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Signal Transduction Differences Between 5-Hydroxytryptamine Type 2A and Type 2C Receptor Systems

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SUMMARY

The cDNAs for human 5-hydroxytryptamine (5-HT)$_{2C}$ and 5-HT$_{2A}$ receptors were stably transfected separately into parent Chinese hamster ovary cells, and cell lines in which levels of transfected receptor protein expression and accumulation of inositol phosphates in response to 5-HT were comparable were chosen for study. The effect of activation of these receptors on 5-HT$_{1B}$-like receptor-mediated responsiveness (i.e., inhibition of forskolin-stimulated cAMP accumulation) was studied. Activation of 5-HT$_{2C}$ receptors with 5-HT (0.1–100 μM) abolished the 5-HT$_{1B}$-like response, which returned when 5-HT$_{2C}$ receptors were blocked with mesulergine (1 μM). Furthermore, the maximal response to 5-carboxytryptamine was reduced in a concentration-dependent manner by the 5-HT$_{2A}$/5-HT$_{2C}$-selective partial agonist (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane. In contrast, activation of 5-HT$_{2A}$ receptors with either 5-HT or (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane did not alter the 5-HT$_{1B}$-like response. The reduction of 5-HT$_{1B}$-like responsiveness produced by 5-HT$_{2C}$ receptor activation was independent of protein kinase C activation and increases in the intracellular calcium concentration. Although 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors are strikingly similar in structure and pharmacology, and the signal transduction systems coupled to these receptors have been thought to be similar, if not identical, these data provide the first evidence for fundamental differences in the signal transduction systems of these 5-HT$_2$ receptor subtypes.

5-HT$_{2A}$ (formerly 5-HT$_2$) and 5-HT$_{2C}$ (formerly 5-HT$_{1C}$) receptors are subtypes of the 5-HT$_2$ receptor family. These G protein-coupled receptors share a high degree of amino acid sequence homology (80% in transmembrane regions), and their pharmacological profiles and signal transduction systems are very similar (1). Activation of both receptors results in PLC-mediated PI lipid hydrolysis (2, 3), which liberates the second messengers diacylglycerol and inositol trisphosphate. Diacylglycerol activates PKC, whereas inositol trisphosphate increases $[Ca^{2+}]_i$ (4, 5). Both increases in $[Ca^{2+}]_i$ and activation of PKC have been observed in response to activation of 5-HT$_{2A}$ (6, 7) and 5-HT$_{2C}$ (8, 9) receptors. In addition to coupling to PLC-mediated PI lipid hydrolysis, 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors have been shown to activate phospholipase A$_2$-mediated arachidonic acid release (10).[2] Furthermore, long term exposure to agonists (11–13) or antagonists (14, 15) causes down-regulation of both receptors, which suggests similarity in the regulation of these receptor systems.

Although the 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor systems are similar, some differences with respect to drug recognition properties have been identified. For example, 5-HT has higher affinity (K_d = 10 nM) for the low affinity state of the 5-HT$_{2C}$ receptor than for the low affinity state of the 5-HT$_{2A}$ receptor (K_d = 100–1000 nM) (16). In addition, these receptor subtypes are differentially sensitive to the antagonists spiperone and ketanserin (16).

To date, no fundamental differences in the signal transduction systems of these receptor subtypes have been identified. Therefore, we tested the hypothesis that 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors couple to the same (identical) signal transduction systems. Here we present evidence that, after stable transfection into CHO cells, activation of 5-HT$_{2C}$ but not 5-HT$_{2A}$ receptors reduces receptor-mediated inhibition of adenylyl cyclase activity. Moreover, this effect of 5-HT$_{2C}$ receptor activation is independent of activation of PKC and increases in $[Ca^{2+}]_i$. These data suggest that 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors may have fundamental differences in their signal transduction

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ABBREVIATIONS: 5-HT, 5-hydroxytryptamine; 5-CT, 5-carboxytryptamine; BAPTA, 1,2-bis(*o*-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid; $[Ca^{2+}]_i$, intracellular calcium concentration; DOI, (±)-1-(2,5-dimethoxy-4-iodophenyl)-2-aminopropane; HBSS, Hanks' balanced salt solution; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; 8-OH-DPAT, (±)-8-hydroxydipropylaminotetralin; IP, inositol phosphates; PI, phosphatidylinositol; PKC, protein kinase C; PLC, phospholipase C; FSCA, forskolin-stimulated cAMP accumulation; CHO, Chinese hamster ovary; AM, acetoxymethyl ester.

systems, which can be revealed through their differential effects on the 5-HT_1-like receptor that is naturally expressed in CHO cells.

Recently we reported that CHO cells naturally express a 5-HT receptor that inhibits adenylyl cyclase activity (17). We present here a more detailed characterization of this CHO 5-HT_1-like receptor, which appears to most closely resemble the 5-HT_{1B} receptor subtype.

Materials and Methods

Transfection. The human 5-HT_{2A} and 5-HT_{2C} genes were isolated as described previously (18). The coding region of the human 5-HT_{2A} or the human 5-HT_{2C} gene was cloned into the mammalian expression vector p198-DHFR-Hygro, a pBR322-based vector that contains a gene for hygromycin resistance, a simian virus 40 early promoter, and a cytomegalovirus enhancer element and simian virus 40 polyadenylation signal for transcribing the receptor cDNA (19). Stable transfection of CHO cells with the mammalian expression vector encoding the respective 5-HT receptor subtype was performed using a commercial calcium phosphate transfection kit (5 Prime→3 Prime, Boulder, CO), following the manufacturer's instructions. Stable clones were selected for their resistance to hygromycin (300 μg/ml), their ability to bind [^{3}H]ketanserin or [^{3}H]mesulergine, and their ability to accumulate IP in response to 5-HT, as described below.

Cell culture. CHO cells (CCL 61), originally obtained from the American Type Culture Collection, were maintained in minimum essential medium-α formulation supplemented with 5% fetal bovine serum and 100 units/ml penicillin/streptomycin. For cell lines stably expressing 5-HT_{2A} or 5-HT_{2C} receptors, 300 μg/ml hygromycin was included in the growth medium. For all experiments, cells were seeded into 24-well tissue culture vessels at a density of 4×10^4 cells/cm^2. After a 24-hr plating period, cells were washed with HBSS and placed into Dulbecco's modified Eagle's medium/F-12 medium (1:1) with 5 μg/ml insulin, 5 μg/ml transferrin, 30 nM selenium, 5.35 μg/ml linoleic acid, 0.1% bovine serum albumin (ITS$^+$; Collaborative Biomedical Products, Bedford, MA), 20 nM progesterone, and 100 μM putrescine (serum-free medium). Cells were grown in serum-free medium for 24 hr before all experiments.

