squares curve-fitting procedures, using the computer programs LIGAND (saturation binding data; Munson & Rodbard, 1980) or KALEIDAGRAPH (competition binding data; Synergy Software).

PI Hydrolysis

Approximately 24 h after transfections, cells were split into six-well dishes (ca. 0.4 × 10^6 cells/well) in culture medium supplemented with 3 μCi/mL [^{3}H]*myo*-inositol (20 Ci/mmol; American Radiolabeled Chemicals Inc.). After a 24-h labelling period, cells were preincubated for 20 min at room temperature in 2 mL of Hank's balanced salt solution containing 20 mM HEPES and 10 mM LiCl. Cells were then stimulated, in the same buffer, with increasing concentrations of the muscarinic agonist, carbachol, for 1 h at 37 °C. After removal of the medium, the reaction was stopped by addition of 0.75 mL of 20 mM formic acid. Cell extracts were collected after a 40 min incubation period at 4 °C and neutralized with 0.25 mL of 60 mM ammonium hydroxide. The inositol monophosphate (IP_1) fraction was then isolated by anion exchange chromatography as described (Berridge *et al*., 1983).

RESULTS

Functional Analysis of Mutant m2 Muscarinic Receptors

The role of the VTIL motif in the m2 muscarinic receptor (corresponding to Val$_{385}$, Thr$_{386}$, Ile$_{389}$, and Leu$_{390}$; Figure

Table 2: Functional Interaction of the Wild Type m2 Muscarinic Receptor with C-Terminally Modified Mutant α_q Subunits[a]

G protein α subunit co-expressed with the wild type m2 receptor	PI hydrolysis	
	maximum increase in IP_1 levels (fold above basal)	EC_{50} (carbachol) (μM)
vector (pcDNAI)	1.8 ± 0.2	nd[b]
q(wt)	2.5 ± 0.1	nd[b]
q(EYNLV→DCGLF) (= qi5)	8.2 ± 1.9	0.29 ± 0.11
q(YNLV→CGLF) (= qi4)	9.2 ± 1.5	0.20 ± 0.06
q(Y→C)	6.3 ± 0.9	0.55 ± 0.19
q(N→G)	6.2 ± 1.1	0.39 ± 0.18
q(V→F)	3.6 ± 0.1	1.29 ± 0.11
q(YN→CG)	6.6 ± 1.5	0.14 ± 0.06
q(YV→CF)	9.5 ± 2.2	0.28 ± 0.13
q(NV→GF)	8.2 ± 1.0	0.40 ± 0.03

[a] COS-7 cells were cotransfected with expression plasmids coding for the wild type m2 muscarinic receptor and various mutant α_q subunits in which distinct amino acids present at the C-terminus of q(wt) were replaced with the corresponding $\alpha_{i1,2}$ residues (Figure 4). Carbachol-induced increases in IP_1 production were determined as described under Experimental Procedures. Data are given as means ± SE of three independent experiments, each carried out in triplicate. [b] Not determinable with sufficient accuracy.

1) in the recognition of the C-terminus of G protein $\alpha_{i/o}$ subunits was explored by systematic site-directed mutagenesis studies. Specifically, these four residues were replaced, either individually or in combination, with the corresponding residues (AALS) present in the $G_{q/11}$-coupled muscarinic receptors (m1, m3, and m5; Figure 1A, Table 1). The resulting mutant m2 receptors were functionally characterized in COS-7 cells after coexpression with different wild type and mutant G protein α subunits (see below). [^{3}H]NMS radioligand binding studies showed that all mutant receptors were expressed at levels comparable to those found with m2-(wt) (approximately 1000 fmol/mg; Table 1).

Consistent with the known G protein coupling preference of m2(wt), carbachol stimulation of m2(wt), when co-transfected with either vector DNA (control) or q(wt), did not result in an efficient stimulation of the PI cascade. The maximum stimulation in inositol phosphate production (above basal levels) mediated by m2(wt), in the absence or presence of co-transfected q(wt), amounted to only 1.5−2.5-fold (Tables 1, 2). Previous studies have shown that this (rather weak) response is due to activation of distinct PLCβ isoforms by G protein $\beta\gamma$ complexes that are released by m2 receptor-mediated activation of G_i proteins (Katz *et al*., 1992). However, coexpression of m2(wt) with a mutant α_q subunit in which the last five amino acids (EYNLV) were replaced with the corresponding α_o sequence (GCGLY; resulting in the hybrid α subunit, qo5) led to a pronounced stimulation (5-fold) of PLC activity (Tables 1; Figure 2). In agreement with published data (Liu *et al*., 1995), replacement of the VTIL motif in m2(wt) with the AALS sequence (Figure 1) resulted in a mutant receptor [m2(VTIL→AALS)] that virtually lost the ability to interact with qo5 (Table 1, Figure 2).

