0026-895X/95/061024-06$3.00/0

MOLECULAR PHARMACOLOGY, 48:1024–1029 (1995).

Multiple Phosphorylation Sites Are Required for Pathway-Selective Uncoupling of the 5-Hydroxytryptamine$_{1A}$ Receptor by Protein Kinase C

PAOLA M. C. LEMBO and PAUL R. ALBERT[1]

Department of Pharmacology and Therapeutics, McGill University, Montreal, Quebec, Canada H3G-1Y6

Received April 14, 1995; Accepted September 5, 1995

SUMMARY

Classically, acute uncoupling of G protein-linked receptors has been presented as a nonselective process in which modification of the receptor by phosphorylation leads to reduction in or the loss of coupling to all effectors. Investigation of multiple signaling pathways has modified this view: for example, when expressed in Ltk$^-$ fibroblasts, the 5-hydroxytryptamine$_{1A}$ (5-HT$_{1A}$) receptor couples to both stimulation of intracellular calcium mobilization (via inositol phosphate generation) and inhibition of cAMP accumulation. Acute pretreatment for 2 min with 100 nM 12-*O*-tetradecanoylphorbol-13-acetate (TPA), an activator of protein kinase C (PKC), abolished the intracellular calcium response induced by 100 nM 5-HT but did not reduce 5-HT$_{1A}$ receptor-mediated inhibition of cAMP. In the present report, mutant 5-HT$_{1A}$ receptors lacking one, two, or three putative PKC phosphorylation sites located in the receptor's third cytoplasmic loop were constructed and transfected separately into Ltk$^-$ cells. The receptor mutants displayed agonist affinities similar to that of the nonmutated receptor. The relative magnitudes of 5-HT-induced changes in intracellular calcium or forskolin-stimulated cAMP levels were also similar to those of the wild-type receptor for all except one of the mutants. In addition, TPA treatment did not change the magnitude or potency of 5-HT-induced inhibition of prostaglandin E$_1$-stimulated cAMP accumulation in the wild-type or triple-mutant 5-HT$_{1A}$ receptor clones. On pretreatment with TPA, there was a progressive recovery to 74% of the control 5-HT-induced increase in calcium mobilization as PKC sites were eliminated from the receptor, indicating that multiple phosphorylation sites are required for PKC-mediated receptor uncoupling. Also, these results indicate that receptor phosphorylation selectively uncouples the 5-HT$_{1A}$ receptor from enhancement of calcium mobilization without reducing receptor-mediated inhibition of cAMP. Thus, phosphorylation by PKC can sculpt receptor signaling via pathway-selective uncoupling.

Desensitization is defined as a decrease in receptor responsiveness on prolonged exposure to agonist (1). Uncoupling, the immediate phase of the desensitization of G protein-coupled receptors, is characterized by the activation of protein kinases and phosphorylation of the receptor protein, leading to impaired activation of G proteins (2–4). The prototypic β-adrenergic receptor, which stimulates adenylyl cyclase, is uncoupled by phosphorylation of distinct sites by protein kinase A and receptor kinases (2–6). Elimination of these sites by receptor mutagenesis reduces the extent of agonist-induced desensitization (7, 8).

The 5-HT$_{1A}$ receptor is a member of the family of "inhibitory" receptors that couple to PTX-sensitive G proteins to inhibit adenylyl cyclase, open potassium channels, and close calcium channels, leading to a decrease in calcium levels in pituitary and neuronal cells (9–13). The 5-HT$_{1A}$ receptor, as well as other 5-HT$_1$ and dopamine-D$_2$ receptors, induces a cell-specific enhancement of PI turnover and increase in intracellular calcium levels when expressed in fibroblast cells such as Ltk$^-$, BALB/c-3T3 cells or HeLa cells; these responses are blocked by PTX (13–17). In these cells, acute (2 min) pretreatment with TPA, which activates several isoforms of PKC, selectively abrogates 5-HT-induced increases in PI turnover and intracellular calcium but not inhibition of cAMP level. The rapid time course and concomitant phosphorylation of the 5-HT$_{1A}$ receptor (18) suggest that the uncoupling could be mediated by receptor phosphorylation. However, several targets phosphorylated by PKC, including the receptor (18), specific G proteins (19), and the effector, PLC-β (20), might be involved in pathway-selective modulation of receptor signaling by PKC. We addressed the mecha-

This work was supported by the Medical Research Council, Canada. P.M.C.L. is the recipient of a Studentship from the Fonds de la Formation de Chercheurs et l'Aide à la Recherche, Québec. P.R.A. is Chercheur Boursier of the Fonds de la Recherche en Santé du Québec.

[1] Current affiliation: Neuroscience Research Institute, University of Ottawa, Ottawa, Canada K1H 8M5.

ABBREVIATIONS: [^{3}H]DPAT, 8-hydroxy-(2-(*N*,*N*-di-[2,3,^{3}H]propylamino)1,2,3,4-tetrahydronaphthalene; PI, phosphatidylinositol; PKC, protein kinase C; PLC, phospholipase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; 5-HT, 5-hydroxytryptamine (serotonin); PTX, pertussis toxin; PGE$_1$, prostaglandin E$_1$; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

nism of PKC action by mutating the receptor and show that PKC activation induces selective uncoupling from calcium mobilization via phosphorylation of the 5-HT$_{1A}$ receptor at multiple sites.