5-HT_{2A} and 5-HT_{2C} receptor binding. Cells were washed twice with HBSS, scraped, and centrifuged at 500 × *g* for 5 min. Cell pellets were flash frozen in liquid nitrogen and stored at −135° until use. All membrane preparation procedures were done at 4°. Cell pellets were thawed, resuspended in buffer A (50 mM HEPES, 2 mM EDTA, 5 mM magnesium acetate, pH 7.4), homogenized (Polytron homogenizer, setting 6, for 10 sec), and centrifuged (40,000 × *g* for 15 min). The resulting membrane pellet was washed once with buffer A and once with buffer B (50 mM Tris, pH 7.4) and was resuspended in buffer B for use in the binding assay. Protein was determined by the method of Lowry *et al.* (20), using bovine serum albumin as a standard. Saturation binding assays using [^{3}H]mesulergine (5-HT_{2C}) or [^{3}H]ketanserin (5-HT_{2A}) in CHO membrane preparations were done as described previously for other radioligands (21), with the exception that incubations were carried out for 1 hr at 37°. Each experiment consisted of 15 concentrations of ligand (in duplicate) over a 3-log unit range. Nonspecific binding was determined in the presence of 1 μM mianserin (5-HT_{2C}) or 1 μM methysergide (5-HT_{2A}).

Measurement of cAMP accumulation. The 5-HT receptor-mediated inhibition of adenylyl cyclase activity was evaluated by measuring the inhibition of cAMP accumulated in response to 1 μM forskolin (15 min, at 37°), in the presence of the phosphodiesterase inhibitor rolipram. The amount of intracellular cAMP accumulated was measured by radioimmunoassay, as described previously (6). Briefly, cells were washed twice with HBSS containing 10 mM HEPES and 4 mM sodium bicarbonate, pH 7.4 (wash buffer). Cells were preincubated in 500 μl of wash buffer/well for 15 min, at 37° in 5% CO_2, before the addition of drugs. Where indicated, antagonists were present during this preincubation period. Forskolin (1 μM final concentration), rolipram (100 μM final concentration), and various drugs (final concentrations as indicated) were added to each well and incubation was continued for 15 min. Incubations were terminated by aspiration of the wash buffer and addition of 500 μl of ice-cold absolute ethanol. The ethanol extracts from individual wells were dried under a gentle stream of air and reconstituted in 500 μl of 50 mM sodium acetate, pH 6.2. The cAMP content was determined by radioimmunoassay of duplicate aliquots from each well. The cellular residue remaining in each well was solubilized with 1 N NaOH, and protein content was measured by the method of Lowry *et al.* (20), using bovine serum albumin as the standard.

Measurement of IP accumulation. Cells were grown for 24 hr in serum-free medium containing 1 μCi/ml *myo*-[^{3}H]inositol. Total IP accumulation in response to agonist stimulation in the presence of 20 mM LiCl for 10 min at 37° was determined as described previously (6), with the exception that termination of the incubation was done with 10 mM formic acid (22). The [^{3}H]IP (inositol monophosphate, inositol bisphosphate, and inositol trisphosphate) formed was separated according to the ion exchange method of Berridge *et al.* (23).

Data analysis. For saturation binding experiments, data were fit to eq. 1, using nonlinear regression analysis, to provide estimates of the parameters B_{max}, K_d, and n,

$$B = \frac{B_{max}}{1 + \left(\frac{K_d}{[D]}\right)^n} + m \cdot [D] \quad (1)$$

where B is the measured amount of radioligand bound (fmol/mg of protein) in the presence of various concentrations of radioligand ([D]), B_{max} is the maximal amount of radioligand bound, K_d is the concentration of radioligand producing half-maximal binding, n is the slope factor, and m is the slope of the linear regression line for "nonspecific" binding.

For IP accumulation experiments, data were expressed as a percentage, relative to basal values [% increase over basal = [(experimental dpm − basal dpm)/basal dpm] · 100], and fit by nonlinear regression to eq. 2, to provide estimates of R_{max}, EC_{50}, and n,

$$R = \frac{R_{max}}{1 + \left(\frac{EC_{50}}{[A]}\right)^n} \quad (2)$$

where R (response) is the accumulation of IP (percentage of basal accumulation) in response to a specified concentration of agonist ([A]), R_{max} is the maximal response produced by agonist, EC_{50} is the agonist concentration producing a half-maximal response, and n is the slope factor.

For cAMP determinations, concentration-response data were fit by nonlinear regression to eq. 3, to provide estimates of R_o, R_i, EC_{50}, and n

$$R = R_o - \frac{R_o - R_i}{1 + \left(\frac{[A]}{EC_{50}}\right)^n} \quad (3)$$

where R is the measured response (pmol of cAMP/mg of protein) at a given agonist concentration ([A]), R_o is the response in the absence of agonist, R_i is the response after maximal inhibition by agonist, EC_{50} is the concentration of agonist that produces a half-maximal response, and n is the slope factor. R_{max} (the maximal inhibition produced by the agonist) was calculated as $R_o - R_i$. Data were normalized for each experiment by defining the response to 1 μM forskolin as 100%. The pA_2 for methiothepin was determined by Schild analysis (24). The Schild regression slope was not significantly different from unity; therefore, the pA_2 was obtained from a regression constrained with a slope set to unity. The Student t test was used for statistical comparisons.

Materials. The following materials were purchased from commercial sources: forskolin (Calbiochem, La Jolla, CA); *myo*-[^{3}H]inositol, ^{125}I-cAMP tracer, [^{3}H]mesulergine, and [^{3}H]ketanserin (New England Nuclear, Boston, MA); anti-cAMP antibody (ICN Immunobiologicals, Lisle, IL); and CGS-12066B, 8-OH-DPAT HBr, 5-HT HCl, 5-CT, (±)-DOI HCl, mesulergine HCl, methiothepin mesylate, mianserin HCl, and quipazine dimaleate (Research Biochemicals, Inc., Natick, MA). All cell culture reagents were from GIBCO (Grand Island, NY). Rolipram was a generous gift from Berlex Laboratories (Cedar Knolls, NJ). CP 93129 was a generous gift from Pfizer (Groton, CT). RU 24969 [5-methoxy-3-(1,2,3,6-tetrahydro-4-pyridinyl)-1*H*-indolesuccinate] was a gift from Roussel-UCLAF (Paris, France) and sumatriptan succinate was a generous gift from Dr. Frank Yocca, Bristol-Myers Squibb (Wallingford, CT). *d*-Lysergic acid diethylamide was obtained from the National Institute on Drug Abuse. All other drugs and chemicals (reagent grade) were purchased from Sigma Chemical Co. (St. Louis, MO).

