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A Conserved Threonine Residue in the Second Intracellular Loop of the 5-Hydroxytryptamine 1A Receptor Directs Signaling Specificity

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SUMMARY

Productive interaction between receptors and G proteins involves multiple intracellular receptor domains, but the role of individual receptor amino acids in directing the selection of specific signaling pathways has not yet been identified. Sequence alignment of several G protein-coupled receptors identified a highly conserved threonine residue in the i2 loop of the 5-hydroxytryptamine 1A (5-HT_{1A}) receptor that is a putative protein kinase C phosphorylation consensus site and is located in a predicted amphipathic α-helical domain. To examine the role of this conserved threonine residue in 5-HT_{1A} receptor coupling to G_i/G_o proteins, this residue was mutated to alanine (T149A mutant). Wild-type and mutant 5-HT_{1A} receptors were stably transfected into both Ltk^- and GH4C1 cells to investigate receptor coupling to multiple signaling pathways. In both cell lines, the T149A mutant displayed similar agonist affinities as the wild-type receptor. In Ltk^- cells, the T149A 5-HT_{1A} receptor inhibited cAMP accumulation by 30% compared with wild-type (83%). A 2.6-fold increase in intracellular calcium (due to phospholipase C-mediated calcium mobilization) was observed for the wild-type receptor upon the addition of 100 nM 5-HT; whereas the T149A 5-HT_{1A} receptor failed to mediate a calcium mobilization response at equivalent receptor levels to wild-type. When transfected in GH4C1 cells, the T149A receptor mutant fully inhibited basal cAMP and partially inhibited G_s-stimulated cAMP accumulation compared with wild-type receptor (57 $\pm$ 14% versus 86 $\pm$ 2%). In contrast, the T149A 5-HT_{1A} receptor mutant failed to block the influx of calcium induced by calcium channel agonist ($\pm$)-Bay K8644, whereas the wild-type 5-HT_{1A} receptor inhibited the calcium influx by 40%. Thus, the Thr149 residue is directly involved in G protein coupling to calcium mobilization (mediated by $\beta\gamma$ subunits of G_{i2}) and to inhibition of calcium channel activation (mediated by $\beta\gamma$ subunits of G_o) but plays a minor role in coupling to α_i-mediated inhibition of cAMP accumulation. The conserved i2 loop threonine may serve as a G protein contact site to direct the signaling specificity of multiple receptors.

A large variety of neurotransmitters, neuropeptides, and autocrine and paracrine factors mediate their biological actions by activation of receptors that are coupled to heterotrimeric G proteins. These receptors have primary sequences that are consistent with a secondary structure composed of seven conserved α-helical transmembrane domains, three intracellular loops, and an intracellular carboxyl-terminal domain. Receptor mutational studies (1–3) and experiments using receptor antibodies (4) or short synthetic peptides that inhibit or mimic receptor interactions with various G proteins (5–10) have identified the i2 loop, the amino- and carboxyl-terminal domains of the i3 loop, and the membrane-proximal portion of the carboxyl-terminal tail as receptor domains that participate in receptor/G protein interactions. However, the role of individual amino acids in determining the selectivity of receptor-mediated signals has not been addressed.

The 5-HT_{1A} receptor is a member of a family of receptors that couple to PTX-sensitive G proteins (G_i/G_o) to initiate inhibitory or stimulatory signal transduction pathways, depending on the cell type in which the receptor is expressed (11, 12). When transfected in GH4C1 pituitary cells, which have characteristics of neuronal cells such as voltage-gated ion channels and regulated secretion of hormones, the 5-HT_{1A} receptor displays an inhibitory signaling phenotype characteristic of receptors endogenously expressed in neurons (13, 14). On activation, the 5-HT_{1A} receptor reduces both basal cAMP and Gs-stimulated cAMP accumulation and inhibits Bay K8644-induced influx of Ca^{2+} to decrease $[Ca^{2+}]_i$.

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; DPAT, 8-hydroxy-(2-(*N*,*N*-di-[2,3,^{3}H]propylamino)-1,2,3,4-tetrahydronaphthalene; AM, acetoxymethyl ester; PI, phosphatidyl inositol; PKC, protein kinase C; PLC, phospholipase C; PTX, pertussis toxin; VIP, vasoactive intestinal peptide; $[Ca^{2+}]_i$, intracellular Ca^{2+} concentration; i2, second intracellular; i3, third intracellular.

These changes are associated with inhibition of secretion and inhibition of cell proliferation. However, when expressed in a variety of fibroblast-derived cells, such as Ltk$^-$, Hela, or Balb/c-3T3, the 5-HT$_{1A}$ receptor enhances PI turnover, releasing intracellular Ca^{2+} stores to increase $[Ca^{2+}]_i$. In fibroblast cells, the 5-HT$_{1A}$ receptor does not alter the basal level of cAMP but inhibits both forskolin- and G_s-stimulated cAMP accumulation (15–17). These responses are associated with increased DNA synthesis and ultimately with oncogenic transformation. Each response mediated by the 5-HT$_{1A}$ receptor is blocked by pretreatment with PTX, indicating the involvement of G_i/G_o proteins. In addition to 5-HT$_{1A}$ receptors, other receptors that couple to G_i/G_o, such as the 5-HT$_{1B}$ and dopamine D_2 (long and short variant) receptors, reproduce this cell-specific pattern of signaling (i.e., inhibitory in pituitary cells versus stimulatory in fibroblast cells) (12, 18). However, the precise amino acids of the receptor that determine coupling to these pathways remain to be elucidated.

The β_2-adrenergic receptor provides the most complete model of structure-function relationships for receptor/G protein interaction. Chimeric and site-directed mutagenesis studies have shown unequivocally that the carboxyl-terminal and i3 loop domains of the β_2-adrenergic receptor are essential for coupling to G_s (1–3), but the role of the i2 loop was unclear. Recent molecular studies focusing on the i2 loop of several G_q-coupled receptors have revealed the importance of this loop in coupling to PLC-linked pathways (19–21). Mutation of the Leu131 residue to alanine in the i2 loop of the human m1 muscarinic receptor decreased PI turnover; mutation of the corresponding Leu174 residue in the human m3 muscarinic receptor had the same effect (19). Mutations in the i2 loop of the Angiotensin II receptor type 1 abolished Angiotensin II-induced stimulation of inositol trisphosphate (20). Furthermore, residues 525–527 and 528–532 of the i2 loop of the TSH receptor were shown to be essential for agonist-induced cAMP and phosphatidylinositol trisphosphate signaling, respectively (21). These observations indicate that the i2 loop of G_q-coupled receptors is critical for activation of PLC.