Experimental Procedures

Materials. Restriction endonucleases and other molecular biology reagents were purchased from Boehringer Mannheim and GIBCO-BRL. Sequenase was obtained from U.S. Biochemicals. TPA, forskolin, 3-isobutyl-1-methylxanthine, 5-HT, and PGE_1 were purchased from Sigma Chemical Co.; [^{3}H]DPAT (135 Ci/mmol) and [α-^{32}P]ATP (2200 Ci/mmol) were obtained from Amersham Chemical Co. Geneticin was obtained from GIBCO, and Fura-2-AM was obtained from Molecular Probes.

Cell culture. All cells were grown as monolayers in α-minimum Eagle's medium supplemented with 5% fetal bovine serum at 37° in a humidified atmosphere with 5% carbon dioxide. Media were changed 12–24 hr before experiments.

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 (12) (containing the mouse metallothionein promoter) was subcloned into p-Select to use as a template for site-directed mutagenesis (Altered-Sites Mutagenesis, Promega). Three putative PKC sites in the third loop were mutagenized individually to T229A, S253G, and T343A using oligonucleotides incorporating point mutations (nucleotides 662–682, ATCCGCAAG*GC*TGTCAGGAAG; nucleotides 749–766, CCAAGAAG*GG*GCCTGAACG; and nucleotides 1018–1038, GAAAGGAAG*GG*CGGTGAAGA, respectively; Ref. 12). Double and triple mutants consisting of T229A/S253G and T229A/S253G/T343A were also constructed. All mutations were confirmed by DNA sequencing. Mutated and wild-type (without metallothionein promoter) 5-HT$_{1A}$ receptor clones were subcloned into the eukaryotic expression vector pcDNA I (Invitrogen) and cotransfected with pSV-Neo in Ltk$^-$ cells using calcium phosphate coprecipitation (12). Neomycin-resistant cells expressing 5-HT$_{1A}$ receptors were selected and grown in α-minimum Eagle's medium supplemented with 10% fetal calf serum and 700 μg/ml geneticin. Isolated clones were screened by Northern blot analysis.

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 rpm 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 TME and [^{3}H]DPAT (Amersham) 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 immediate washing with 3 × 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) and quantified by liquid scintillation counting. Protein was assayed with the Bio-Rad protein assay kit with bovine serum albumin as standard.

cAMP assay. Measurement of cAMP was performed as described previously (12). Briefly, cells plated into six-well 35-mm dishes were washed twice with 1 ml of HBBS buffer (118 mM NaCl, 4 mM KCl, 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) as described (13). 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 experiments.

Intracellular calcium measurement. As described previously (13), cells were harvested by incubation in HBBS buffer plus 5 mM EDTA and 0.05% trypsin and incubated with Fura-2 for 20 min at 37°.The cells were centrifuged, washed twice with HBBS plus 1 mM $CaCl_2$, and placed in a fluorescence cuvette. Change in fluorescence ratio was recorded on a Perkin-Elmer LS-50 spectrofluorometer and analyzed by computer, based on a K_d 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 test solutions as indicated in the figures.

Results

The close association between PKC-induced phosphorylation and desensitization of the 5-HT$_{1A}$ receptor (18) suggested that TPA may act by inducing receptor phosphorylation rather than via phosphorylation of downstream components (eg., G proteins or PLC). We addressed this possibility by generating 5-HT$_{1A}$ receptors mutated at PKC consensus phosphorylation sites located in the third intracellular loop of the receptor (Fig. 1). The single point mutants included T229A, S253G, and T343A, which substitute alanine and glycine for the phosphate acceptors threonine and serine, respectively. A double mutant (T229A/S253G) and triple mutant were also constructed. These mutants were stably transfected into receptor-negative Ltk$^-$ cells, and positive clones expressing receptor RNA were selected for further characterization. Membranes prepared from positive clones were subjected to saturation binding analysis using [^{3}H]DPAT, a selective 5-HT$_{1A}$ receptor agonist (12). The levels of 5-HT$_{1A}$ receptor expression of the various clones varied due to different efficiencies of transfection expression, with B_{max} values of 0.30–0.76 nmol/mg protein compared with 1.64 nmol/mg protein for the wild-type receptor. The K_d values calculated for the mutant receptors were all in the nanomolar range (Table 1), similar to the affinity of the nonmutated receptor (12). Thus, the mutations did not greatly alter