Results and Discussion

Characterization of human 5-HT$_{2A}$ and 5-HT$_{2C}$ receptors stably expressed in CHO cells. Parent CHO cells were screened for the presence of 5-HT$_2$ receptor subtypes by measuring IP accumulation in response to various 5-HT receptor agonists. No increases in IP accumulation were detected in response to 10 μM 5-HT, 1 μM DOI, 1 μM 5-CT, 100 nM *d*-lysergic acid diethlyamide, or 100 μM quipazine. After transfection with the human cDNA for the 5-HT$_{2A}$ or 5-HT$_{2C}$ receptor subtypes, several cloned lines were screened by functional responses, as well as ligand binding, for stable expression of the respective subtype. Two cell lines with comparable levels of receptor expression and IP accumulation, CHO-FA3 (5-HT$_{2A}$) and CHO-1C19 (5-HT$_{2C}$), were chosen for study. Analysis of mianserin-sensitive [^{3}H]mesulergine binding revealed that the CHO-1C19 cell line expressed a receptor density of 201 ± 48 fmol/mg of protein (mean ± standard error, three experiments). The pK_d value for mesulergine was 8.95 ± 0.09 (1.12 nM). Similarly, the CHO-FA3 cell line expressed a receptor density of 260 ± 98 fmol/mg of protein (mean ± standard error, three experiments), as measured by methysergide-sensitive [^{3}H]ketanserin binding. The pK_d for ketanserin was 9.47 ± 0.1 (0.34 nM). As illustrated in Fig. 1A, maximal 5-HT-mediated accumulation of IP was comparable between the two cell lines. The maximum percentage increase above basal levels for 5-HT in 5-HT$_{2C}$-expressing cells was 860 ± 134%, compared with 759 ± 137% for 5-HT$_{2A}$-expressing cells (mean ± standard error, four experiments). 5-HT was more potent at the 5-HT$_{2C}$ receptor than at the 5-HT$_{2A}$ receptor. The pEC$_{50}$ for 5-HT for 5-HT$_{2C}$ receptor activation was 7.55 ± 0.10 (28 nM), compared with 6.16 ± 0.04 (692 nM) for 5-HT$_{2A}$ receptor activation. For both the 5-HT$_{2C}$ and 5-HT$_{2A}$ receptor-containing cell lines there appeared to be an absence of receptor reserve. In cells treated for 30 min with 500 nM *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline, the maximal IP accumulation in response to 5-HT was reduced by 47% in CHO-1C19 cells and by 28% in CHO-FA3 cells, with no shift in EC$_{50}$ in either cell line (data not shown).

In contrast to 5-HT-elicited responses, the maximal level of IP accumulation in response to the partial agonist DOI was much greater in 5-HT$_{2C}$-expressing cells than in 5-HT$_{2A}$-expressing cells (521 ± 64% versus 127 ± 38%; mean ± standard error, three experiments) (Fig. 1B). The pEC$_{50}$ for DOI was indistinguishable between receptor systems, being 6.93 ± 0.09 (117 nM) and 6.85 ± 0.19 (141 nM) for 5-HT$_{2C}$ and 5-HT$_{2A}$ receptors, respectively.

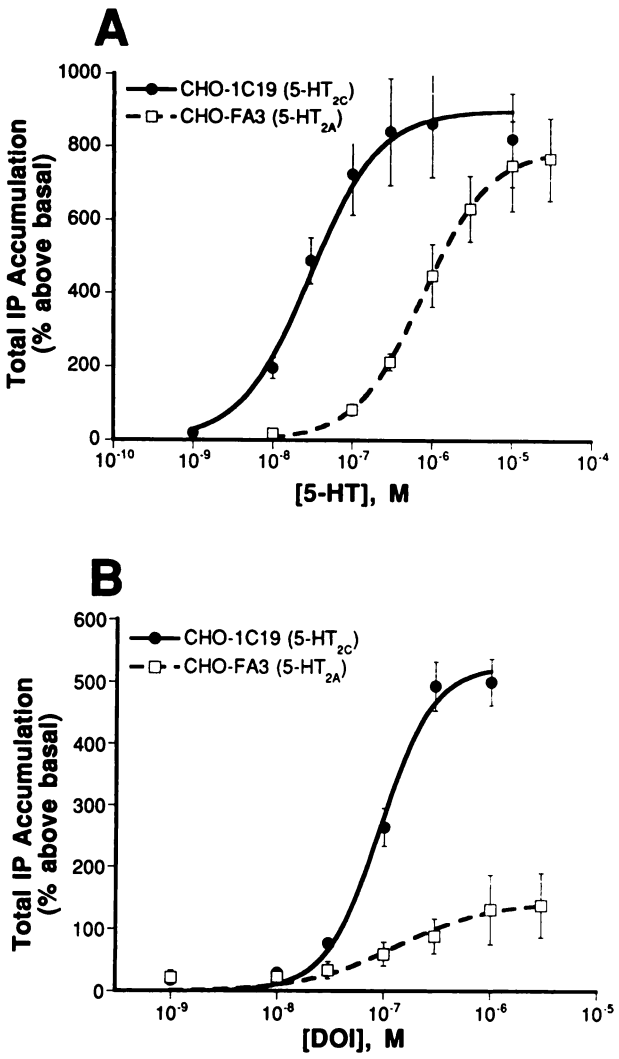

Fig. 1. IP accumulation in response to 5-HT (A) or DOI (B) in CHO cells transfected with human 5-HT$_{2C}$ (CHO-1C19 cells) or 5-HT$_{2A}$ (CHO-FA3 cells) receptor cDNA. Cells were labeled with 1 μCi/ml *myo*-[^{3}H]inositol for 24 hr. After washing, cells were incubated at 37° with 20 mM LiCl and the indicated concentrations of 5-HT or DOI. Accumulation of [^{3}H]IP was measured (in triplicate) after 10 min. Data, expressed as percentage of basal IP accumulation, were fit by nonlinear regression to eq. 2 (see Materials and Methods). Data shown are the mean ± standard error of three (DOI) or four (5-HT) experiments. Basal IP accumulation for CHO-1C19 and CHO-FA3 cells was 694 ± 110 and 597 ± 117 dpm (mean ± standard error), respectively. The mean EC$_{50}$ values in CHO-1C19 (5-HT$_{2C}$) and CHO-FA3 (5-HT$_{2A}$) cells were 28 and 692 nM for 5-HT and 140 and 117 nM for DOI, respectively.