Initially, single point mutations were introduced into the VTIL motif in m2(wt) (substitution with the corresponding residues present in the $G_{q/11}$-coupled muscarinic receptors; Figure 1A), and the resulting m2 mutant receptors were studied for their ability to functionally interact with q(wt) or qo5. Similar to m2(wt), none of the resulting m2 receptor single point mutants was able to productively interact with q(wt) (maximum PLC stimulation: 1.5−2.5-fold; data not shown). Strikingly, three of the studied mutant receptors [m2(V→A), m2(T→A), and m2(I→L)] were clearly less efficient than m2(wt) in activating the hybrid α subunit, qo5 (Table 1, Figure 2A). The m2(V→A) and m2(T→A) mutant receptors showed a considerably reduced maximum PLC response, while m2(I→L), similar to m2(V→A), showed a drastic reduction (approximately 75-fold) in carbachol potency in inducing qo5-mediated PI hydrolysis. However, since m2(I→L) also displayed an about 6-fold reduction in carbachol binding affinity (as determined in [^{3}H]NMS competition binding studies; Table 1), the loss in functional carbachol potency observed with this mutant receptor appears to be at least partially due to impaired receptor binding. In contrast to m2(V→A), m2(T→A), and m2(I→L), the m2-(L→S) mutant receptor displayed a functional profile that was virtually identical to that of m2(wt) (Table 1, Figure 2A).

To examine whether the detrimental functional effects caused by single point mutations within the VTIL motif were additive, four m2 receptor double point mutants were constructed (Table 1). As shown in Figure 2B and Table 1, the m2(VT→AA) and m2(VI→AL) mutant receptors virtually completely lost their ability to functionally interact with qo5. On the other hand, m2(IL→LS) and m2(TL→AS) showed unchanged or slightly reduced maximum PLC responses, respectively [as compared to m2(wt)]. As shown in Table 1, m2(IL→LS) showed a 25-fold reduction in carbachol potency in mediating qo5-dependent PLC activation. The functional carbachol potency observed with m2-(TL→AS) was approximately 10-fold increased [as compared with m2(wt)] which, however, may be due, at least partially, to the increase in carbachol binding affinity seen with this mutant receptor (Table 1).

Co-Expression of Mutant m2 Muscarinic Receptors with α_{15}

As outlined in the previous paragraph, several mutant m2 receptors were identified that were functionally severely impaired or unable to activate the hybrid α subunit, qo5. To exclude the possibility that the lack of functional activity found with these mutant receptors was due to improper folding of the intracellular receptor surface (as opposed to the disruption of specific receptor/G protein contact sites), all mutant m2 receptors [as well as m2(wt)] were also coexpressed with (mouse) α_{15} (Wilkie *et al*., 1991). It has been shown that α_{15} (as well as the human homolog, α_{16}) can be activated by almost all G protein-coupled receptors including m2(wt) (Offermanns & Simon, 1995), resulting in the breakdown of PI lipids mediated by $\alpha_{15/16}$-dependent activation of distinct PLCβ isoforms (Lee *et al*., 1992). PI assays showed that all mutant m2 receptors [except m2-(VI→AL)] were able to productively interact with coexpressed α_{15}, in a fashion similar to m2(wt) (Figure 3).

Functional Interaction of the Wild Type m2 Muscarinic Receptor with Mutant α_q Subunits

As outlined in the introduction, the second major aim of this study was to examine which specific amino acids within the C-terminal segment of $\alpha_{i/o}$ subunits are critical for the predicted interaction with the VTIL motif in m2(wt).