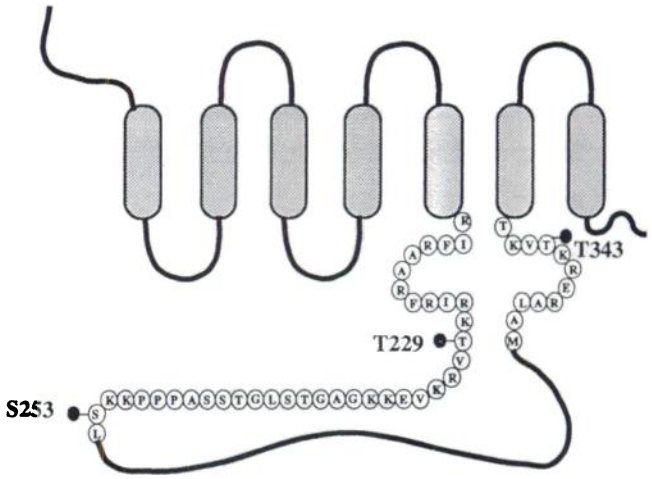

Fig. 1. Model of targeted mutants of the 5-HT$_{1A}$ receptor showing the proposed structure of the rat 5-HT$_{1A}$ receptor consisting of seven hydrophobic transmembrane domains forming three intracellular loops. ●, The three putative PKC consensus phosphorylation sites that were mutated.

TABLE 1

Binding characteristics of the wild-type and mutant 5-HT_{1A} receptors

K_d and B_{max} values were determined by nonlinear regression analysis of saturation binding data derived with the ligand program. Values shown are the mean ± standard error of data from at least three independent experiments. Membranes were prepared from Ltk$^-$ cell clones stably transfected with wild-type or mutant 5-HT_{1A} receptors and subjected to saturation binding analysis with [^{3}H]DPAT (see Experimental Procedures).

5-HT_{1A} receptor mutant	B_{max}	K_d
	pmol/mg	*nM*
Wild-type	1.64 ± 0.38	2.74 ± 0.18
T229A	0.34 ± 0.12	5.12 ± 1.5
S253G	0.76 ± 0.10	1.31 ± 1.3
T343A	0.62 ± 0.10	7.8 ± 2.5
T229A/S253G	0.30 ± 0.06	7.9 ± 2.1
T229A/S253G/T343A	0.31 ± 0.04	1.4 ± 0.3

agonist affinity, consistent with previous observations that mutations of cytoplasmic domains have little effect on ligand binding in other monoamine receptors (21, 22).

The ability of wild-type and mutant 5-HT_{1A} receptors to inhibit adenylyl cyclase was examined by measuring cAMP accumulation in transfected clones in the absence and presence of forskolin, an activator of adenylyl cyclase. Neither mutant nor wild-type receptors inhibited basal cAMP level (without forskolin) when expressed in Ltk$^-$ cells (data not shown), as observed previously for the wild-type receptor (13). The wild-type 5-HT_{1A} receptor markedly inhibited forskolin-stimulated cAMP level by >80% (Table 2). Each of the mutants was approximately as effective at inhibiting forskolin-induced cAMP (70–75%) despite a 50–70% lower receptor number (B_{max}) in cell lines expressing the mutant receptors (Table 1). Only the T229A mutant (30% inhibition) had a markedly reduced coupling, a result that was confirmed in three independent clones. This 5-HT_{1A} receptor mutant was atypical as the double (T229A/S253G) and triple mutants inhibited cAMP levels to a similar extent as the wild-type receptor. The reduced coupling of the T229A mutant may be due to a confirmational change introduced by the alanine substitution in an intracellular segment of the receptor believed to be important for coupling (3), which could be reversed when the double and triple mutations were reintroduced.

The effect of TPA on 5-HT-induced inhibition of cAMP accumulation in both wild-type and triple-mutant 5-HT_{1A} receptor clones was determined. Cells were incubated with PGE_1, which activates a G_s-coupled enhancement of cAMP