Receptor mutagenesis studies have also indicated a potential role for the i2 loop in coupling to certain G_i-linked pathways. A chimeric receptor in which the i3 loop of the G_i-coupled muscarinic m2 receptor was replaced with the i3 loop of the β_2-adrenergic receptor coupled to both G_s and G_i. Hence, the ability to stimulate G_i did not reside solely in the i3 loop. In the same report, a nine-amino acid peptide representing the carboxyl-terminal sequence of the i2 loop stimulated high affinity GTPase activity and inhibited forskolin-stimulated adenylyl cyclase in membranes (22). Moreover, synthetic peptides corresponding to the entire i2 loop of the 5-HT$_{1A}$ receptor strongly inhibited forskolin-stimulated adenylyl cyclase activity (23). On the basis of the above results suggesting a role for i2 domains in G_i-mediated signaling, we examined the possibility that part of the i2 loop of the 5-HT$_{1A}$ receptor might be involved in G_i/G_o coupling to its effectors. We therefore mutated to alanine a threonine residue located in the i2 loop of the 5-HT$_{1A}$ receptor that forms part of a consensus sequence that is conserved in multiple $G_i/G_o/G_q$-coupled receptors. This site also forms part of a putative PKC consensus phosphorylation site. The wild-type and mutant 5-HT$_{1A}$ receptors were transfected in Ltk$^-$ fibroblasts and GH4C1 pituitary cells to investigate their potential role in receptor G_i/G_o protein coupling to cell-specific effectors.

Experimental Procedures

Materials. Restriction endonucleases and other molecular biology reagents were purchased from Boehringer-Mannheim Biochemicals (Indianapolis, IN) and GIBCO-BRL (Gaithersburg, MD). Sequenase was from United States Biochemical (Cleveland, OH). Forskolin, 3-isobutyl-1-methylxanthine, 5-HT, and VIP were from Sigma Chemical (St. Louis, MO). [^{3}H]DPAT (228 Ci/mmol) and [α-^{32}P]ATP (2200 Ci/mmol) were obtained from Amersham (Arlington Heights, IL). Geneticin was purchased from GIBCO, and Fura-2/AM was from Molecular Probes (Eugene, OR).

Cell culture. Ltk$^-$ cells were grown as a monolayer in α-minimum essential medium, and GH4C1 cells were grown in F-10 medium supplemented with 8% fetal bovine serum at 37° in a humidified atmosphere with 5% carbon dioxide. Media were changed 12–24 hr before experimentation.

Construction and expression of 5-HT$_{1A}$ receptor mutants. The *Bam*HI/*Xba*I fragment of the rat 5-HT$_{1A}$ receptor gene in the pZEM-3 vector (15) (containing the mouse metallothionein promoter) was subcloned into p-Select to use as a template for site-directed mutagenesis (Altered-Sites Mutagenesis, Promega, Madison, WI). The putative PKC site in the second loop was mutated at T149 to an alanine using an oligonucleotide (AACAAAAGG*G*AGCCCCGGC) incorporating the point mutation. The mutation was confirmed by DNA sequencing. Mutated and wild-type 5-HT$_{1A}$ receptor cDNAs were subcloned into the eukaryotic expression vector pcDNA I (InVitrogen, San Diego, CA) and cotransfected with pSV-Neo in Ltk$^-$ cells and GH4C1 cells using Ca^{2+} phosphate coprecipitation (14). Neomycin-resistant cells expressing 5-HT$_{1A}$ receptors were selected and grown in α-minimum essential medium or F-10 medium supplemented with 10% fetal calf serum and 700 μg/ml geneticin. Clones were screened by Northern blot analysis.

Transient transfection. To incorporate the T149A mutation into a more convenient vector for transient transfection, the 1.6-kb *Bst*XI/*Xba*I of the T149A mutant was subcloned into *Bst*XI/*Xba*I-cut pcDNA3 (InVitrogen)-DBX (containing the 1.9-kb *Bam*HI/*Xba*I fragment of wild-type 5-HT$_{1A}$ receptor) and verified by DNA sequencing. Ltk$^-$ cells (10^{-7}/15-cm dish) in 12 ml of α-minimum essential medium plus 1% serum were transfected with 15 μg of DNA in the presence of 200 μg/ml DEAE-dextran (molecular mass, 500,000 Da) for 4 hr at 37° in 5% CO_2. Cells were then treated with 12 ml of phosphate-buffered saline plus 10% dimethylsulfoxide for 1 min. After washing with 12 ml of phosphate-buffered saline, the cells were cultured for 2 days in growth medium before Ca^{2+} and binding assays were performed, as described below.

Ligand binding. Cell membranes were prepared from 10- or 15-cm dishes by replacing the growth medium with ice-cold hypotonic buffer (15 mM Tris, pH 7.4, 2.5 mM $MgCl_2$, 0.2 mM EDTA). After swelling for 10–15 min at 4°, the cells were scraped from the plates, sonicated on ice, centrifuged (20,000 × *g* for 10 min), and resuspended in ice-cold TME buffer (75 mM Tris, pH 7.4, 12.5 mM $MgCl_2$, 1 mM EDTA). Aliquots of thawed and sonicated membrane preparation were added to tubes containing 200 μl of TME, [^{3}H]DPAT, and indicated drugs. 5-HT (10 μM) was used to define nonspecific binding, which was <10% of total binding at concentrations of radioligand near the K_d value. Incubations with six to eight different concentrations of [^{3}H]DPAT (in triplicate) were initiated by the addition of 100 μg of membrane protein, carried out at room temperature for 30 min and stopped by filtration through GF/C (Whatman) filters and immediately washed with three washes of 4 ml of ice-cold buffer (50 mM Tris, pH 7.4). Radioactivity retained on the filter was dissolved in 5 ml of HiSafe3 (Wallac, Gaithersburg, MD) and quantified by liquid scintillation counting. For binding assays of membranes prepared from transient transfections, a saturating concentration of [^{3}H]DPAT (20 nM) was used. Protein was assayed with the BioRad (Her-

cules, CA) protein assay kit using bovine serum albumin as a standard.

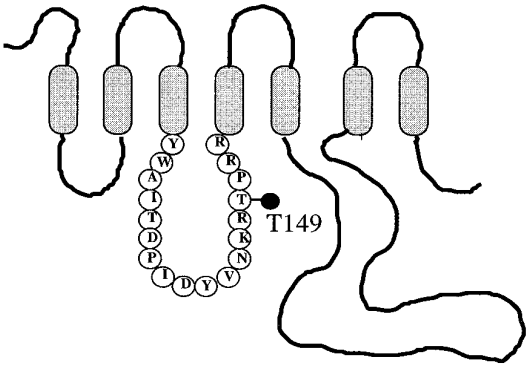
cAMP assay. Measurement of cAMP was performed as previously described (11). Briefly, cells plated onto six-well 35-mm dishes were washed twice with 1 ml of HBBS/Ca^{2+} (118 mM NaCl, 4 mM KCl, 1 mM $CaCl_2$, 10 mM D-glucose, 20 mM HEPES, pH 7.2) containing 100 μM 3-isobutyl-1-methylxanthine and resuspended with 1 ml of buffer containing various test compounds for test incubation of 20 min at 37°. The buffer was collected and stored at −20° until assayed for cAMP by a specific radioimmunoassay (ICN Biomedicals, Cleveland, OH). Standard curves displayed average IC_{50} values of 0.5 ± 0.2 pmol using cAMP as standard. Data for cAMP assays are presented as mean ± standard error for triplicate wells.

Intracellular Ca^{2+} measurement. As described previously (11), cells were harvested by incubation in HBBS plus 5 mM EDTA and 0.05% trypsin (for Ltk^- cells) or HBBS plus EDTA (5 mM) (for GH4C1 cells) and incubated with Fura-2 for 20–30 min at 37°. The cells were centrifuged, washed twice with HBBS/Ca^{2+}, and placed in a fluorescence cuvette. Change in fluorescence ratio was recorded on a Perkin-Elmer Cetus (Buckinghamshire, UK) LS-50 spectrofluorometer and analyzed by computer, based on a K_d value of 227 nM for the Fura-2/Ca^{2+} complex. Calibration of R_{max} was performed by the addition of 0.1% Triton X-100 and 20 mM Tris base and of R_{min} by the addition of 10 mM EGTA. All experimental compounds were added directly to the cuvette from 200-fold concentrated solutions.