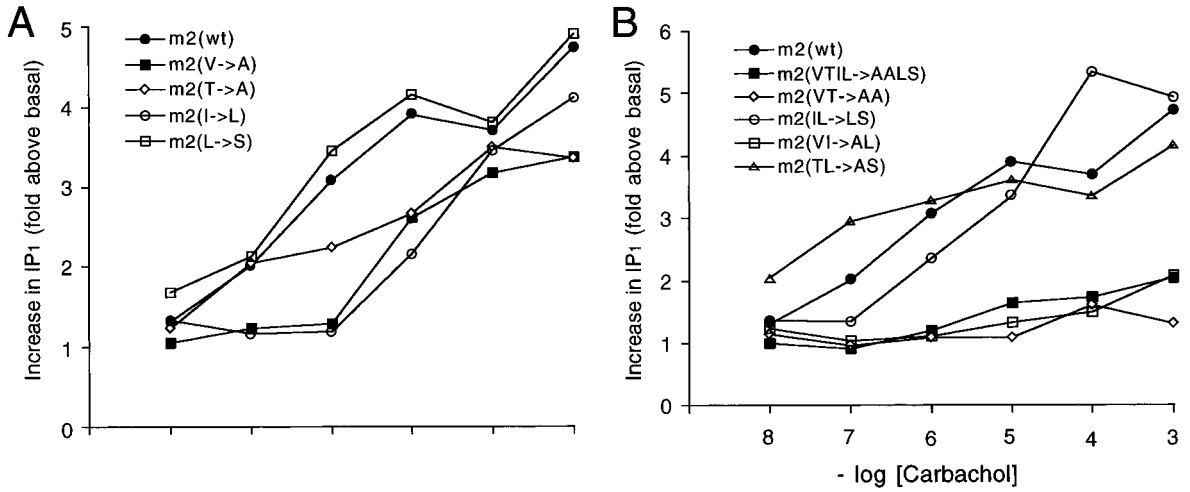

FIGURE 2: Functional interaction of mutant m2 muscarinic receptors with the mutant α_q subunit, qo5. COS-7 cells were co-transfected with expression plasmids coding for the wild type or mutant m2 muscarinic receptors containing single (A) or multiple (B) point mutations with the mutant α_q subunit, qo5, in which the last five amino acids of q(wt) were replaced with the corresponding α_o sequence (Figure 4). The structure of the different mutant receptors in which distinct residues at the i3 loop/TM VI junction were replaced with the corresponding m1 (= m3, m5) receptor residues, is shown in Figure 1. Carbachol-induced increases in intracellular inositol monophosphate (IP_1) levels were determined as described under Experimental Procedures. Basal IP_1 levels (no carbachol added) were similar for the wild type m2 and the different mutant receptors (data not shown). Data are given as mean values (SE typically less than 10%) from three to five independent experiments.

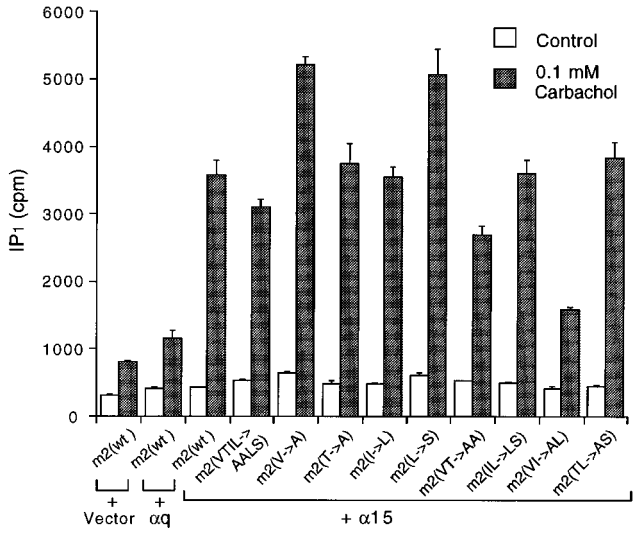

FIGURE 3: Functional interaction of mutant m2 muscarinic receptors with G_{15}. COS-7 cells were co-transfected with α_{15} (Wilkie *et al*., 1991) or vector DNA (pcDNAI) and the indicated receptor constructs. Carbachol-induced (0.1 mM) increases in intracellular IP_1 levels were determined as described under Experimental Procedures. Data are given as means ± SE and are representative of two independent experiments, each carried out in triplicate.

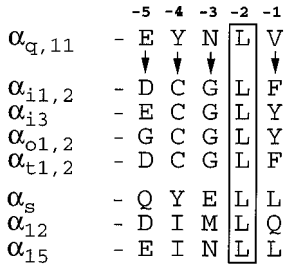

FIGURE 4: Comparison of the C-terminal five amino acids of selected G protein α subunits. Mutant α_q subunits were created by replacing the highlighted α_q residues (arrows), either individually or in combination, with the corresponding $\alpha_{i1,2}$ residues. The single-letter amino acid code is used. The boxed leucine residue (position −2) is conserved among all known mammalian α subunits.

Toward this goal, specific residues at the C-terminus of q(wt) were replaced, either alone or in combination, with the corresponding $\alpha_{i1,2}$ residues (Figure 4). Western analysis using a monoclonal antibody directed against the HA-epitope tag present in all G protein constructs (Wedegaertner *et al*., 1993) showed that all mutant α_q subunits were expressed at similar levels as q(wt) (data not shown).

Consistent with its known G protein coupling preference, m2(wt) could productively interact not only with qo5 but also with qi5, a mutant α_q subunit in which the last five amino acids of q(wt) (EYNLV) were replaced with the corresponding $\alpha_{i1,2}$ sequence (DCGLF) (Liu *et al*., 1995; Table 2). As shown in Table 2 and Figure 5, the mutant α_q subunit, qi4, in which only the last four amino acids of q(wt) were replaced with the corresponding $\alpha_{i1,2}$ sequence, could be activated by m2(wt) in a fashion very similar to qi5. Since the C-terminal four amino acids of α_q differ in only three residues from the corresponding $\alpha_{i1,2}$ sequence (the leucine residue at position −2 is conserved among all mammalian α subunits), three α_q single point mutants were prepared [q(Y→C), q(N→G), and q(V→F); Figure 4]. Interestingly, PI assays showed that either of these three mutant α_q subunits gained the ability to be activated by m2(wt), although with different efficiencies. As shown in Figure 5 and Table 2, q(Y→C) and q(N→G) were able to mediate a rather robust (6-fold) increase in PLC activity (as compared to an 8−9-fold increase observed with qi4 or qi5). Co-expression of q(V→F) with m2(wt) resulted in a relatively modest second messenger response (3−4-fold increase in PLC activity), which, however, was always significantly greater than that seen after coexpression of q(wt) with m2(wt) (Figure 5, Table 2).