These data are consistent with other reports that have shown that 5-HT is more potent at 5-HT$_{2C}$ than at 5-HT$_{2A}$ receptors (3, 25) and that DOI is a partial agonist with similar affinities for the two receptor subtypes (26). Because at equivalent receptor occupancy DOI activation of 5-HT$_{2C}$ receptors produced a much greater response (PI hydrolysis) than did DOI activation of 5-HT$_{2A}$ receptors, these data suggest that the efficacy of DOI at 5-HT$_{2C}$ receptors is greater than that at 5-HT$_{2A}$ receptors in these CHO cell lines.

Pharmacology of the naturally expressed 5-HT receptor in CHO cells. We have found that CHO cells naturally

express a 5-HT receptor coupled to the inhibition of adenylyl cyclase activity. Accumulation of cAMP in response to 1 μM forskolin (15 min at 37°) in parent CHO cells was inhibited by 5-CT and other 5-HT agonists in a concentration-dependent manner (Fig. 2; Table 1), with a rank order of agonist potency of 5-HT = 5-CT ≥ RU 24969 > CP 93129 > sumatriptan = pindolol > 8-OH-DPAT > CGS 12066B. The putative 5-HT_{1B} receptor agonist CP 93129 (27) was a full agonist and inhibited cAMP accumulation with an EC_{50} of 16.5 nM (Fig. 2; Table 1). Pindolol, sumatriptan, and 8-OH-DPAT displayed partial agonist activity. Neither FScA nor 5-CT-mediated inhibition was altered by the antagonists spiperone, mesulergine, or ketanserin (at 1 μM) (data not shown). However, concentration-response curves for 5-CT were shifted to the right by the antagonist methiothepin, in a parallel and surmountable manner (Fig. 3), with a pA_2 value of 7.9 (11 nM). In addition, the 5-CT-mediated inhibition of FScA was reduced from 74% (19.7 ± 5 pmol/mg of protein) to 0% (68 ± 16 pmol/mg of protein) (i.e., no inhibition) after 24-hr treatment with 50 ng/ml pertussis toxin. Neither basal cAMP levels nor FScA alone were altered by pertussis toxin treatment (8.2 ± 2.6 versus 6.6 ± 1.5 pmol/mg of protein and 77 ± 15 versus 63 ± 13 pmol/mg of protein, respectively; three experiments). These data are consistent with G_i/G_o protein mediation of inhibition of adenylyl cyclase activity, as has been shown for other 5-HT_1-like receptors (28–31).

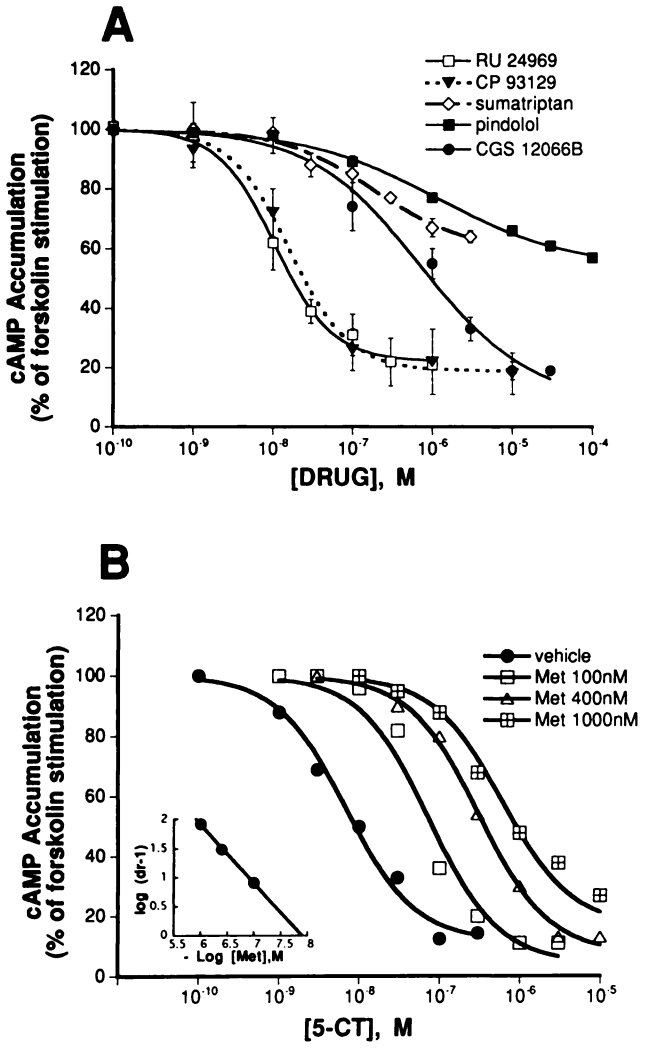

Fig. 2. Pharmacology of the 5-HT_{1B}-like receptor expressed naturally in CHO cells. A, Inhibition of FScA in parent CHO cells by selected agonists. Parent CHO cells were incubated with 1 μM forskolin and the indicated concentrations of agonists for 15 min, in the presence of the phosphodiesterase inhibitor rolipram (0.1 mM). cAMP accumulation was measured (in triplicate) by radioimmunoassay. Individual concentration-response data were fit by nonlinear regression to eq. 3 (see Materials and Methods). Data shown represent the mean of three to five experiments. FScA was 87 ± 11 pmol/mg of protein (31 experiments). The rank order of agonist potency is most consistent with that for a 5-HT_{1B} receptor subtype (also see Table 1). B, Antagonism by methiothepin (*Met*) of 5-CT-mediated inhibition of FScA. Parent CHO cells were preincubated with the indicated concentrations of methiothepin for 15 min before further incubation (15 min) with 1 μM forskolin and the indicated concentrations of 5-CT. Data were fit to eq. 3 by nonlinear regression, as described in Materials and Methods. Mean EC_{50} values for 5-CT were 6.5 (control), 59 (100 nM methiothepin), 227 (400 nM methiothepin), and 549 nM (1000 nM methiothepin) (three experiments). *Inset*, Schild regression analysis of methiothepin-induced shifts in 5-CT concentration-response curves. Data points represent the mean ± standard error of three experiments. The regression slope was not different from unity (1.006); therefore, a pA_2 of 7.95 (11 nM) was calculated with the slope constrained to unity.

TABLE 1

Effects of various 5-HT agonists on inhibition of FScA in parent CHO cells

Concentration-response data were fit to eq. 3 as described in Materials and Methods. Results shown are mean ± standard error of individual curve fits for the indicated number of experiments (*n*). FScA alone was 87 ± 11 pmol/mg of protein (mean ± standard error, 31 experiments).