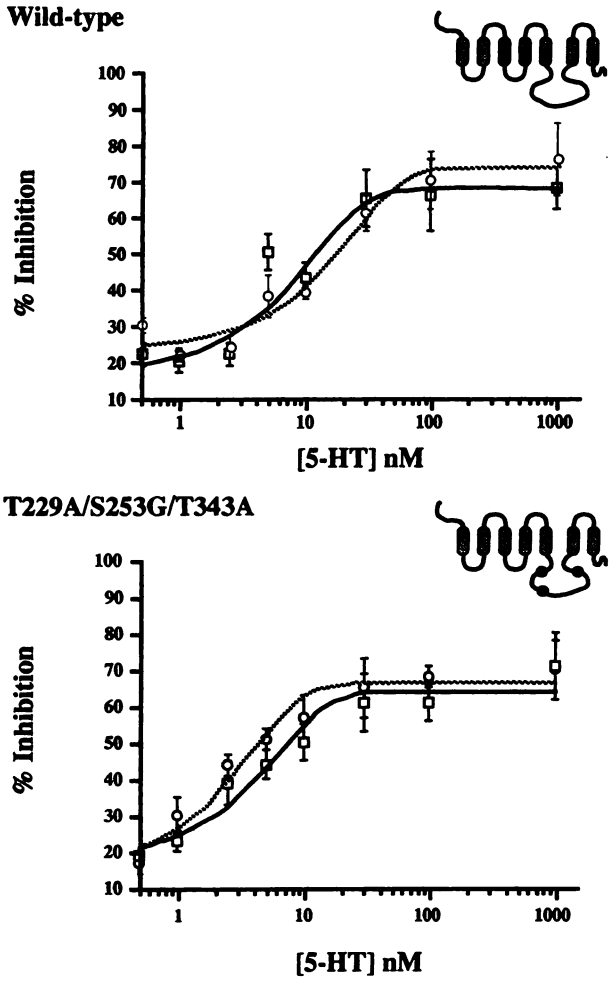

Fig. 2. The effect of TPA on 5-HT-induced inhibition of PGE_1-stimulated cAMP accumulation in wild-type and T229A/S253G/T343A 5-HT_{1A} receptor clones. The indicated concentrations of 5-HT were added in the absence (○, *hatched line*) and presence (□, *solid line*) of 100 nM TPA. Values are given as mean ± standard error of triplicate samples and are plotted as percent inhibition of cAMP accumulation induced by 1 μM PGE_1 versus the concentration of 5-HT; curves were fit mathematically using the Kaleidograph program. A, Cells transfected with wild-type 5-HT_{1A} receptor: EC_{50} = 6.0 nM, EC_{50} (+TPA) = 5.1 nM. The EC_{50} values from three experiments for wild-type were 4.4 ± 1.3 nM and (+TPA) 4.1 ± 0.8 nM. B, Cells transfected with T229A/S253G/T343A 5-HT_{1A} receptor: EC_{50}= 1.9 nM, EC_{50} (+TPA) = 2.9 nM. The EC_{50} values from three experiments for the triple mutant were EC_{50}= 1.9 ± 0.1 nM and EC_{50} (+TPA) = 3.2 ± 0.5 nM.

TABLE 2

Inhibition of cAMP accumulation for wild-type and mutant 5-HT_{1A} receptor

The percent inhibition of (10 μM) Forskolin-stimulated cAMP accumulation by 5-HT (10 μM) is tabulated as the mean ± standard error of at least three independent experiments. Calculation for inhibition of forskolin-stimulated cAMP accumulation was [(F − FS)/F] × 100, where the level of cAMP after forskolin (F) or forskolin and serotonin (FS) was measured.

5-HT_{1A} receptor mutant	Inhibition
	%
Wild-type	83 ± 10
T229A	31 ± 10
S253G	74 ± 10
T343A	70 ± 8
T229A/S253G	73 ± 5
T229A/S253G/T343A	75 ± 8

levels, and the potency of 5-HT to inhibit this response was examined in the absence and presence of TPA (Fig. 2). The magnitude and potencies of the wild-type and the triple-mutant receptors did not differ greatly in the presence and absence of TPA. These results confirm and extend the conclusion that acute PKC activation does not uncouple the 5-HT_{1A} receptor from inhibition of adenylyl cyclase in this cell line (13).

Coupling of wild-type and mutant 5-HT_{1A} receptors to calcium mobilization and its modulation by PKC were examined by monitoring cytosolic free calcium levels in cells loaded with the calcium indicator Fura-2. 5-HT (100 nM) induced an immediate 2.6-fold peak increase in intracellular calcium levels in Ltk$^-$ cells transfected with wild-type 5-HT_{1A} recep-