Results

For a more complete understanding of the molecular mechanisms governing the specificity of receptor/G protein coupling, the specific amino acids that are required for interactions between the receptor and G proteins must be identified. Although chimeric approaches provide insight into the function of amino acids that diverge among different receptors, we have chosen the point mutagenesis approach to address the roles of single conserved amino acids in receptor function (24). In particular, examination of the molecular structure of the 5-HT_{1A} receptor revealed a potential PKC phosphorylation site located at T149 in the i2 loop (Fig. 1A). An alignment of peptide sequences corresponding to this region in other G protein-coupled receptors revealed a striking conservation of the threonine residue. Furthermore, in receptors that couple to G_i/G_o proteins, a BB**T**XBB (X = P/T/S, B = basic residue) consensus PKC phosphorylation sequence is well conserved. The related AA**T**XBB (A = aliphatic residue) sequence was identified in several G_s- and G_q-coupled receptors. Based on the potential role of the i2 loop in receptor signaling, we addressed the role of this conserved threonine residue in 5-HT_{1A} receptor function. The 5-HT_{1A} receptor mutant

A)



B)

$G_{\alpha i}$	r5-HT1A	Y	V	N	K	R	**T**	P	R	R
	m5-HT1B	Y	S	A	K	R	**T**	P	K	R
	h5-HT1D	Y	S	K	R	R	**T**	A	G	H
	rM2	Y	P	V	K	R	**T**	T	K	M
	hM4	Y	P	A	R	R	**T**	T	K	M
	hD2	Y	N	T	R	Y	**S**	S	K	R
	hα-2A	Y	N	L	K	R	**T**	P	R	R
	hα-2B	Y	N	L	K	R	**T**	P	R	R
	rCannabinoid	Y	K	R	I	V	**T**	R	P	K
$G_{\alpha s}$	hβ1	Y	Q	S	L	L	**T**	R	A	R
	rβ2	Y	Q	S	L	L	**T**	K	N	K
	hD1	Y	E	R	M	M	**T**	P	K	A
	rD5	Y	K	R	K	M	**T**	Q	M	
$G_{\alpha q}$	hM1	Y	R	A	K	R	**T**	P	R	R
	rM3	Y	R	A	K	R	**T**	T	K	R
	hM5	Y	R	A	K	R	**T**	P	K	R
	hmα-1	Y	P	T	L	V	**T**	R	R	K
	mTRH A		Q	F	L	C	**T**	F	S	R
	bAngiotensin II	S	R	L	R	R	**T**	M	L	

Fig. 1. A conserved threonine consensus sequence in the i2 loop of the 5-HT_{1A} receptor. A, 5-HT_{1A} receptor i2 loop. The proposed structure of the rat 5-HT_{1A} receptor used in this study consists of seven hydrophobic transmembrane domains forming three intracellular loops and a short cytoplasmic carboxy-terminal tail. ●, Location of the putative PKC consensus site in the second loop. B, A conserved threonine in G protein-coupled receptors. Amino acid sequence alignment of an i2 loop domain of selected receptors that have a positionally conserved threonine or serine (*boxed*) in the i2 loop flanked by basic amino acid residues (*outlined*). *r*, rat; *m*, mouse; *h*, human; *hm*, hamster; *b*, bovine.

T149A was generated, eliminating the hydroxyl side chain that may serve as a contact point for G proteins or as a phosphate acceptor site for PKC (Fig. 1A). The wild-type and mutant receptors were stably transfected in receptor-negative Ltk$^-$ cells and GH4C1 cells to investigate multiple pathways of signal transduction and G protein coupling.

Ligand binding. Membranes prepared from positive clones were subjected to saturation isotherm binding analyses using [^{3}H]DPAT, a selective 5-HT$_{1A}$ agonist (15). The affinity values (K_d) calculated for the mutant receptors were in the nanomolar range (Table 1) in both Ltk$^-$ and GH4C1 cell lines and similar to the affinity of the wild-type receptor. Thus, the mutation did not greatly alter agonist affinity, which is consistent with earlier reports that mutations in the cytoplasmic portions of G protein-coupled receptors have a minor influence on ligand binding (1–3). In Ltk$^-$ cells, two independent transfections yielded clones with lower receptor levels than wild-type. We included a previously characterized T343A (i3 loop) 5-HT$_{1A}$ receptor mutant with a more similar receptor level for functional comparisons with the T149A receptor in these cells (24). The level of 5-HT$_{1A}$ mutant receptor expression in GH4C1 clones examined was higher than that in GH4ZD10 cells expressing wild-type receptor (2.71 versus 1.10 pmol/mg of protein, respectively).

Receptor coupling to the adenylyl cyclase pathway. The wild-type 5-HT$_{1A}$ receptor couples negatively to the adenylyl cyclase effector system. We compared the ability of wild-type and mutant 5-HT$_{1A}$ receptors to inhibit adenylyl cyclase by measuring cAMP accumulation in transfected clones in the absence and presence of stimulators of adenylyl cyclase: forskolin in Ltk$^-$ cells and VIP in GH4C1 cells. When expressed in Ltk$^-$ cells, neither of the T149A mutant clones, the T343A mutant, nor the wild-type receptor significantly inhibited basal (without forskolin) cAMP level. The wild-type 5-HT$_{1A}$ receptor inhibited the forskolin-stimulated cAMP level by >80%, whereas the T343A clone with fewer receptors inhibited by 65%, which is lower than but not significantly different from wild-type receptor. Both of the T149A mutants in Ltk$^-$ cells reduced forskolin-stimulated cAMP accumulation by 30%, which is significantly less pronounced ($p < 0.001$) compared with wild-type receptor (Fig. 2A and Table 2). This reduction in T149A receptor efficacy may have been due in part to the lower levels of receptor compared with wild-type (Table 1). However, other threonine mutants (e.g., the T343A mutant) retained receptor efficacy when expressed at similar levels (24), suggesting a partial impairment of T149A 5-HT$_{1A}$ receptors in coupling to inhibition of forskolin-stimulated cAMP accumulation in these cells. When expressed in GH4C1 cells, the T149A mutant inhibited basal cAMP levels to the same extent as the wild-type receptor (38 ± 8% versus 33 ± 3%). The wild-type receptor markedly inhibited VIP-stimulated cAMP accumulation by 86 ± 2% (Fig. 2B and Table 2), whereas the T149A mutant decreased VIP-stimulated cAMP accumulation by 57%, which is not significantly different from wild-type. ($p > 0.05$). These results suggest that in both Ltk$^-$ and GH4C1 cells, the T149 residue plays a partial role to mediate inhibition of G_s- and forskolin-induced enhancement of cAMP levels.