To examine whether the functional effects caused by these single amino acid substitutions were additive, three α_q double point mutants [q(YN→CG), q(YV→CF), and q(NV→GF)] were constructed and functionally analyzed. When co-expressed with m2(wt), q(YV→CF) and q(NV→GF) were able to activate the PI cascade in a fashion very similar to qi4 or qi5 (Table 2), indicating that m2(wt) was able to interact with these two α_q double point mutants more

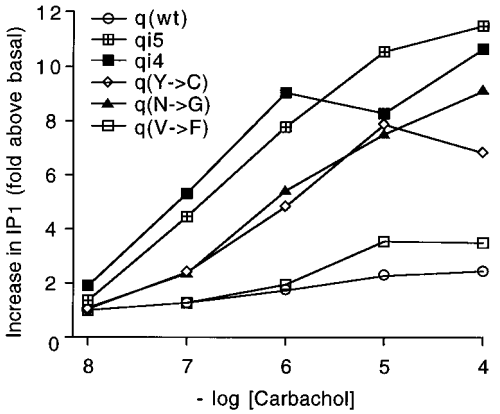

FIGURE 5: Functional interaction of the wild type m2 muscarinic receptor with C-terminally modified mutant α_q subunits. COS-7 cells were co-transfected with expression plasmids coding for the wild type m2 muscarinic receptor and the indicated G protein constructs. The structure of the different α_q mutants, in which one or more amino acids at the C-terminus of q(wt) were replaced with the corresponding $\alpha_{i1,2}$ residues, is shown in Figure 4. In qi4 and qi5, the last four or five amino acids of q(wt), respectively, were replaced with the corresponding $\alpha_{i1,2}$ sequence. Carbachol-induced increases in intracellular IP_1 levels were determined as described under Experimental Procedures. Basal IP_1 levels (no carbachol added) were similar for the different wild type and mutant α_q constructs (data not shown). Results (mean values) from a representative experiment, carried out in triplicate, are shown; two additional experiments gave similar results.

efficiently than with either of the corresponding α_q single point mutants [q(Y→C), q(N→G), or q(V→F)]. The q(YN→CG) mutant subunit (upon co-expression with m2-(wt) and incubation with carbachol) could stimulate PLC activity to a maximum extent similar to that found with q(Y→C) and q(N→G), but with significantly increased (3−4-fold) carbachol potency (Table 2).

Functional Interaction of a Mutant m3 Muscarinic Receptor Containing the VTIL Motif with Mutant α_q Subunits

We have shown previously (Liu *et al*., 1995) that replacement of the N-terminal portion of the i3 loop of the rat m3 muscarinic receptor (residues 252−387) with the corresponding m2 receptor sequence yields a hybrid receptor (referred to as CR2) that does not couple to either q(wt) or mutant α_q subunits such as qi5 or qo5. However, consistent with the proposed role of the VTIL motif in recognition of the C-terminus of $\alpha_{i/o}$ subunits, substitution of the VTIL sequence into CR2 resulted in a mutant receptor (CR15; Figure 6A) that gained the ability to couple to qo5 and qi5 [but not to q(wt); Liu *et al*., 1995]. If our assumption that the VTIL motif directly contacts the C-terminus of $\alpha_{i,o}$ subunits is correct, one would expect that CR15 should be able to interact with the different mutant α_q subunits described in the previous paragraph in a fashion analogous to m2(wt). Figure 6B shows that this is in fact the case. The overall activity profile found upon co-expression of this hybrid receptor with the different mutant α_q subunits qualitatively resembled that obtained for m2(wt). However, maximum PI responses mediated by CR15 were generally smaller than the corresponding m2(wt) effects, and in two cases [q(Y→C) and q(V→F)] no significant increase in PLC activity was observed [as compared with the responses seen after co-expression of CR15 with vector DNA or q(wt)]. One possible reason for the reduced coupling efficiencies observed