Drug	pEC_{50}	EC_{50}	E_{max}	*n*
		nM	% inhibition	
5-HT	8.31 ± 0.10	4.9	81 ± 4	4
5-CT	8.47 ± 0.22	3.4	85 ± 5	5
RU 24969	8.10 ± 0.14	7.9	77 ± 6	3
CP 93129	7.78 ± 0.11	16.6	82 ± 6	4
Sumatriptan	7.04 ± 0.19	91.2	36 ± 3	4
Pindolol	6.87 ± 0.24	135.0	43 ± 5	5
8-OH-DPAT	6.57 ± 0.22	269.0	36 ± 4	3
CGS 12066B	6.09 ± 0.12	813.0	83 ± 6	3

The rank order of agonist potency for inhibition of FScA with this naturally expressed 5-HT receptor in CHO cells most closely resembles that for inhibition of FScA with the transfected rat 5-HT_{1B} subtype in Y-1 cells (5-CT > RU 24969 > 5-HT > pindolol = sumatriptan > CGS 12066B) (32). 5-HT_{1B} receptors coupled to the inhibition of adenylyl cyclase have been identified in only a few species, i.e., mouse (30), rat (28), opossum (31), and hamster (33), and are considered to be the rodent homologues of the 5-$HT_{1D\beta}$ receptor that is found in humans (28). Conclusive identification of the naturally expressed 5-HT receptor in CHO cells as the 5-HT_{1B} subtype is difficult, because the rank order of EC_{50} values for serotonergic agonists in these cells differs somewhat from the established rank order of EC_{50} values for inhibition of forskolin-stimulated adenylyl cyclase activity in rat substantia nigra membranes (34) and from the rank order of K_i values determined from binding experiments in rat cortical homogenates (35) and in human embryonic kidney 293 cells transfected with rat 5-HT_{1B} receptor cDNA (36). We are in the process of cloning the CHO 5-HT receptor to confirm subtype identity. Until results of cloning experiments are known, we will refer to the 5-HT receptor expressed naturally in CHO cells as a 5-HT_{1B}-like receptor subtype.

Reduction of 5-HT_{1B}-like receptor-mediated responsiveness by activation of 5-HT_{2C} receptors. In contrast to its effect in parent CHO cells (Table 1), 5-HT (at concentra-

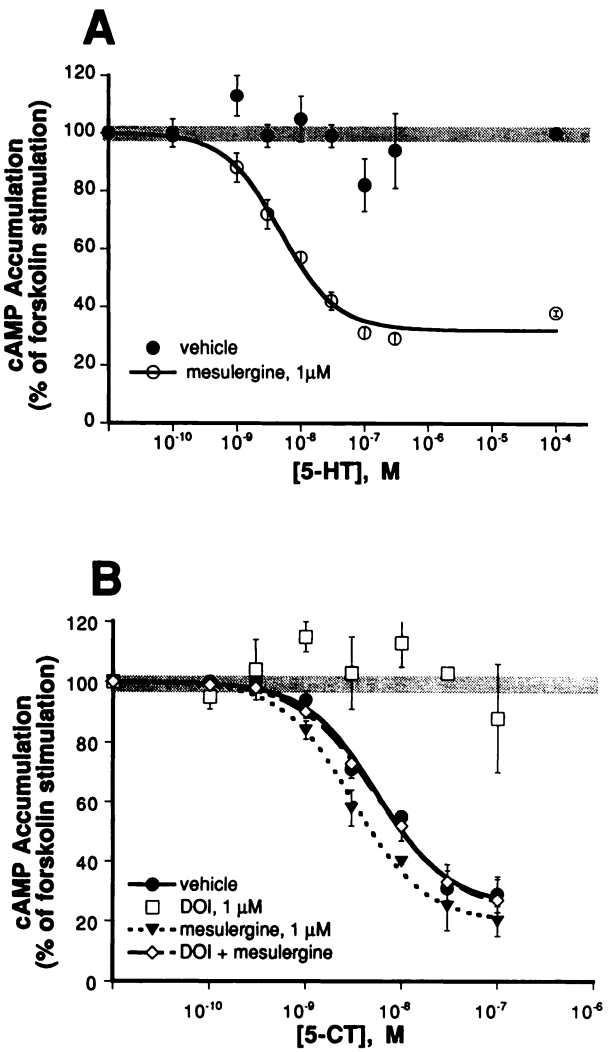

Fig. 3. Abolition of 5-HT$_{1B}$-like receptor-mediated inhibition of FScA by coactivation of 5-HT$_{2C}$ receptors. A, CHO-1C19 (5-HT$_{2C}$) cells were incubated for 15 min at 37° with 1 μM forskolin and the indicated concentrations of 5-HT, in the absence or presence of mesulergine (1 μM). 5-HT$_{1B}$-like receptor-mediated inhibition of FScA occurred only in the presence of mesulergine (to block 5-HT$_{2C}$ receptors). The pEC$_{50}$ for 5-HT in the presence of mesulergine was 8.23 ± 0.09 (5.8 nM) and maximal inhibition was 83 ± 2% (mean ± standard error, four experiments). Data represent the mean ± standard error of four or five experiments. B, CHO-1C19 (5-HT$_{2C}$) cells were incubated for 15 min at 37° with 1 μM forskolin and the indicated concentrations of 5-CT, in the absence or presence of DOI (1 μM) and/or mesulergine (1 μM; K_d = 1 nM). In the presence of DOI, 5-CT did not inhibit FScA. The pEC$_{50}$ values (mean ± standard error, three experiments) for 5-CT were 8.26 ± 0.07 (5.4 nM) for control, 8.50 ± 0.09 (3.1 nM) for mesulergine, and 8.27 ± 0.13 (5.7 nM) for mesulergine plus DOI. The maximal inhibition was 75 ± 8, 80 ± 6, and 77 ± 9% (mean ± standard error) for control, mesulergine, and mesulergine plus DOI, respectively. FScA was unaltered by addition of either DOI or mesulergine. cAMP levels (mean ± standard error) were 80 ± 10 pmol/mg of protein for forskolin alone and 95 ± 19 pmol/mg of protein for forskolin plus mesulergine (A) and 108 ± 23 pmol/mg of protein for forskolin alone, 106 ± 27 pmol/mg of protein for forskolin plus DOI, 114 ± 33 pmol/mg of protein for forskolin plus mesulergine, and 111 ± 34 pmol/mg of protein for forskolin plus both DOI and mesulergine (B).