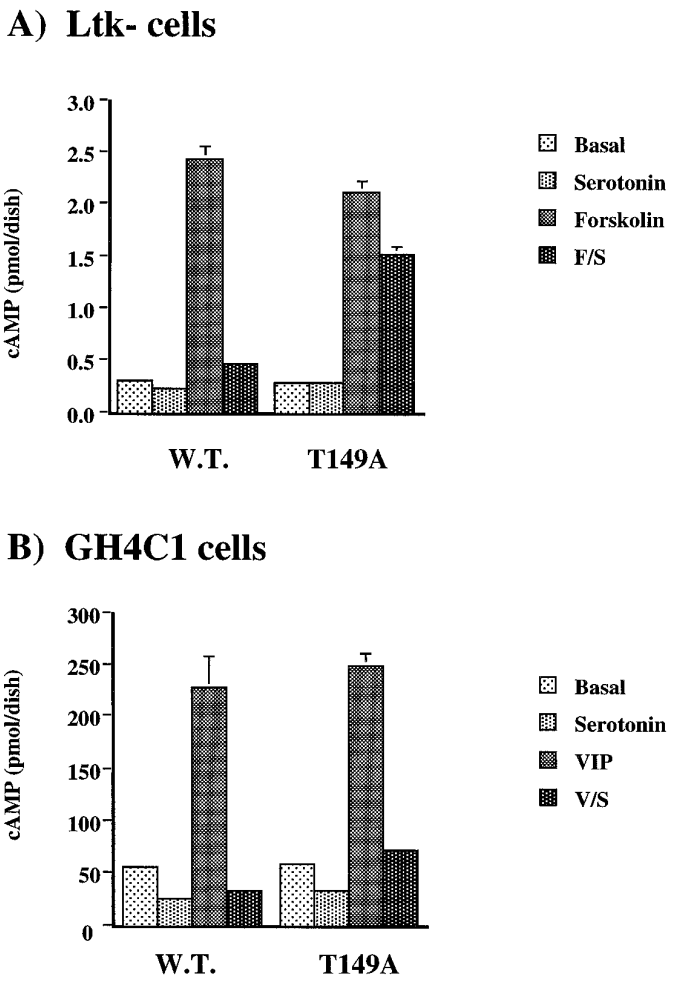


Fig. 2. Inhibition of cAMP accumulation by wild-type and T149A 5-HT$_{1A}$ receptors in Ltk$^-$/GH4C1 cells. cAMP accumulation was measured in media harvested from Ltk$^-$ or GH4C1 cells transfected with wild-type (*W.T.*) or T149A 5-HT$_{1A}$ receptor cDNAs after the addition of experimental compounds. The data are expressed as mean ± standard error of triplicate determinations from a single experiment. These results were repeated in three or four independent experiments. Concentrations used (A) Ltk$^-$ clones, 10 μM 5-HT and forskolin (*F/S*, coaddition of 5-HT and forskolin) and (B) GH4C1 clones, 500 nM VIP and 10 μM 5-HT (*V/S*, coaddition of VIP and 5-HT).

TABLE 1

Binding characteristics of Ltk$^-$ GH4C1 cells expressing transfected wild-type and mutant 5-HT$_{1A}$ receptors

Membranes from clones stably transfected with wild-type or mutant 5-HT$_{1A}$ receptors were prepared and subjected to saturation binding analysis using [^{3}H]DPAT as a selective agonist (see text). K_d and B_{max} values were determined by nonlinear regression analysis of saturation binding data derived with the RECEPTOR FIT (Lundon program). Values shown are the mean ± standard error of data from at least three independent experiments except for Ltk$^-$: T149A$_2$ (two experiments).

5-HT$_{1A}$ receptor	B_{max}	K_d
	pmol/mg of protein	*nM*
Ltk$^-$: Wild-type	1.64 ± 0.38	2.74 ± 0.38
Ltk$^-$: T343A[a]	0.62 ± 0.10	7.80 ± 2.50
Ltk$^-$: T149A$_1$	0.36 ± 0.06	5.40 ± 0.30
Ltk$^-$: T149A$_2$	0.34 ± 0.09	4.30 ± 0.06
GH$_4$: Wild-type	1.10 ± 0.45	5.62 ± 1.50
GH$_4$: T149A	2.71 ± 0.90	3.57 ± 1.10

[a] Data from Ref. 24.

Receptor coupling to mobilization of intracellular Ca^{2+}. Coupling of wild-type and mutant T149A 5-HT$_{1A}$ receptors to Ca^{2+} mobilization in Ltk$^-$ cells was examined by monitoring $[Ca^{2+}]_i$ in cells loaded with the Ca^{2+} indicator

TABLE 2

Inhibition of cAMP accumulation in Ltk⁻/GH4C1 cells expressing wild-type and mutant 5-HT_{1A} receptors

The percent inhibition of forskolin- (in Ltk⁻ cells) or VIP-stimulated (in GH4C1 cells) cAMP accumulation by 10 μM 5-HT is tabulated as the mean ± standard error of at least three independent experiments. Calculation for inhibition of forskolin- and VIP-stimulated cAMP accumulation: Inhibitory activity = [(A − AS)/A] × 100, where the level of cAMP after (A) activator of adenylyl cyclase, forskolin or VIP, or activator and serotonin (AS) was measured. * p value < 0.001: † p value > 0.059.

5-HT_{1A} receptor	Adenylyl cyclase activity
	% of forskolin inhibition
Ltk⁻: Wild-type	83 ± 10
Ltk⁻: T343A	65 ± 6
Ltk⁻: $T149A_1$	30 ± 12[a]
Ltk⁻: $T149A_2$	31 ± 3[a]
GH_4: Wild-type	86 ± 2
GH_4: T149A	57 ± 14[b]

[a] $p < 0.001$.
[b] $p > 0.059$.

Fura-2. On addition of 100 nM 5-HT, an immediate 2.6-fold peak increase in $[Ca^{2+}]_i$ was induced in cells expressing the wild-type 5-HT_{1A} receptor (Fig. 3A). For comparison, the addition of 100 nM 5-HT to the T343A mutant clone induced a 1.7-fold increase in $[Ca^{2+}]_i$ (Fig. 3B). The slight reduction in the Ca^{2+} response compared with the wild-type clone may reflect the lower receptor levels in the T343A clone. On the other hand, both of the T149A mutant clones (Fig. 3, C and D) failed to elicit any Ca^{2+} response after activation with ≤100-fold higher (10 μM) 5-HT, even though inhibition of forskolin-stimulated adenylyl cyclase was observed (Fig. 2).

To further document the uncoupling of the T149A mutant from Ca^{2+} signaling, we used transient transfection of Ltk⁻ cells with equal amounts of T149A and wild-type 5-HT_{1A} receptor plasmid DNA to express the receptors at equal levels. When expressed at equal densities (Fig. 4), the wild-type receptor coupled to increase $[Ca^{2+}]_i$ by an average of 1.72 ± 0.10-fold basal (five experiments), whereas the T149A mutant elicited no significant increase (1.08 ± 0.07-fold basal; six experiments). In contrast, ATP (acting via endogenous receptors) elicited identical responses in both transfections, indicating that the cells were equally responsive with regard to receptor-mediated Ca^{2+} mobilization. These results indicate that the T149 residue of the i2 loop of the 5-HT_{1A} receptor is a critical site for G protein coupling to Ca^{2+} mobilization in Ltk⁻ cells.