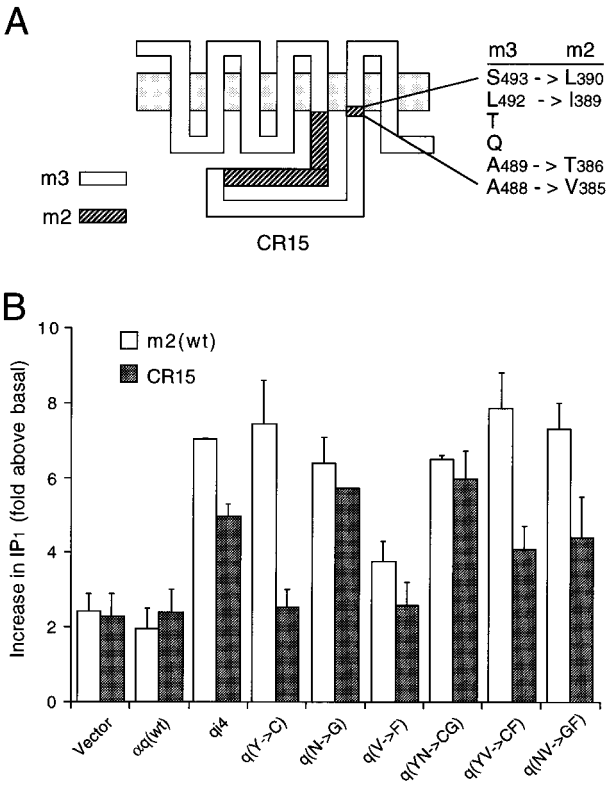

FIGURE 6: Functional interaction of a hybrid m2/m3 muscarinic receptor (CR15) with C-terminally modified mutant α_q subunits. For comparison, responses obtained with the wild type m2 receptor are also shown. (A) Structure of CR15 (for precise amino acid composition, see Experimental Procedures). (B) COS-7 cells were co-transfected with expression plasmids coding for CR15 or the wild type m2 muscarinic receptor and the indicated mutant α_q constructs. The structure of the different mutant α_q subunits, in which one or more amino acids at the C-terminus of q(wt) were replaced with the corresponding $\alpha_{i1,2}$ residues, is given in Figure 4. Carbachol-induced (0.1 mM) increases in intracellular IP_1 levels were determined as described under Experimental Procedures. Basal IP_1 levels (no carbachol added) were similar in all cases (data not shown). Data are given as means ± SD of two independent experiments, each carried out in triplicate.

with CR15 is that this mutant receptor was expressed at approximately 5-fold lower levels (B_{max}) than m2(wt) (as determined in [^{3}H]NMS saturation binding studies; data not shown). Another possibility is that CR15, due to its chimeric structure, may not be folded in a fashion that is optimal for interactions with G proteins.

DISCUSSION

Structural Analysis of the VTIL Motif

Previous mutagenesis studies (Liu *et al*., 1995, 1996) strongly suggest that a short sequence motif in the m2 muscarinic receptor (VTIL; corresponding to Val_{385}, Thr_{386}, Ile_{389}, and Leu_{390}), located at the i3 loop/TM VI junction, can functionally interact with the C-terminal segment of $\alpha_{i/o}$ subunits. To examine which specific amino acids within the VTIL motif in m2(wt) are of particular importance for recognition of the C-terminus of $\alpha_{i/o}$ subunits, each of these four m2 receptor residues was replaced, either individually or in combination, with the corresponding residues present in the $G_{q/11}$-coupled muscarinic receptors (AALS). We found that three of the four m2 receptor single point mutants examined [m2(V→A), m2(T→A), and m2(I→L)] were clearly less efficient than m2(wt) in mediating qo5-dependent

PLC activation (loss in efficacy and/or carbachol potency). However, we could demonstrate that these three mutant receptors (similar to most other receptor constructs examined in this study; Figure 3) retained the ability to productively couple to G_{15} (upon co-expression with α_{15}), a G protein known to be activated by most G protein-coupled receptors including m2(wt) (Offermanns & Simon, 1995). This observation strongly suggests that the inability of m2(V→A), m2(T→A), and m2(I→L) to efficiently interact with qo5 is not caused by a generalized misfolding of the intracellular receptor surface, but is most likely due to the disruption of specific receptor/G protein contact sites.

On the other hand, the m2(L→S) point mutant was able to couple to qo5 in a fashion virtually identical to that found with m2(wt) (Table 1), suggesting that Leu_{390} does not play a critical role in the recognition of the C-terminus of $\alpha_{i/o}$ subunits. This notion is also consistent with the observation that Leu_{390} is replaced with a different residue (phenylalanine) in the m4 muscarinic receptor (Figure 1A) which is known to display the same G protein coupling preference as m2-(wt) (Peralta *et al*., 1988; Wess, 1996).

The results obtained with a series of m2 receptor double point mutants (VT→AA, VI→AL, IL→LS, and TL→AS) generally support the conclusions drawn based on the functional analysis of the single point mutants. For example, two of these mutant receptors [m2(VT→AA) and m2-(VI→Al)] almost completely lost the ability to functionally interact with the mutant α_q subunit, qo5 (Figure 2B), consistent with the key role of the targeted residues (Val_{385}, Thr_{386}, and Ile_{389}) in recognition of the C-terminus of $\alpha_{i/o}$ subunits. It is therefore likely that each of these residues is engaged in specific interactions with the C-terminus of $\alpha_{i/o}$ subunits, and that each of these contact sites contributes to the specificity and efficiency of receptor/G protein coupling.