tions up to 100 μM) did not inhibit FScA in the CHO-1C19 (5-HT$_{2C}$) cell line (Fig. 3A). However, when 5-HT$_{2C}$ receptors were blocked with the antagonist mesulergine, 5-HT produced a concentration-dependent inhibition of FScA, with a pEC$_{50}$ of 8.23 ± 0.09 (5.8 nM) (mean ± standard error, six experiments). Similarly, activation of 5-HT$_{2C}$ receptors with DOI (1 μM) also abolished 5-CT-mediated inhibition of FScA (Fig. 3B). When activation of 5-HT$_{2C}$ receptors was blocked with mesulergine, 5-CT-mediated inhibition of FScA was similar to the response seen in the absence of DOI and to the responses measured in parent cells (see Table 1). In the presence of mesulergine alone the E_{max} and pEC$_{50}$ values for 5-CT were 80 ± 6% and 8.50 ± 0.09 (3.1 nM), respectively, and in the presence of mesulergine and DOI the respective values were 77 ± 9% and 8.27 ± 0.13 (5.7 nM). DOI alone had no effect on FScA (108 ± 24 versus 106 ± 27 pmol of cAMP/mg of protein; mean ± standard error, four experiments). In the parent CHO cell line, DOI had no measurable effect on FScA or 5-CT-mediated inhibition (data not shown). Furthermore, in CHO-1C19 cells forskolin (1 μM) had no effect on either 5-HT- or DOI-mediated IP accumulation and activation of 5-HT$_{1B}$-like receptors with 5-CT (100 nM) did not alter IP accumulation in response to DOI (data not shown).

Lack of reduction of 5-HT$_{1B}$-like receptor-mediated responsiveness by activation of 5-HT$_{2A}$ receptors. In contrast to cells expressing 5-HT$_{2C}$ receptors (Fig. 3A), 5-HT inhibited FScA in cells transfected with human 5-HT$_{2A}$ receptors (Fig. 4A). In CHO-FA3 cells, maximal 5-HT$_{1B}$-like receptor-mediated inhibition of cAMP accumulation was observed within the full range of 5-HT concentrations (0.1–100 μM) needed to activate 5-HT$_{2A}$ receptors (see Fig. 1A). The 5-HT$_{2A}$ antagonist ketanserin (1 μM) did not alter the 5-HT-mediated inhibition of FScA or FScA itself. Fig. 4B shows that activation of 5-HT$_{2A}$ receptors with a maximal concentration of DOI (1 μM) (Fig. 1B) had no effect on 5-CT-mediated inhibition of FScA. The E_{max} and pEC$_{50}$ values for 5-CT alone were 72 ± 7% and 8.66 ± 0.22 (2 nM), respectively, and the values did not change in the presence of DOI [72 ± 13% and 8.52 ± 0.31 (3 nM), respectively]. The presence of the antagonist ketanserin (1 μM) had no effect on 5-CT-mediated inhibition of FScA either in the presence or in the absence of 1 μM DOI (data not shown). DOI had no effect on FScA (77 ± 14 versus 86 ± 16 pmol of cAMP/mg of protein; mean ± standard error, three experiments), and forskolin (1 μM) had no effect on 5-HT- or DOI-mediated IP accumulation in CHO-FA3 cells.

Maximal activation of 5-HT$_{2C}$ receptors with the partial agonist DOI produced a greater level of PI hydrolysis, compared with that obtained from 5-HT$_{2A}$ receptor activation (see Fig. 1B). To provide a stimulus for the 5-HT$_{2C}$ receptor pathway that is equivalent to maximal activation of the 5-HT$_{2A}$ pathway with DOI (IP accumulation of ≈130% above basal levels in CHO-FA3 cells), we examined the effect of 30 nM DOI (IP accumulation of ≈100% above basal levels in CHO-1C19 cells) on 5-CT-mediated inhibition of FScA. As shown in Fig. 5, 30 nM DOI shifted the EC$_{50}$ for 5-CT to the right approximately 7–8-fold (four experiments; $p < 0.005$), with no effect on the maximal inhibition. DOI at 100 nM produced a 10-fold shift to the right in EC$_{50}$ and reduced the maximal inhibition by 5-CT from 70% to 47% (four experiments; $p < 0.05$). As shown before (Fig. 3B), 1 μM DOI completely abolished 5-HT$_{1B}$-like receptor-mediated responsiveness.

Lack of effect of activation of PKC and elevation of [Ca^{2+}]$_i$ on 5-HT$_{1B}$-like receptor responsiveness. Two consequences of PI hydrolysis, i.e., liberation of diacylglycerol and inositol trisphosphate, lead to activation of the multifunctional

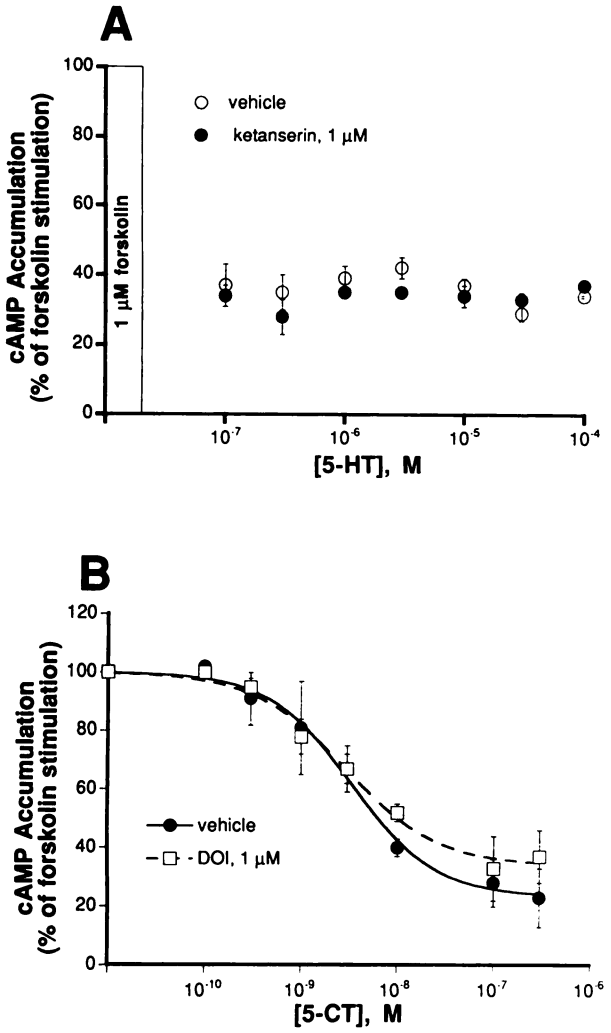

Fig. 4. Lack of effect of coactivation of 5-HT_{2A} receptors on 5-HT_{1B}-like receptor-mediated inhibition of FScA. A, CHO-FA3 (5-HT_{2A}) cells were incubated for 15 min at 37° with 1 μM forskolin and the indicated concentrations of 5-HT, in the absence or presence of ketanserin (1 μM; K_d = 0.3 nM). At the concentrations of 5-HT used to activate 5-HT_{2A} receptors (EC_{50} ≈ 700 nM) (see Fig. 1), 5-HT_{1B}-like receptor-mediated inhibition of FScA was maximal (EC_{50} ≈ 5 nM) (see Table 1). Data represent the mean ± standard error of three experiments. B, CHO-FA3 (5-HT_{2A}) cells were incubated for 15 min at 37° with 1 μM forskolin and the indicated concentrations of 5-CT, in the absence or presence of DOI (1 μM). DOI did not alter the 5-HT_{1B}-like receptor-mediated inhibition of FScA. The E_{max} and pEC_{50} values for 5-CT alone were 72 ± 7% and 8.66 ± 0.22 (2.2 nM) and for 5-CT in the presence of DOI were 72 ± 13% and 8.52 ± 0.31 (3 nM) (mean ± standard error, four experiments), respectively. Neither ketanserin nor DOI altered FScA. The cAMP levels (mean ± standard error) were 77 ± 14 pmol/mg of protein with forskolin alone, 78 ± 8 pmol/mg of protein in the presence of 1 μM ketanserin, and 86 ± 16 pmol/mg of protein in the presence of 1 μM DOI.