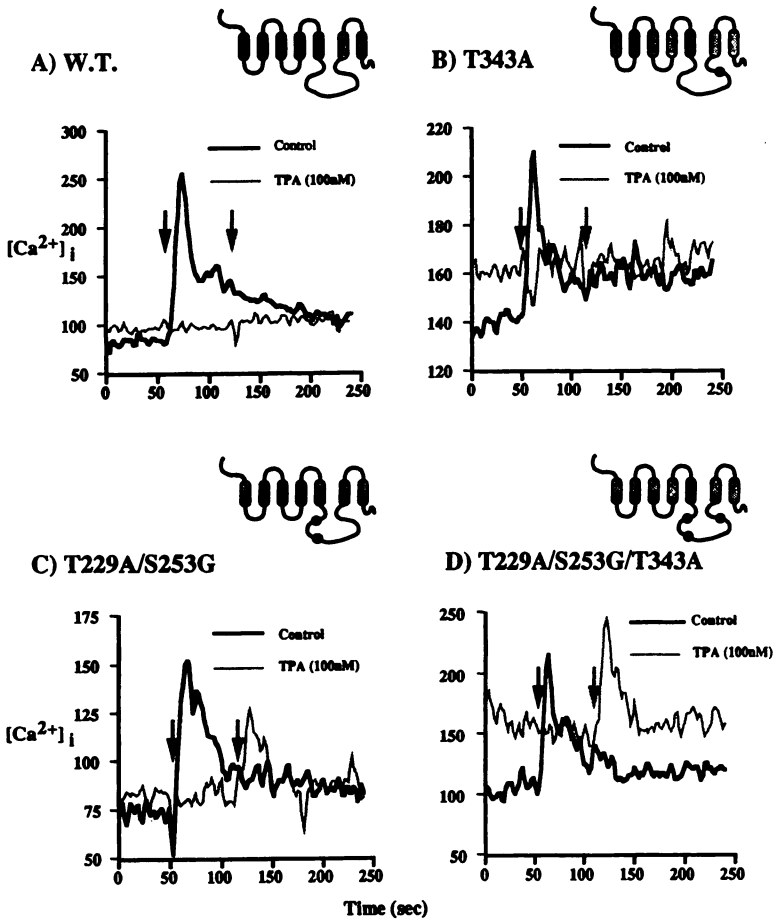

Fig. 3. Calcium mobilization by PKC site mutants of the 5-HT$_{1A}$ receptor transfected in Ltk$^-$ cells. *Dark tracing*, control samples: 5-HT (100 nM) was added at 60 sec (*dark arrow*). *Light tracing*, TPA-treated samples: TPA (100 nM) was added at time 0 and 5-HT (100 nM) at 120 sec (*shaded arrow*). A, Wild-type receptor; in TPA-treated samples (*light tracing*) the serotonin-mediated calcium response was completely inhibited. B, T343A (TPA completely blocked the 5-HT response). C, T229A/S253G; after treatment with TPA, 50% of the 5-HT-induced calcium response was recovered. D, T229A/S253G/T343A; 74% of 5-HT-mediated calcium response was recovered after TPA. All curves were generated by a computer and were from a single experiment that was repeated at least three times and with at least two independent clones that gave similar results.

TABLE 3

Effect of PKC on 5-HT-induced changes in $[Ca^{2+}]_i$

The influence of acute pretreatment of TPA in LtK$^-$ cells transfected with wild-type and mutant receptors. Serotonin-induced changes in $[Ca^{2+}]_i$ are indicated as -fold basal level of $[Ca^{2+}]_i$ and represent mean ± standard error of at least three independent determinations in at least two different clones. *Percent of control* represents the percent of the serotonin-induced response recovered after TPA treatment.

5-HT$_{1A}$ receptor mutant	5-HT induced increase in $[Ca^{2+}]_i$	Post-TPA treatment 5-HT-induced increase in $[Ca^{2+}]_i$
	-fold basal	*% of control*
Wild-type	2.6 ± 0.30	NR
T229A	1.4 ± 0.03	NR
S253G	3.0 ± 0.20	NR
T343A	1.9 ± 0.10	NR
T229A/S253G	2.1 ± 0.10	54 ± 7
T229A/S253G/T343A	1.8 ± 0.10	74 ± 6

NR, no response to 5-HT.

tor (Fig. 3A and Table 3). The response elicited by activation of the mutant receptors was in the same range and varied from 1.9-fold to 3.0-fold basal level, except for the atypical mutant T229A, which was only 1.4-fold basal (Table 3). Thus, the mutations did not eliminate coupling of the 5-HT$_{1A}$ receptor to enhance calcium mobilization. Acute pretreatment (for 2 min) with 100 nM TPA to activate PKC abolished the action of 5-HT to enhance calcium levels for the wild-type receptor and for each of the single-mutant 5-HT$_{1A}$ receptors (Fig. 3 and Table 3). However, on acute pretreatment with TPA of cells expressing the double (T229A/S253G) and triple mutant, the 5-HT-induced calcium response was still observed (54 ± 6% and 74 ± 7% of treated; Table 3). The potency of TPA action on calcium mobilization for the wild-type receptor was 20 nM, whereas the EC_{50} value for the triple mutant was >1 μM (data not shown). These results indicate that the 5-HT$_{1A}$ receptor mutants with multiple mutations became progressively more resistant to PKC as the number of available PKC sites on the receptor decreased. Thus, the action of PKC to uncouple the 5-HT$_{1A}$ receptor from stimulation of calcium mobilization may involve multiple phosphorylation sites located on the receptor. In contrast, TPA did not change receptor-mediated inhibition of cAMP in either wild-type or triple-mutant receptor, indicating that PKC preferentially uncouples the calcium mobilization pathway.

Discussion

Activation of the multiple subtypes of PKC by TPA or diacylglycerol (23), the endogenous product of PLC activation, results in the phosphorylation of substrate proteins at the minimal consensus sequences (S/T)-X-(K/R) or (K/R)-X-(S/T) (24, 25). Phosphorylation of these consensus sequences found in the intracellular domains of the β-adrenergic receptor has been suggested to account for the action of acute TPA treatment in reducing receptor potency (26, 27). Likewise, the 5-HT$_{1A}$ receptor is rapidly phosphorylated after the addition of TPA, which correlates with a rapid and complete uncoupling from PLC activation (18). However, the site of action of PKC has not been identified. Although PKC-dependent phosphorylation of downstream signaling proteins (G_{i2}

[19, 28, 29] and PLC-β [20]) has been demonstrated, the functional importance of these events in receptor signaling in intact cells is not known. The increasing insensitivity to PKC activation of 5-HT_{1A} receptors mutated at one, two, or three consensus phosphorylation sites indicates that phosphorylation of the 5-HT_{1A} receptor serves as the primary site for uncoupling mediated by PKC activation. The residual inhibitory effect of PKC activation on the triple mutant could be mediated by phosphorylation at other sites on the receptor, G proteins, or effector. These results represent the first evidence that elimination of multiple sites for receptor phosphorylation blocks the complete uncoupling of a receptor signaling pathway induced by acute activation of PKC.