Secondary structure prediction algorithms (Strader *et al*., 1989) as well as mutagenesis studies (Berstein *et al*., 1995; Blin *et al*., 1995) suggest that the receptor region at the i3 loop/TM VI junction is α-helically arranged. If this is correct, Val_{385}, Thr_{386}, and Ile_{389} are predicted to form a contiguous surface located on one side of an amphiphilic α-helix (Figure 1B). It should be noted that the corresponding region at the i3 loop/TM VI junction in the photoreceptor, rhodopsin, shares a high degree of sequence identity with the corresponding m2 (m4) muscarinic receptor sequence (Figure 1A). For example, Val_{250} and Thr_{251} in (bovine) rhodopsin directly correspond to Val_{385} and Thr_{386} in the m2 receptor (Figure 1A). Interestingly, a site-directed spin labeling study (in which individual residues at the i3 loop/TM VI junction in rhodopsin were replaced with cysteine residues, followed by covalent modification of the cysteine residues with a spin marker) recently provided more direct structural evidence that this region (that includes Val_{250} and Thr_{251}) does in fact adopt an α-helical secondary structure (Altenbach *et al*., 1996). Given the high degree of sequence identity between rhodopsin and the m2 (m4) muscarinic receptors within this region, it is highly likely that the model depicted in Figure 1B is correct.

We could recently show that the insertion of one or more extra alanine residues immediately C-terminal of Val_{385} in m2(wt) leads to mutant m2 receptors that can interact with the proper G proteins even in the absence of activating ligands (Liu *et al*., 1996). On the other hand, deletion of the residue (Ala_{391}) C-terminal of Val_{385} in m2(wt) completely abolished m2 receptor activity (in the absence or presence of agonist ligands; Liu *et al*., 1996). These data, taken together with the results of the present study, are consistent with a model of receptor activation that involves a ligand-induced rotational and/or translational movement of TM VI, thus exposing the critical, previously inaccessible residues, Val_{385}, Thr_{386}, and Ile_{389}, for interactions with the C-terminus of G protein $\alpha_{i/o}$ subunits.

Baldwin (1993) recently proposed a model of the packing arrangement of the seven transmembrane helices present in all G protein-coupled receptors that is compatible with the results of a vast number of mutagenesis studies as well as with a low-resolution electron density map of rhodopsin (Schertler *et al*., 1993). Based on this model, Val_{385}, Thr_{386}, and Ile_{389} are predicted to project into the interior of the helical bundle where they may be engaged in tertiary interactions with residues located on other transmembrane helices. Interestingly, spin labeling studies have shown that several residues at the i3 loop/TM VI junction in bovine rhodopsin (particularly Val_{250} and Thr_{251}) undergo large increases in mobility upon photoexcitation (Altenbach *et al*., 1996). In addition, studies with mutant rhodopsin molecules modified with two different spin markers (Farrens *et al*., 1996) recently showed that this mobility increase is most likely due to a ligand-induced rotational movement of TM VI (accompanied by an "outward" movement of TM VI, as has also been observed for bacteriorhodopsin; Subramaniam *et al*., 1993). These findings, therefore, provide direct experimental support for our previously proposed model (Liu *et al*., 1995, 1996) in which ligand binding to the m2 receptor protein is predicted to lead to a rotational and/or translational movement of TM VI, thus enabling the C-terminus of $\alpha_{i/o}$ subunits to interact with the functionally critical residues (Val_{385}, Thr_{386}, and Ile_{389}) aligned along the inner face of the i3 loop/TMVI junction.

Structural Modification of the C-terminus of α_q

The second major goal of the present study was to investigate which structural elements (amino acids) within the C-terminal portion of $\alpha_{i/o}$ subunits are critical for the functional interaction with the VTIL motif in m2(wt). Co-expression experiments showed that m2(wt) could interact with qi4 [a mutant α_q subunit in which the last four amino acids of q(wt) (YNLV) were replaced with the corresponding $\alpha_{i1,2}$ sequence (CGLF)] with the same high efficiency as observed with qi5 (Figure 5). This observation indicates that the presence of the aspartate residue at position −5 in $\alpha_{i1,2}$ is not critical for m2(wt)-mediated activation of $\alpha_{i1,2}$ subunits. This notion is also supported by the observation that this aspartate residue is replaced with glutamate and glycine in α_{i3} and $\alpha_{o1,2}$, respectively (Figure 4).