Receptor coupling to dihydropyridine-sensitive Ca^{2+} channels. The role of T149 in coupling the 5-HT_{1A} receptor to inhibition of voltage-dependent Ca^{2+} influx was examined in GH4C1 cells by using the dihydropyridine channel agonist (±)-Bay K8644 to activate L-type Ca^{2+} channels. The addition of 1 μM (±)-Bay K8644 to GH4C1 cells induced a 2.3-fold increase in $[Ca^{2+}]_i$, and activation of the wild-type 5-HT_{1A} receptor with 1 μM 5-HT inhibited the change in $[Ca^{2+}]_i$ by 40% (Fig. 5A). However, the T149A mutant clone failed to respond to 1 μM 5-HT after pretreatment with 1 μM (±)-Bay K8644 (Fig. 5B). These results suggest that the T149

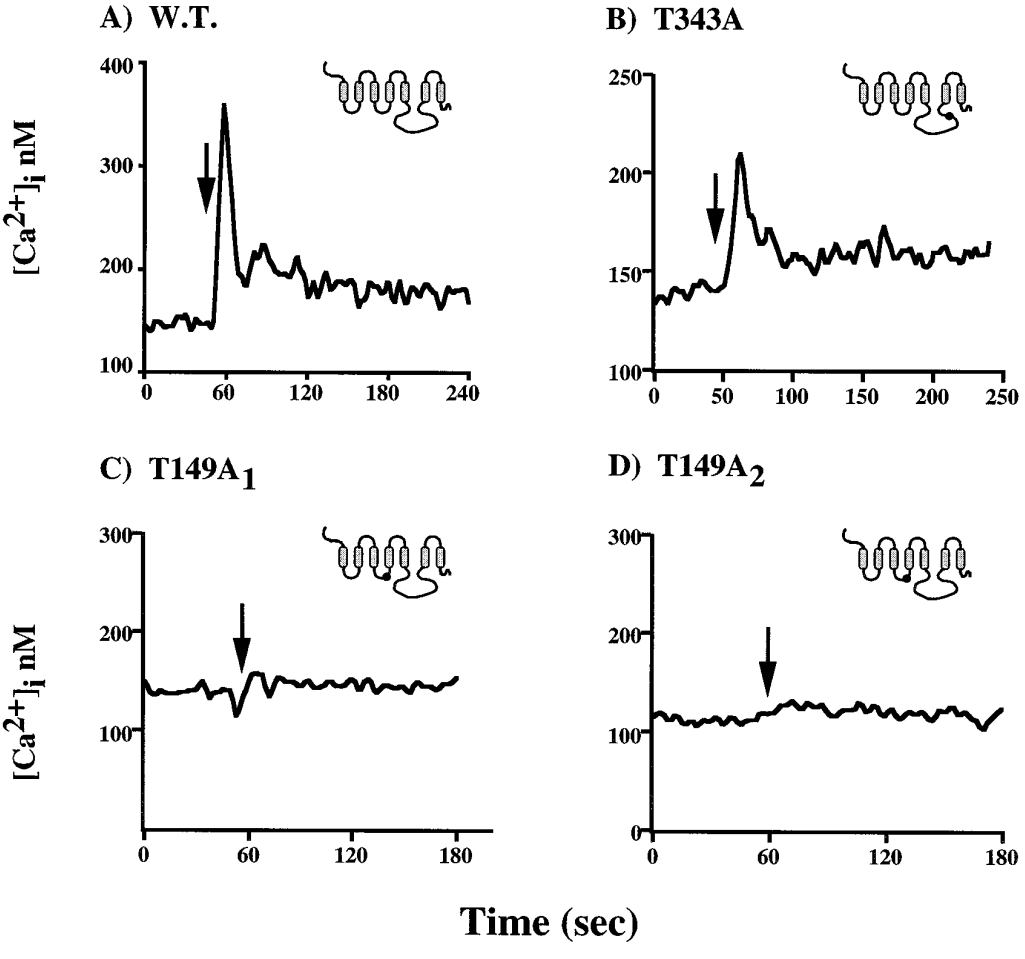


Fig. 3. Actions of wild-type and mutated 5-HT_{1A} receptors on $[Ca^{2+}]_i$ in Ltk⁻ cells. A, Wild-type (*W.T.*) 5-HT_{1A} receptor in Ltk⁻ cells. *Arrow*, addition of 100 nM 5-HT induced a 2.6-fold increase in intracellular Ca^{2+} when added at 60 sec. B, T343A mutant. *Arrow*, 100 nM 5-HT induced a 1.7-fold increase in $[Ca^{2+}]_i$. C, $T149A_1$ mutant. *Arrow*, 10 μM 5-HT at 60 sec failed to induce a Ca^{2+} response. This result was repeated four times with similar results. D, $T149A_2$ mutant. *Arrow*, 10 μM 5-HT was added and failed to induce a change in $[Ca^{2+}]_i$. This result was repeated three times.

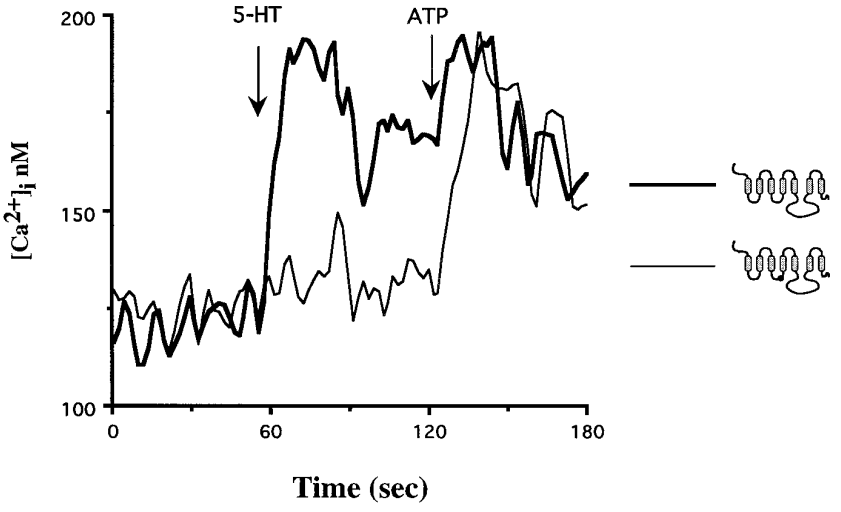


Fig. 4. Uncoupling of the T149A 5-HT$_{1A}$ receptor mutant from Ca^{2+} mobilization in transiently transfected Ltk$^-$ cells. Ltk$^-$ cells were transfected separately with 20 μg each of wild-type (*bold trace*) or T149A (*light trace*) mutant 5-HT$_{1A}$ receptor expression plasmids, and $[Ca^{2+}]_i$ was measured. Additions were 1 μM 5-HT at 60 sec and 50 μM ATP at 120 sec (*arrows*). The levels of specific [^{3}H]DPAT binding measured in parallel in membranes prepared from the transfected cells were 1.12 $\pm$ 0.11 (three experiments) pmol/mg of protein (wild-type 5-HT$_{1A}$) and 0.97 $\pm$ 0.03 (three experiments) pmol/mg of protein (T149A mutant).