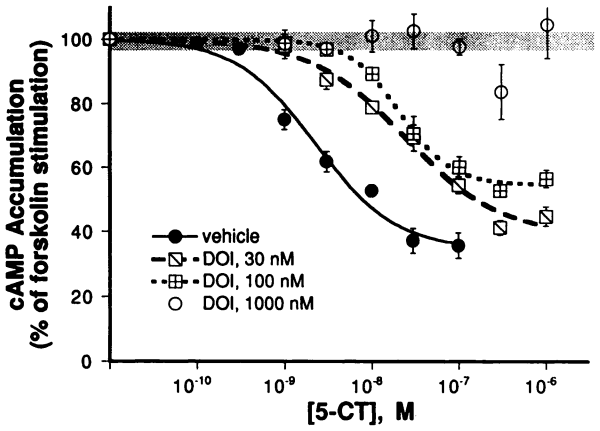

Fig. 5. Effect of activation of 5-HT_{2C} receptors with DOI on the 5-HT_{1B}-like receptor-mediated inhibition of FScA. CHO-1C19 (5-HT_{2C}) cells were incubated with the indicated concentrations of 5-CT in the presence or absence of DOI at 30 nM, 100 nM, or 1 μM. Activation of 5-HT_{2C} receptors with 30 nM DOI produced the same degree of PI hydrolysis as did 1 μM DOI acting on 5-HT_{2A} receptors (see Fig. 1B). DOI at 30 nM shifted the 5-CT concentration-response curve to the right by ~8-fold. The E_{max} and pEC_{50} values for 5-CT were 70 ± 7% and 8.50 ± 0.13 (3 nM) for 5-CT alone, 61 ± 4% and 7.64 ± 0.12 (23 nM) for 5-CT plus 30 nM DOI, and 47 ± 3% and 7.63 ± 0.12 (24 nM) for 5-CT plus 100 nM DOI, respectively. As shown in Fig. 3B, 1 μM DOI complete blocked 5-HT_{1B}-like receptor-mediated inhibition. Data shown are the mean ± standard error of four experiments.

protein kinase PKC and increases in $[Ca^{2+}]_i$, respectively. Because both 5-HT_{2A} and 5-HT_{2C} receptors increase PI hydrolysis in CHO cells (Fig. 1) and have been shown to activate PKC and increase $[Ca^{2+}]_i$ (6, 8, 9), it is unlikely that either PKC or increased $[Ca^{2+}]_i$ mediates the reduction in 5-HT_{1B}-like responsiveness produced by 5-HT_{2C} receptors. Consistent with this hypothesis, coincubation with the phorbol ester phorbol-12-myristate-13-acetate (1 μM), the synthetic diacylglycerol *sn*-1,2-dioctanoylglycerol (100 μM), or the calcium ionophore A23187 (1 μM) did not alter 5-CT-mediated inhibition of FScA in either CHO-1C19 cells (Fig. 6A) or parent CHO cells (data not shown). Furthermore, the presence of the PKC inhibitor staurosporine (1 μM) or the membrane-permeable calcium chelator BAPTA/AM (10 μM) did not alter the reduction of 5-CT-mediated inhibition of FScA by 100 nM DOI (Fig. 6B). Although more studies are needed to determine the involvement of PLC activation, these data indicate that the 5-HT_{2C} receptor-mediated reduction in the 5-HT_{1B}-like response is independent of two consequences of PI hydrolysis (i.e., activation of PKC and increases in $[Ca^{2+}]_i$). Furthermore, to the best of our knowledge, this is the first report of a PKC- and calcium-independent modulation of a G_i-linked receptor system by a PLC-coupled receptor.

These data suggest that, after transfection into CHO cells, 5-HT_{2A} and 5-HT_{2C} receptors couple to different signal transduction systems. Equivalent activation of the 5-HT_{2A} and 5-HT_{2C} receptor pathways produced qualitatively different responses in CHO cells. Activation of 5-HT_{2A} receptors with DOI (1 μM) did not alter the 5-HT_{1B}-like response, whereas equivalent activation of 5-HT_{2C} receptors (with 30 nM DOI) attenuated the 5-HT_{1B}-like response. Furthermore, maximal activation of 5-HT_{2C} receptors with 5-HT completely abolished the 5-HT_{1B}-like response, whereas maximal activation of 5-HT_{2A} receptors with 5-HT (which produced a degree of PI hydrolysis equivalent to that for the 5-HT_{2C} pathway) did not alter the 5-HT_{1B}-like response. This lack of effect of maximal activation of 5-HT_{2A} receptors with 5-HT on the 5-HT_{1B}-like response cannot be explained on the basis that the high concentration of 5-HT used (100 μM), relative to the 5-HT_{1B}-like EC_{50} (≈5 nM), surmounted, and therefore masked, a 5-HT_{2A}-mediated effect. A reduction in the maximal 5-HT_{1B}-like response occurred upon 5-HT_{2C} activation with 5-HT (Fig. 3) or with DOI at >30 nM (Fig. 5). Moreover, the 5-HT_{1B}-like response was abolished when 100 μM 5-HT was used to maximally activate