Since the C-terminal four amino acids of α_q differ in only three residues from the corresponding $\alpha_{i1,2}$ sequence (the leucine residue at position −2 is conserved among all mammalian α subunits; Figure 4), three α_q single point mutants [q(Y→C), q(N→G), and q(V→F)] were prepared and tested for their ability to functionally interact with co-expressed m2(wt). We made the surprising observation that either of these three mutant α_q subunits could be activated by m2(wt), although with different efficiencies. Whereas co-expression of m2(wt) with q(V→F) resulted in a modest but reproducible increase in PLC activity [3−4-fold as

compared to a 2.5-fold increase observed with q(wt)], coexpression with q(Y→C) or q(N→G) led to a more robust activation of the PI cascade (6−7-fold increase in maximum PLC activity). The involvement of the C-terminal phenylalanine residue (position −1) of $\alpha_{i1,2}$ in determining receptor coupling specificity is also highlighted by the observation that m2(wt) was able to interact with the q(YV→CF) and q(NV→GF) double point mutants with significantly greater efficiency than with the corresponding single point mutants, q(Y→C) and q(N→G), respectively, which display a valine residue at their extreme C-terminus (Table 2). Consistent with these results, the C-terminal phenylalanine (or tyrosine) residue is highly conserved only within the $\alpha_{i/o}$ protein family (Figure 4). The ability of q(V→F) to functionally interact with $G_{i/o}$-coupled receptors remained undetected in previous studies using A1 adenosine and D2 dopamine receptors as potential coupling partners (Conklin *et al*., 1993, 1996). Since the mutant G protein was coexpressed with multiple receptor DNAs in these experiments (which were carried out in HEK-293 cells), one likely reason for this observed discrepancy is the increased sensitivity of the COS-7 cell expression system used in the present study where only one receptor DNA [m2(wt)] is co-transfected with the different α subunits.

As shown in Figure 4, the last five amino acids of α_t (α subunit of transducin), another member of the $\alpha_{i/o}$ protein family, are identical with the corresponding $\alpha_{i1,2}$ sequence. Consistent with the results of our gain-of-function mutagenesis study, loss-of-function mutagenesis experiments (Garcia *et al*., 1995; Osawa & Weiss, 1995) as well as biochemical studies with short peptides derived from the C-terminal portion of α_t (Dratz *et al*., 1993; Martin *et al*., 1996) suggest that the cysteine residue at −4 position (which is the site of pertussis toxin-mediated ADP-ribosylation; West *et al*., 1985) and the glycine residue at the −3 position are required for efficient stimulation of transducin by light-activated rhodopsin. Interestingly, NMR studies on a C-terminal α_t peptide suggest that the last four amino acids of $\alpha_{i/o}$ subunits form a type II′ β-turn (which depends on the presence of the conserved glycine residue at the −3 position and cannot exist in the presence of any other natural amino acid; Figure 4) which is broken upon interaction with the ligand-occupied receptor (Dratz *et al*., 1993). However, our observation that the q(Y→C) and q(YV→CF) mutant α_q subunits (which do not contain this glycine residue) can be efficiently activated by m2(wt) suggests that a glycine residue at the −3 position (and, therefore, a type II′ β-turn; Dratz *et al*., 1993) is not absolutely essential for receptor-mediated activation of $\alpha_{i/o}$ subunits.

The ability of three different α_q point mutants to productively interact with m2(wt) may be indicative of the fact that each of the three introduced $\alpha_{i1,2}$ residues can participate in specific interactions with amino acids located at the i3 loop/TM VI junction in $G_{i/o}$-coupled receptors [such as Val_{385}, Thr_{386}, and Ile_{389} in m2(wt)]. Consistent with this notion, we could demonstrate that substitution of the VTIL motif into a mutant m3 muscarinic receptor unable to couple to q(wt) yielded a hybrid receptor (CR15; Figure 6A) that gained the ability to efficiently couple to most mutant α_q subunits investigated in this study displaying an activity pattern that was qualitatively similar to that observed with m2(wt) (Figure 6B).

CONCLUSIONS

In conclusion, the present study provides novel insight into the molecular basis of receptor/G protein coupling selectivity. Most remarkably, our data provide the first example that the coupling specificity of G protein α subunits can be changed by single amino acid substitutions. The approach described here (involving the co-expression of mutant G protein-coupled receptors with mutant G protein α subunits) should be generally applicable to identify other major, functionally relevant receptor/G protein contact sites.