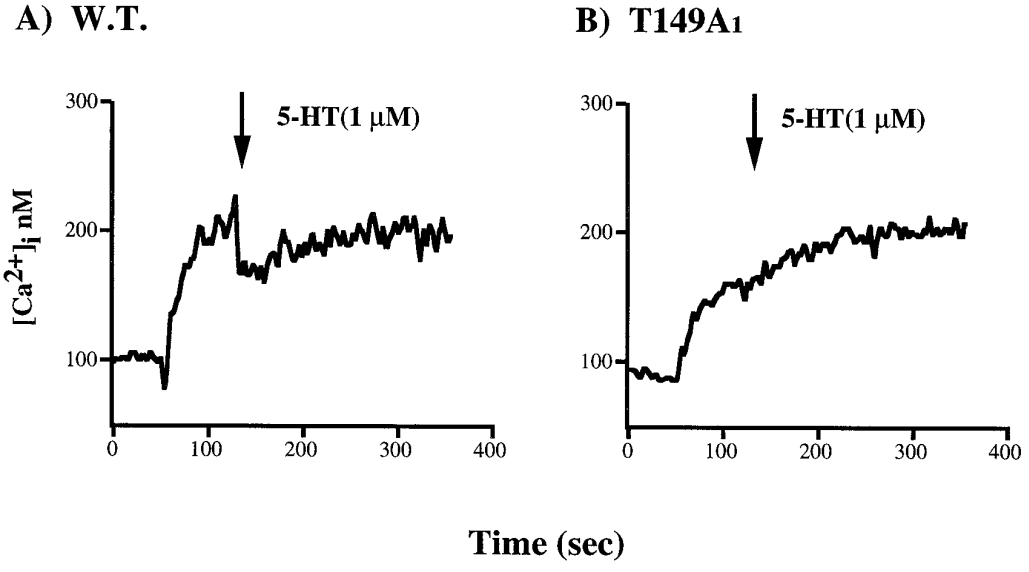


Fig. 5. Actions of wild-type and T149A mutant 5-HT$_{1A}$ receptors on $[Ca^{2+}]_i$ in GH4C1 cells. A, Wild-type (W.T.) 5-HT$_{1A}$ receptor in GH4C1 cells. At 60 sec, the addition of 1 μM ($\pm$)-Bay K8644 induced a 2.3-fold increase in Ca^{2+} influx, and the addition of 1 μM 5-HT (*arrow*) caused a 40% decrease in $[Ca^{2+}]_i$. B, T149A 5-HT$_{1A}$ receptor: 1 μM ($\pm$)-Bay K8644 induced a 2-fold enhancement of $[Ca^{2+}]_i$; the subsequent addition of 1 μM 5-HT (*arrow*) failed to decrease $[Ca^{2+}]_i$ in these cells. All curves were from a single experiment that was repeated at least three times with similar results.

residue in the i2 loop of the receptor is a critical site for inhibitory coupling to Ca^{2+} channels in GH4C1 cells.

Discussion

In previous studies, we and others have identified multiple signals mediated by the coupling of receptors, including the 5-HT$_{1A}$ receptor, to the PTX-sensitive G$_i$/G$_o$ family of G proteins. We focused on three of these pathways: 1) inositol trisphosphate-mediated Ca^{2+} mobilization in fibroblasts, 2) inhibition of Ca^{2+} channel opening in pituitary cells, and 3) inhibition of cAMP levels in both cell types. When expressed in fibroblast cells (12, 15, 16, 17) and endogenously in lymphoid cells (26), the 5-HT$_{1A}$ receptor enhances PI turnover to mobilize intracellular Ca^{2+} . The identification of PLC-β2 and PLC-β3 as subtypes that respond to $\beta\gamma$ subunits derived from G protein heterotrimers (27, 28) suggests a hypothesis in which the release of $\beta\gamma$ subunits on G protein activation by 5-HT$_{1A}$ receptors mediates PI turnover and Ca^{2+} mobilization. Concomitant release of α_i subunits would mediate the bifurcating pathway of inhibition of adenylyl cyclase. The specific G protein that mediates the PI response is unclear, although ablation of G$_{i2}$ (but not G$_{i1}$, G$_{i3}$, or G$_o$) by overexpression of full-length antisense α subunit RNA selectively inhibited 5-HT-induced Ca^{2+} mobilization in L cells.[1] However, it seems that multiple subtypes are required because the PI response is not reconstituted by any single subtype of G protein (29). This is consistent with the higher levels of $\beta\gamma$ relative to α_q subunits required to activate PLC *in vitro* (27) and the higher sensitivity of the PLC pathway to the level of receptor expression in various cell types (12).

Inhibition of cAMP accumulation seems to be mediated by the G$_i$ class (especially G$_{i2}$), but not by G$_o$, in GH4C1 cells, as determined by antisense experiments (30). Receptor-mediated inhibition of Ca^{2+} channel opening in pituitary cells is mediated by G$_o$, although the specific combination of subunits is receptor dependent (30–33). Specific ablation of α_o protein using antisense strategies blocked inhibitory coupling to Ca^{2+} channels but did not alter receptor coupling to inhibition of cAMP (30). Thus, G$_i$/G$_o$-coupled receptors like the 5-HT$_{1A}$ receptor mediate a variety of cell-specific responses via discrete G protein-effector systems: 1) in fibroblasts, the $\beta\gamma$ subunits of multiple G$_i$/G$_o$ proteins seem to be necessary to stimulate the isoforms of PLC that are present;

[1] Y. F. Liu and P. R. Albert, unpublished observations.

2) in pituitary cells, G_o proteins [possibly via release of $\beta\gamma$ subunits (34, 35)] mediate the closing of endogenously expressed Ca^{2+} channels; and 3) in both cell types, α_i proteins mediate the inhibition of basal and G_s-stimulated adenylyl cyclase activity.

The experiments presented here provide evidence that a single threonine residue (T149) in the i2 loop of the 5-HT_{1A} receptor plays a crucial role in direction of the signaling of the receptor. Mutation of this residue to alanine entirely uncouples the receptor from Ca^{2+} mobilization in fibroblasts and from inhibition of Ca^{2+} influx in pituitary cells and partially attenuates the adenylyl cyclase pathway in both cell types. In contrast, 5-HT_{1A} receptors with point mutations of threonine residues located in the i3 loop (e.g., T343) mediate both Ca^{2+} mobilization and cAMP inhibition pathways (24). The T149A (i2 loop) mutant lacked completely a Ca^{2+} response despite the presence of a consistent cAMP response. Because G_{i2} seems to be essential for the Ca^{2+} response, impaired interaction with G_{i2} may reduce the mobilization of $\beta\gamma$ subunits on activation of the i2 receptor mutant, whereas inhibition of adenylyl cyclase persists because other α_i subunits seem to substitute for α_{i2}. In pituitary GH4C1 cells, the T149A mutant failed to inhibit Ca^{2+} channel activation but did inhibit cAMP accumulation nearly as strongly as the wild-type receptor. These data suggest that the i2 loop is an important contact point for G_o, which mediates Ca^{2+} channel inhibition. Interestingly, recent data indicate that inhibition of Ca^{2+} channels by G_o is mediated by direct interaction of $\beta\gamma$ subunits with the channel protein (34, 35). Thus, the i2 loop of the 5-HT_{1A} receptor seems to be important for $\beta\gamma$-mediated coupling via G_o (to Ca^{2+} channels in GH4C1 cells) and G_{i2} (to PLC-β2/β3). However, α_i proteins (which mediate inhibition of adenylyl cyclase) do not rely as heavily on the same i2 site.