Suppression of receptor-mediated calcium mobilization by acute activation of PKC occurs for a large variety of receptors that couple to PLC activation and may serve as an important negative feedback pathway (23). Several receptors couple via PTX-sensitive G proteins (G_o/G_i) to enhance PLC activity and calcium mobilization in cells of mesenchymal origin (16). For example, both transfected (16) and endogenously expressed 5-HT_{1B} receptors in opossum kidney cells (30, 31) couple similarly to induce calcium mobilization. In Ltk^- cells transfected with 5-HT_{1B} receptor, acute TPA treatment completely inhibits coupling to calcium mobilization without blocking receptor-mediated inhibition of cAMP accumulation. Because the PKC sites we mutated in the 5-HT_{1A} receptor are conserved in the human and opossum 5-HT_{1B} receptors, these sites may also mediate PKC-induced uncoupling of the 5-HT_{1B} receptor from PLC. Whether the mechanisms implicated for the 5-HT_{1A} receptor can be extended to regulation of this or other receptor subtypes remains to be seen. Findings using PKC inhibitors have suggested that PKC may not mediate homologous receptor desensitization of the G_q protein-coupled α_{1B}-adrenergic receptor (32). In contrast, mutational analyses of the gastrin-releasing peptide receptor indicate that PKC sites mediate homologous desensitization of this G_q protein-coupled receptor (33). Using an antisense approach, Shih and Malbon have shown that the importance of second messenger-activated kinases (PKC) and receptor kinases can vary depending on the cell type studied (34). Thus, further investigation of the role of PKC in 5-HT_{1A} receptor desensitization may be more appropriately addressed in cells that endogenously express and regulate the receptor. Nevertheless, PKC-induced receptor phosphorylation may be a general signal to selectively inactivate receptor coupling to the PLC pathway.

Results obtained using 5-HT_{1A} receptor mutants indicate that receptor phosphorylation by PKC selectively modulates one pathway (calcium mobilization) without altering the other pathway (inhibition of cAMP level; Ref. 13 and Fig. 2). This may represent an amplified version of a negative feedback mechanism in which the product (diacylglycerol) inhibits its own generation via PLC but not receptor coupling to other effectors (i.e., adenylyl cyclase). This novel "pathway-selective" view of desensitization (16) contrasts with β-adrenergic receptor uncoupling, in which one signaling pathway (enhancement of adenylyl cyclase) has been investigated exhaustively but other pathways have not been examined (1–8). We demonstrated that the pathway-selective modulation of the 5-HT_{1A} receptor by PKC involves phosphorylation sites on the receptor, suggesting that receptor phosphorylation can sculpt receptor signaling, in this case converting a mixed phenotype into a strictly inhibitory one.

The selective action of TPA on the pathways could result from an apparently greater receptor reserve for the cAMP pathway than the calcium pathway of the 5-HT_{1A} receptor (35). Receptor reserve is observed when a maximal response is elicited by an agonist at a concentration that does not occupy all of the available receptors, resulting in a higher agonist potency (EC_{50} = 4.4 ± 1.3 versus 20 nM for calcium response) and lower dependence on receptor number (Ref. 16; compare Tables 2 and 3), which was observed for the cAMP response. Hypothetically, incomplete receptor phosphorylation by PKC could result in a population of nonphosphorylated 5-HT_{1A} receptors capable of mediating the cAMP response but insufficient to transduce a calcium response. However, the presence of PKC during the 20-min cAMP assay is sufficient time to allow maximal 5-HT_{1A} receptor phosphorylation by PKC (18). Thus, maximal activation of PKC for 20 min would be predicted to increase agonist EC_{50} or decrease maximal response but had no effect on 5-HT-induced inhibition of cAMP (Fig. 3). This observation argues against change in receptor reserve as an explanation for the selectivity of PKC action. Alternatively, use of different domains of the receptor to conduct separate signaling pathways may explain the pathway selectivity of PKC action (see below).

The structural basis for the specificity of PKC to selectively block receptor-mediated calcium mobilization but not cAMP accumulation has not been specifically addressed in the present study. The third cytoplasmic loop is clearly important for G protein coupling, and discrete modifications (by phosphorylation or point mutation) in this domain modify receptor (3, 22) signaling. In the β-adrenergic receptor, protein kinase A sites in the third cytoplasmic loop and carboxyl-terminal tail have been implicated in cAMP-mediated receptor uncoupling from adenylyl cyclase (7). Point mutations in the third loop adjacent to transmembrane domain VI cause constitutive activation in several receptors (36, 37), including the thyrotropin receptor. The A623I mutation of the thyrotropin receptor selectively activated coupling to adenylyl cyclase, and no constitutive activation of PI turnover was observed (37). On the other hand, mutation of A623 to a charged residue (E or K) resulted in uncoupling from PLC but not adenylyl cyclase activation (38). Thus, side-chain modifications at a single amino acid can drastically alter the selection of receptor signaling pathways.