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REFERENCES

Altenbach, C., Yang, K., Farrens, D. L., Khorana, H. G., & Hubbell, W. L. (1996) *Biochemistry 35*, 12470−12478.
Baldwin, J. M. (1993) *EMBO J. 12*, 1693−1703.
Berridge, M. J., Dawson, M. C., Downs, C. P., Heslop, J. P., & Irvine, R. F. (1983) *Biochem. J. 212*, 473−482.
Berstein, G., Blank, J. L., Smrcka, A. V., Higashijima, T., Sternweis, P. C., Exton, J. H., & Ross, E. M. (1992) *J. Biol. Chem. 267*, 8081−8088.
Blin, N., Yun, J., & Wess, J. (1995) *J. Biol. Chem. 270*, 17741−17748.
Bonner, T. I., Buckley, N. J., Young, A. C., & Brann, M. R. (1987) *Science 237*, 527−532.
Bradford, M. M. (1976) *Anal. Biochem. 72*, 248−254.
Burstein, E. S., Spalding, T. A., Hill-Eubanks, D., & Brann, M. R. (1995) *J. Biol. Chem. 270*, 3141−3146.
Conklin, B. R., & Bourme, H. R. (1993) *Cell 73*, 631−641.
Conklin, B. R., Farfel, Z., Lustig, K. D., Julius, D., & Bourne, H. R. (1993) *Nature 363*, 274−276.
Conklin, B. R., Herzmark, P., Ishida, S., Voyno-Yasenetskaya, T. A., Sun, Y., Farfel, Z., & Bourne, H. R. (1996) *Mol. Pharmacol. 50*, 885−890.
Cullen, B. R. (1987) *Methods Enzymol. 152*, 684−704.
Dohlman, H. G., Thorner, J., Caron, M. G., & Lefkowitz, R. J. (1991) *Annu. Rev. Biochem. 60*, 653−688.
Dörje, F., Wess, J., Lambrecht, G., Tacke, R., Mutschler, E., & Brann, M. R. (1991) *J. Pharmacol. Exp. Ther. 256*, 727−733.
Dratz, E. A., Furstenau, J. E., Lambert, C. G., Thireault, D. L., Rarick, H., Schepers, T. Pakhlevaniants, S., & Hamm, H. E. (1993) *Nature 363*, 276−281.
Farrens, D. L., Altenbach, C., Yang, K., Hubbell, W. L., & Khoranta H. G. (1996) *Science 274*, 768−770.
Garcia, P. D., Onrust, R., Bell, S. M., Sakmar, T. P., & Bourne, H. R. (1995) *EMBO J. 14*, 4460−4469.
Gudermann, T., Kalkbrenner, F., & Schultz, G. (1996) *Annu. Rev. Pharmacol. Toxicol. 36*, 429−459.
Hedin, K. E., Duerson, K., & Clapham, D. E. (1993) *Cell. Signalling 5*, 505−518.
Higuchi, R. (1989) in *PCR Technology* (Erlich, H. A., Ed.) pp 61−70, Stockton Press, New York.
Lee, C. H., Park, D., Wu, D., Rhee, S. G., & Simon, M. I. (1992) *J. Biol. Chem. 267*, 16044−16047.
Liu, J., Conklin, B. R., Blin, N., Yun, J., & Wess, J. (1995) *Proc. Natl. Acad. Sci. U.S.A. 92*, 11642−11646.
Liu, J., Blin, N., Conklin, B. R., & Wess, J. (1996) *J. Biol. Chem. 271*, 6172−6178.
Martin, E. L., Rens-Domiano, S., Schatz, P. J., & Hamm, H. (1996) *J. Biol. Chem. 271*, 361−366.
Munson, P. J., & Rodbard, D. (1980) *Anal. Biochem. 107*, 220−239.
Neer, E. J. (1995) *Cell 80*, 249−257.
Offermanns, S., & Simon, M. I. (1995) *J. Biol. Chem. 270*, 15175−15180.

Offermanns, S., Wieland, T., Homann, D., Sandmann, J., Bombien, E., Spicher, K., Schultz, G., & Jakobs, K. H. (1994) *Mol. Pharmacol. 45*, 890−898.
Osawa, S., & Weiss, E. R. (1995) *J. Biol. Chem. 270*, 31052−31058.
Parker, E. M., Kameyama, K., Higashijima, T., & Ross, E. M. (1991) *J. Biol. Chem. 266*, 519−527.
Peralta, E. G., Ashkenazi, A., Winslow, J. W., Ramachandran, J., & Capon, D. J. (1988) *Nature 334*, 434−437.
Rens-Domiano, S., & Hamm, H. (1995) *FASEB J. 9*, 1059−1066.
Sanger, F., Nicklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
Savarese, T. M., & Fraser, C. M. (1992) *Biochem. J. 283*, 1−19.
Spiegel, A. M. (1994) in *G Proteins* (Spiegel, A. M., Jones, T. L. Z., Simonds, W. F., & Weinstein, L. S., Eds.) pp 25−31, R. G. Landes Company, Austin, TX.
Strader, C. D., Sigal, I. S., & Dixon, R. A. F. (1989) *FASEB J. 3*, 1825−1832.
Strader, C. D., Fong, T. M., Tota, M. R., Underwood, D., & Dixon, R. A. F. (1994) *Annu. Rev. Biochem. 63*, 101−132.
Subramaniam, S., Gerstein, M., Oesterhelt, D., & Henderson, R. (1993) *EMBO J.* 12, 1−8.
Wedegaertner, P. B., Chu, D. H., Wilson, P. T., Levis, M. J., & Bourne, H. R. (1993) *J. Biol. Chem. 268*, 25001−25008.
Wess, J. (1996) *Crit. Rev. Neurobiol. 10*, 69−99.
West, R. E., Moss, J., Vaughan, M., Liu, T., & Liu, T. Y. (1985) *J. Biol. Chem. 260*, 14428−14430.
Wilkie, T. M., Scherle, P. A., Strathmann, M. P., Slepak, V. Z., & Simon, M. I. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 10049−10053.

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