Current scientific evidence indicates that the i3 loop is not the sole determinant of G protein selectivity and that other cytoplasmic domains of the receptor must also contribute. For example, using a chimeric substitution approach, residues of the i2 loop domain that lie adjacent to the T149 equivalent residue are implicated in coupling of m3 receptors to α_q to stimulate PLC (36). Furthermore, the i2 loop of the gonadotropin-releasing hormone receptor was critical for coupling of via G_q to signal transduction: the L147A and L147D mutants showed a significant impairment of gonadotropin-releasing hormone-stimulated IP production (37). The results of current report complement this work by probing the role of the conserved threonine residue, which was not changed in the above chimeric substitutions. The T149 residue in the i2 loop is conserved among several G_s-, G_i-, and G_q-coupled receptors (Fig. 1) and is a critical site for coupling of the 5-HT_{1A} receptor to PI hydrolysis in Ltk^- cells and for the closing of L-type voltage-gated Ca^{2+} channels in GH4C1 cells but not for inhibition of cAMP levels. Consistent with our results, Van Koppen *et al.* (38) have recently shown that the equivalent Thr145 in the i2 loop of the m4 muscarinic acetylcholine receptor could be mutated to alanine without alteration of inhibition of adenylyl cyclase. In agreement with their studies, we find that the i2 loop plays a lesser role in coupling to α_i and a more important role in coupling to PLC-linked pathways. The putative role of this i2 domain in coupling to G_i/G_o proteins is supported by evidence with synthetic peptides derived from the i2 loop of G_i/G_o-coupled 5-HT_{1A} and muscarinic m2 receptors, which potently inhibited adenylyl cyclase *in vitro* (22, 23). It is thus likely that multiple interactions of G_i proteins at both i2 and i3 loops contribute to coupling of the 5-HT_{1A} receptor to inhibition of cAMP accumulation.

The selective uncoupling of 5-HT_{1A} receptor-mediated Ca^{2+} mobilization but not inhibition of cAMP accumulation by acute (2 min) activation of PKC in Ltk^- cells suggested that distinct coupling mechanisms may induce these signaling pathways (11). Previously, we reported that the cumulative elimination of i3 phosphorylation sites on the 5-HT_{1A} receptor progressively reduced PKC-induced inhibition of Ca^{2+} mobilization, suggesting the involvement of these residues in mediating the 5-HT-induced PI response (24). These results are consistent with a requirement for multiple G_i/G_o proteins in Ca^{2+} mobilization because impaired interaction of the receptor with any G protein would attenuate coupling to this pathway. However, the insensitivity of receptor-mediated inhibition of cAMP to PKC indicates that phosphorylation of these i3 residues is not sufficient to uncouple all responses initiated by the receptor. The current results indicate that mutation of T149 in the i2 loop reproduces the selective inactivation of Ca^{2+} mobilization observed after PKC treatment. Thus, phosphorylation of this i2 loop threonine, in addition to the i3 sites, may mediate the action of PKC to block selectively receptor-induced Ca^{2+} mobilization.

In this regard, Liu *et al.* (39) identified four amino acids (VTIL) in the carboxyl-terminal segment of the i3 loop of the m2 muscarinic receptor that are essential for receptor interactions with the carboxyl-terminal pentapeptide domain of G_i/G_o proteins. The threonine of the VTIL motif is conserved among several G_i/G_o-coupled receptors and may be a specific contact site for coupling to G_i/G_o proteins, although single-point mutations were not performed to determine the specific amino acid coupling site. The carboxyl-terminal i3 threonine residue of the VTIL motif in the m2-muscarinic receptor lies in a predicted α-helical domain that protrudes into the cytoplasm to permit interaction with the carboxyl-terminal pentapeptide domain of α_i and α_o. Similarly, the T149 residue in the i2 loop of the 5-HT_{1A} receptor could be a specific contact site for determining G protein coupling specificity in Ltk^- and GH4C1 cells. The T149 residue is located at the center of a particularly hydrophilic portion of the i2 loop domain that has among the highest surface probability and antigenic indices of the receptor protein. It is predicted to have an amphipathic α-helical structure that protrudes into the cytoplasmic milieu (40). Thus, T149 is well situated to allow hydrogen bonding interactions with intracellular proteins, G proteins, and other intracellular receptor domains. The mutation of this residue to alanine did not greatly alter the predicted secondary structural properties (e.g., flexibility, surface probability, antigenicity) of this domain and presumably acts by eliminating hydrogen bonding interactions with the threonine hydroxyl side chain. The phosphorylation of this residue (e.g., by PKC) would similarly disrupt hydrogen bonding interactions by placing a negatively charged ionic phosphate moiety at this site.

In conclusion, the T149 defines a novel conserved hydrophilic core residue of the i2 loop amphipathic α-helical domain of the 5-HT_{1A} receptor that directs specific interactions of the receptor which result in cell-specific coupling to Ca^{2+} mobilization or inhibition of Ca^{2+} entry, with a partial role in coupling to inhibition of cAMP levels. Our results further

indicate that distinct receptor domains underlie coupling to α_o, α_i, and $\beta\gamma$ subunits within the G_i/G_o family.