Although the mutations we introduced did not greatly affect coupling of the 5-HT_{1A} receptor to various effectors, the capacity for receptor phosphorylation at a few residues selectively inhibits one pathway but not the other. The finding that peptides derived from the second intracellular loop of the 5-HT_{1A} receptor couple to inhibit adenylyl cyclase suggests that this region may be more important for coupling to this pathway; perhaps the third loop is more important for coupling to calcium mobilization. However, the latter suggestion must be interpreted with care because peptide-mediated actions were not sensitive to PTX (39).

In conclusion, multiple PKC consensus phosphorylation sites located in the third cytoplasmic loop of the 5-HT_{1A} receptor mediate pathway-selective uncoupling of the receptor from calcium mobilization by acute PKC activation.

Acknowledgments

We thank Dr. B. Collier, Dr. S. Morris, Marc Pinard, and Mohammad Ghahremani for critical analysis of the manuscript.

References

1. Collins, S., M. G. Caron, and R. J. Lefkowitz. Regulation of adrenergic receptor responsiveness through modulation of receptor gene expression. *Annu. Rev. Physiol.* **53:**497–508 (1991).
2. Lefkowitz, R. J., W. P. Hausdorff, and M. G. Caron. Role of phosphorylation in desensitization of the β-adrenoceptor. *Trends Pharmacol. Sci.* **11:** 190–194 (1990).
3. Kobilka, B. Adrenergic receptors as models for G protein-coupled receptors. *Annu. Rev. Neurosci.* **15:**87–114 (1992).
4. Lefkowitz, R. J. G protein-coupled receptor kinases. *Cell* **74:**409–412 (1993).
5. Inglese, J., N. J. Freedman, W. J. Koch, and R. J. Lefkowitz. Structure and mechanisms of the G-protein-coupled receptor kinases. *J. Biol. Chem.* **269:**23735–23738 (1993).
6. Lohse, M. J., S. Andexinger, J. Pitcher, S. Trukawinski, J. Codina, J. P. Faure, M. G. Caron, and R. J. Lefkowitz. Receptor-specific desensitization with purified proteins: kinase dependence and receptor specificity of β-arrestin and arrestin in the β-adrenergic receptor and the rhodopsin systems. *J. Biol. Chem.* **267:**8558–8564 (1992).
7. Bouvier, M., W. P. Hausdorff, A. De Blaisi, B. F. O'Dowd, B. K. Kobilka, M. G. Caron, and R. J. Lefkowitz. Removal of phosphorylation sites from the β2-adrenergic receptor delays onset of agonist-promoted desensitization. *Nature (Lond.)* **333:**370–373 (1988).
8. Hausdorff, W. P., M. Bouvier, B. F. O'Dowd, G. P. Irons, M. G. Caron, and R. J. Lefkowitz. Phosphorylation sites on two domains of the β2-adrenergic receptor are involved in distinct pathways of receptor desensitization. *J. Biol. Chem.* **264:**12657–12665 (1989).
9. Innis, R. B., and G. H. Aghajanian. Pertussis-toxin blocks 5-HT1A receptor and GABAB receptor-mediated inhibition of serotonergic neurons. *Eur. J. Pharmacol.* **143:**195–204 (1987).
10. Andrade, R., and R. A. Nicoll. Pharmacologically distinct actions of serotonin on single pyramidal neurones of the rat hippocampus recorded *in vitro*. *J. Physiol. (Lond.)* **394:**99–124 (1987).
11. Pennington, N. J., and J. S. Kelly. Serotonin receptor activation reduces calcium current in an acutely dissociated adult central neuron. *Neuron* **4:**751–758 (1990).
12. Albert, P. R., Q. Y. Zhou, H. H. M. VanTol, 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).
13. Liu, Y. F., and P. R. Albert. Cell-specific signaling of the 5-HT1A receptor: modulation by PK C and PK A. *J. Biol. Chem.* **266:**23689–23697 (1991).
14. 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).
15. 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).
16. Albert, P. R. Heterologous expression of G protein-linked receptors in pituitary and fibroblast cell lines. *Vitam. Horm.* **48:**59–109 (1994).
17. 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).
18. Raymond, J. R. Protein kinase C induces phosphorylation and desensitization of the human 5-HT1A receptor. *J. Biol. Chem.* **266:**14747–14753 (1991).
19. Strassheim, D., and C. C. Malbon. Phosphorylation of G_{i2} attenuates inhibitory adenylyl cyclase in neuroblastoma/glioma (NG 108–15) cells. *J. Biol. Chem.* **269:**14307–14313 (1994).
20. Ryu, S. H., U. Kim, M. I. Wahl, A. B. Brown, G. Carpenter, K. Huang, and S. G. Rhee. Feedback regulation of PLC-β by protein kinase C. *J. Biol. Chem.* **265:**17941–17945 (1990).