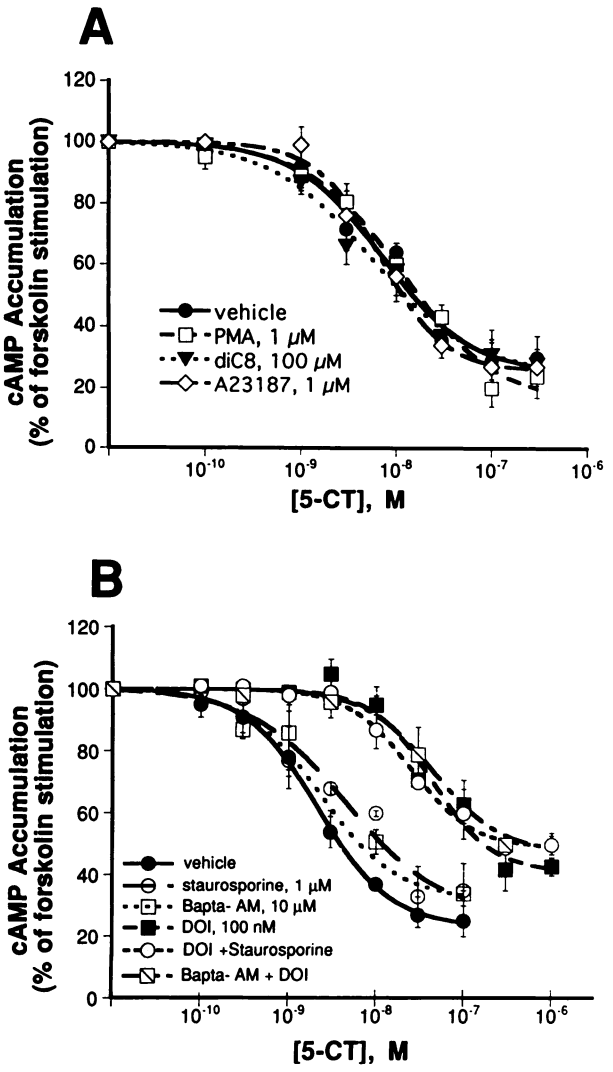

Fig. 6. Lack of effect of activation of PKC or increases in $[Ca^{2+}]_i$ (A) or the presence of the PKC inhibitor staurosporine or the calcium chelator BAPTA/AM (B) on 5-HT$_{1B}$-like receptor-mediated inhibition of FScA. A, CHO-1C19 (5-HT$_{2C}$) cells were incubated for 15 min at 37° with 1 μM forskolin, the indicated concentrations of 5-CT, and either dimethylsulfoxide vehicle (0.01%), the phorbol ester phorbol-12-myristate-13-acetate (*PMA*) (1 μM), the diacylglycerol analog *sn*-1,2-dioctanoylglycerol (*diC8*) (100 μM), or the calcium ionophore A23187 (1 μM). The E_{max} and pEC$_{50}$ values for 5-CT were 73 ± 8% and 8.29 ± 0.2 (5.1 nM), 72 ± 10% and 8.07 ± 0.08 (8.5 nM), 73 ± 4% and 8.29 ± 0.15 (5.2 nM), and 75 ± 6% and 8.16 ± 0.11 (7 nM) (mean ± standard error, four experiments) for dimethylsulfoxide control, phorbol-12-myristate-13-acetate, *sn*-1,2-dioctanoylglycerol, and A23187, respectively. FScA was not altered by any of the treatment conditions (110 ± 32, 115 ± 29, 138 ± 28, and 127 ± 21 pmol/mg of protein for control, phorbol-12-myristate-13-acetate, *sn*-1,2-dioctanoylglycerol, and A23187, respectively). Data represent the mean ± standard error of four experiments. B, CHO-1C19 (5-HT$_{2C}$) cells were preincubated for 5 min with staurosporine (1 μM) or dimethylsulfoxide vehicle or for 30 min with BAPTA/AM (10 μM). Concentration-response curves for 5-CT were obtained in the presence or absence of 100 nM DOI. The pEC$_{50}$ values (mean ± standard error, three experiments) for 5-CT with the indicated drug additions were 8.78 ± 0.23 (1.7 nM) for control, 8.59 ± 0.11 (2.6 nM) for staurosporine, 8.91 ± 0.34 (1.2 nM) for BAPTA/AM, 7.52 ± 0.17 (30 nM) for DOI, 7.32 ± 0.24 (48 nM) for DOI plus staurosporine, and 7.45 ± 0.27 (35 nM) for DOI plus BAPTA/AM. The maximal inhibition of FScA by 5-CT was 76 ± 6%, 68 ± 11%, 67 ± 2%, 51 ± 9%, 52 ± 3%, and 50 ± 4% for control, staurosporine, BAPTA/AM, DOI, DOI plus staurosporine, and DOI plus BAPTA/AM, respectively. None of these treatments significantly altered FScA, which averaged 80 ± 31 pmol/mg of protein. Data represent the mean ± standard error of three experiments.

the 5-HT$_{2C}$ receptor system. Therefore, if 5-HT$_{2A}$ receptors coupled to the same signal transduction pathway as did 5-HT$_{2C}$ receptors in CHO cells, we should have seen a reduction in the 5-HT$_{1B}$-like response when the 5-HT$_{2A}$ receptor system was activated maximally with 5-HT. However, the possibility remains that 5-HT$_{2A}$ receptors activate the signaling pathway that is inhibitory to the 5-HT$_{1B}$-like response with very low efficacy.

The mechanism by which activation of 5-HT$_{2C}$ receptors inhibits the 5-HT$_{1B\text{-like}}$ response is not clear. The simplest explanation is that 5-HT$_{2C}$ receptors couple to an additional effector mechanism, other than the PLC-mediated PI lipid hydrolysis system, that 5-HT$_{2A}$ receptors do not share. Perhaps 5-HT$_{2C}$ and 5-HT$_{2A}$ receptors couple to different G protein subtypes (e.g., different α and/or $\beta\gamma$ subunits). Although IP accumulation in response to 5-HT in both CHO-FA3 and CHO-1C19 cells was insensitive to pertussis toxin (data not shown), this does not define the G protein, because several different pertussis toxin-insensitve G proteins have been identified (e.g., $G_{\alpha q}$ and $G_{\alpha 11}$) (37). Furthermore, in light of recent reports indicating that $\beta\gamma$ subunits influence various signal transduction pathways [e.g., inhibition or stimulation of adenylyl cyclase activity (38), activation of phospholipase A_2 (39, 40), and activation of PLC (41–43)], it is intriguing to speculate that $\beta\gamma$ subunits released upon 5-HT$_{2C}$, but not 5-HT$_{2A}$, receptor activation may be involved in the reduction of 5-HT$_{1B}$-like receptor-mediated inhibition of adenylyl cyclase activity in CHO cells. Experiments to identify a 5-HT$_{2C}$-specific effector system in CHO-C19 cells are in progress.

In conclusion, to our knowledge the data presented here provide the first evidence for fundamental differences in the signal transduction pathways of 5-HT$_{2A}$ and 5-HT$_{2C}$ receptor subtypes. Identification of these differences in signal transduction may help explain differences in the physiological effects elicited by various drugs (e.g., hallucinogens versus nonhallucinogens) acting at these 5-HT receptor subtypes.

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