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References

1. Ostrowski, J., M. A. Kjelsberg, M. G. Caron, and R. J. Lefkowitz. Mutagenesis of the β_2-adrenergic receptor: how structure elucidates function. *Annu. Rev. Pharmacol. Toxicol.* **32:**167–183 (1992).
2. Hedin, K. E., K. Duerson, and D. E. Clapham. Specificity of receptor G-protein interaction: searching for the structure behind the signal. *Cell Signal.* **5:**505–518 (1993).
3. Strader, C. D., T. M. Fong, M. R. Tota, D. Underwood, and R. A. F. Dixon. The stucture and function of G-protein coupled receptors. *Annu. Rev. Biochem.* **63:**101–132 (1994).
4. Weiss, E. R., D. J. Kelleher, and G. L. Johnson. Mapping sites of interaction between rhodopsin and transducin using rhodopsin antipeptide antibodies. *J. Biol. Chem.* **263:**6150–6154 (1988).
5. Konig, B., A. Arendt, J. H. McDowell, M. Kahlert, P. A. Hargrave, and K. P. Hofmann. Three cytoplasmic loops of rhodopsin interact with transducin. *Proc. Natl. Acad. Sci. USA* **86:**6878–6882 (1989).
6. Dalman, H. M., and R. R. Neubig. Two peptides from the α_{2A}-adrenergic receptor alter receptor G protein coupling by distinct mechanisms. *J. Biol. Chem.* **266:**11025–11029 (1991).
7. Okamoto, T., Y. Murayama, Y. Hayashi, M. Inagaki, E. Ogata, and I. Nishimoto. Identification of a Gs activator region of the *beta*-2-adrenergic receptor that is autoregulated via protein kinase A-dependent phosphorylation. *Cell* **67:**723–730 (1991).
8. Luttrell, L. M., J. Ostrowski, S. Cotecchia, H. Kendall, and R. J. Lefkowitz. Antagonism of catecholamine receptor signaling by expression of cytoplasmic domains of the receptors. *Science (Washington D. C.)* **259:**1453–1457 (1993).
9. Munch, G., C. Dees, M. Hekman, and D. Palm. Multisite contacts involved in coupling of the *beta*-adrenergic receptor with the stimulatory guanine-nucleotide-binding regulatory protein: structural and functional studies by *beta*-receptor-site-specific synthetic peptides. *Eur. J. Biochem.* **198:**357–364 (1991).
10. Malek, D., G. Munch, and D. Palm. Two sites in the third inner loop of the dopamine D2 receptor are involved in functional G protein-mediated coupling to adenylate cyclase. *FEBS Lett.* **325:**215–219 (1993).
11. Liu, Y. F., and P. R. Albert. R. Cell-specific signaling of the 5-HT1A receptor: modulation by PK C and PK A. *J. Biol. Chem.* **266:**23689–23697 (1991).
12. Albert, P. R. Heterologous expression of G protein-linked receptors in pituitary and fibroblast cell lines. *Vitamins Hormones* **48:**59–109 (1994).
13. Innis, R. B., and G. H. Aghajanian. Pertussis-toxin blocks 5-HT1A receptor: a $GABA_B$ receptor-mediated inhibition of serotonergic neurons. *Eur. J. Pharmacol.* **143:**195–204 (1987).
14. Penington, N. J., and J. S. Kelly. Serotonin receptor activation reduces calcium current in an acutely dissociated adult central neuron. *Neuron* **4:**751–758 (1990).
15. Albert, P. R., Q. Y. Zhou, H. H. M. Van Tol, J. Bunzow, and O. Civelli. Cloning, functional expression and mRNA tissue distribution of the rat 5-HT1A receptor gene. *J. Biol. Chem.* **265:**5825–5832 (1990).
16. Fargin, A., J. R. Raymond, J. W. Regan, S. Cotecchia, R. J. Lefkowitz, and M. G. Caron. Effector coupling mechanisms of the cloned 5-HT1A receptor. *J. Biol. Chem.* **264:**14848–14852 (1989).
17. Abdel-Baset, H., V. Bozovic, M. Szyf, and P. R. Albert. Conditional transformation mediated via a pertussis toxin-sensitive receptor signaling pathway. *Mol. Endocrinol.* **6:**730–740 (1992).
18. Liu, Y. F., O. Civelli, Q. Y. Zhou, and P. R. Albert. Differential sensitivity of the short and long human dopamine-D2 receptor subtypes to protein kinase C. *J. Neurochem.* **59:**2311–2317 (1992).
19. Moro, O., J. Lameh, P. Hogger, and W. Sadee. Hydrophobic amino acid in the i2 loop plays a key role in receptor-G protein coupling. *J. Biol. Chem.* **268:**22273–22276 (1993).
20. Ohyama, K., Y. Yamano, S. Chaki, T. Kondo, and T. Inagami. Domains for G-protein coupling in angiotensin II receptor type 1: studies by site-directed mutagenesis. *Biochem. Biophys. Res. Commun.* **189:**677–683 (1992).
21. Kosugi, S., L. D. Kohn, T. Akamixu, and T. Mori. The middle portion in the second cytoplasmic loop of the thyrotropin receptor plays a crucial role in adenylate cyclase activation. *Mol. Endocrinol.* **8:**498–509 (1994).
22. McClue, S., B. M. Baron, and B. A. Harris. Activation of Gi protein peptide structures of the muscarinic M2 receptor second intracellular loop. *Eur. J. Pharmacol.* **267:**185–193 (1994).
23. Varrault, A., D. L. Nguyen, S. McClue, B. Harris, P. Jouin, and J. Bockaert. 5-HT 1A receptor synthetic peptides: mechanisms of adenylyl cyclase inhibition. *J. Biol. Chem.* **269:**16720–16725 (1994).
24. Lembo, P. M. C., and P. R. Albert. Multiple phosphorylation sites are required for pathway-selective uncoupling of the 5-hydroxytryptamin$_{1A}$ receptor by protein kinase C. *Mol. Pharmacol.* **48:**1024–1029 (1995).
25. Aune, T. M., K. M. McGrath, T. Sarr, M. P. Bombara, and K. A. Kelley. Expression of 5-HT1A receptors on activated human T cells: regulation of cyclic AMP levels and T cell proliferation by 5-hydroxytryptamine. *J. Immunol.* **151:**1175–1183 (1993).
27. Clapman, D. E., and E. J. Neer. New roles for G-protein $\beta\gamma$-dimers in transmembrane signaling. *Nature (Lond.)* **365:**403–406 (1993).
28. Clapman, D. E. The G-protein nanomachine. *Nature (Lond.)* **379:**297–299 (1996).
29. Ghahremani, M. H., and P. R. Albert. Signalling pathways of the dopamine D2S receptor: dissection using pertussis toxin-insensitive G protein mutants. *Proc. Soc. Neurosci.* **21:**1863 (1995).
30. Liu, Y. F., Jakobs, K. H., Rasenick, M. M., Albert, P. R. G-protein specificity in receptor-effector coupling. *J. Biol. Chem.* **269:**13880–13886 (1994).
31. Kleuss, C., H. Scherubl, G. Schultz, and B. Wittig. Different β-subunits determine G-protein interaction with transmembrane receptors. *Nature (Lond.)* **358:**424–426 (1992).
32. Kleuss, C., H. Scherubl, G. Schultz, and B. Wittig. Selectivity in signal transduction determined by γ-subunits of heterotrimeric G-proteins. *Science (Washington D. C.)* **259:**832–834 (1993).
33. Albert, P. R., and S. J. Morris. Antisense knockouts: molecular scalpels for the dissection of signal transduction. *Trends Pharmacol. Sci.* **15:**250–254 (1994).
34. Ikeda, S. R. Voltage-dependent modulation of N-type calcium channels by G-protein $\beta\gamma$ subunits. *Nature (Lond.)* **380:**255–258 (1996).
35. Herlitze, S., D. E. Garcia, K. Mackie, B. Hille, T. Scheuer, and W. A. Catterall. Modulation of Ca^{2+} channels by G protein $\beta\gamma$ subunits. *Nature (Lond.)* **380:**259–262 (1996).
36. Blin, N., J. Yun, and J. Wess. Mapping of single amino acid residues required for selective activation of Gq/11 by the m3 muscarinic acetylcholine receptor. *J. Biol. Chem.* **270:**17741–17748 (1995).
37. Arora, K. K., A. Sakai, and K. J. Catt. Effects of second intracellular loop mutations on signal transduction and internalization of the gonadotropin-releasing hormone receptor. *J. Biol. Chem.* **270:**22820–22826 (1995).
38. Van Koppen, C. J., W. Lenz, J. P. L. Nunes, C. Y. Zhang, M. Schmidt, and K. H. Jakobs. The role of membrane proximal threonine residues conserved among guanine-nucleotide-binding-protein-coupled receptors in internalization of the m4 ACh receptor. *Eur. J. Biochem.* **234:**536–541 (1995).
39. Liu, J., B. R. Conklin, N. Blin, J. Yun, and J. Wess. Identification of a receptor/G-protein contact site critical for signaling specificity and G-protein activation. *Proc. Natl. Acad. Sci. USA* **92:**11642–11646 (1995).
40. Sylte, I., O. Edvardsen, and S. G. Dahl. Molecular dynamics of the 5-HT1A receptor and ligands. *Protein Eng.* **6:**691–700 (1993).

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