21. Hausdorff, W. P., M. Hnatowich, B. F. O'Dowd, M. G. Caron, and R. J. Lefkowitz. A mutation of the β2-adrenergic receptor impairs agonist activation of adenylyl cyclase without affecting high affinity agonist binding: distinct molecular determinants of the receptor are involved in physical coupling to and functional activation of Gs. *J. Biol. Chem.* **265:**1388–1393 (1990).
22. Ostrowski. J., M. A. Kjelsberg, M. G. Caron, and R. J. Lefkowitz. Mutagenesis of the β2-adrenergic receptor: how structure elucidates function. *Annu. Rev. Pharmacol. Toxicol.* **32:**167–183 (1992).
23. Nishizuka, Y. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature (Lond.)* **334:**661–665 (1988).
24. Kemp, B. E., and R. B. Pearson. Protein kinase recognition sequence motifs. *Trends Biochem. Sci.* **15:**342–346 (1990).
25. Kennelly, P. J., and E. G. Krebs. Consensus sequences as substrate specificity determinants for protein kinases and protein phosphatases. *J. Biol. Chem.* **266:**15555–15558 (1991).
26. Bouvier, M., N. Guibault, and H. Bonin. Phorbol-ester induced phosphorylation of the β2-adrenergic receptor decreases its coupling to Gs. *FEBS Lett.* **279:**243–248 (1991).
27. Yuan, N., J. Friedman, B. S. Whaley, and R. B. Clark. cAMP-dependent protein kinase and protein kinase C consensus site mutations of the β2-adrenergic receptor. *J. Biol. Chem.* **269:**23032–23038 (1994).
28. Bushfield, M., G. J. Murphy, B. E. Lavan, P. J. Parker, V. J. Hruby, G. Milligan, and M. D. Houslay. Hormonal regulation of G_{i2} α-subunit phosphorylation in intact hepatocytes. *Biochem. J.* **268:**449–457 (1990) .
29. Yatomi, Y., Y. Arata, S. Tada, S. Kume, and M. Ui. Phosphorylation of the inhibitory guanine-nucleotide-binding protein as a possible mechanism of inhibition by protein kinase C of agonist-induced Ca^{2+} mobilization in human platelet. *Eur. J. Biochem.* **205:**1003–1009 (1992).
30. Cerutis, D. R., N. A. Hass, L. J. Iversen, and D. B. Bylund. The cloning and expression of an OK cell cDNA encoding a 5-HT1B receptor. *Mol. Pharmacol.* **45:**20–28 (1993).
31. Lembo, P., and P. R. Albert. 5-HT1B receptors mediate a stimulatory calcium signaling opossum kidney (OK) cells: negative regulation by protein kinase C. *Can. J. Physiol. Pharmacol.* **72 (suppl. 1):**536 (1994).
32. Lattion, A. L., D. Divani, and S. Cotecchia. Truncation of the receptor carboxyl terminus impairs agonist-dependent phosphorylation and desensitization of the α_{1B}-adrenergic receptor. *J. Biol. Chem.* **269:**22887–22893 (1994).
33. Benya, R. V., T. Kusui, J. F. Battey, and R. T. Jensen. Chronic desensitization and down-regulation of the gastrin-releasing peptide receptor are mediated by a protein kinase C-dependent mechanism. *J. Biol. Chem.* **270:**3346–3352 (1995).
34. Shih, M., and C. C. Malbon. Oligodeoxynucleotides antisense to mRNA encoding protein kinase reveal distinctive cell-type-specific roles in agonist-induced desensitization. *Proc. Natl. Acad. Sci. USA* **91:**12193–12197 (1994).
35. Hoyer, D., and H. W. G. M. Boddeke. Partial agonists, full agonists, antagonists: dilemmas of definition. *Trends Pharmacol. Sci.* **14:**270–275 (1993).
36. Kjelsberg, M. A., S. Cotecchia, J. Ostrowski, M. G. Caron, and R. J. Lefkowitz. Constitutive activation of the α-1B adrenergic receptor by all amino acids substitutions at a single site: evidence for a region which contains receptor activation. *J. Biol. Chem.* **267:**1430–1433 (1992).
37. Kosugi, S., F. Okajima, T. Ban, A. Hidaka, A. Shenker, and L. D. Kohn. Mutation of alanine 623 in the third cytoplasmic loop of the rat thyrotropin (TSH) receptor results in a loss in the phosphoinositide but not cAMP signal induced by TSH and receptor autoantibodies. *J. Biol. Chem.* **267:** 24153–24156 (1992).
38. Parma, J., L. Duprez, J. Van Sande, P. Cochaux, C. Gervy, J. Mockel, J. Dumont, and G. Vassart. Somatic mutations in the thyrotropin receptor gene cause hyperfunctioning thyroid adenomas. *Nature (Lond.)* **365:**649–651 (1993).
39. Varrault, A., D. L. Nguyen, S. McClue, B. Harris, P. Jouin, and J. Bockaert. 5-Hydroxytryptamine 1A receptor synthetic peptides: mechanisms of adenylyl cyclase inhibition. *J. Biol. Chem.* **269:**16720–16725 (1994).

Send reprint requests to: Dr. Paul R. Albert, Department of Pharmacology and Therapeutics, McGill University, 3655 Drummond Street, Montreal, Quebec H3G-1Y6, Canada.