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Dual Coupling of Cloned Human 5-Hydroxytryptamine$_{1D\alpha}$ and 5-Hydroxytryptamine$_{1D\beta}$ Receptors Stably Expressed in Murine Fibroblasts: Inhibition of Adenylate Cyclase and Elevation of Intracellular Calcium Concentrations via Pertussis Toxin-Sensitive G Protein(s)

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SUMMARY

The second messenger coupling of cloned human 5-hydroxytryptamine (5-HT)$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptors stably expressed in murine fibroblasts (LM (tk^-)) was investigated. Clonal cell lines expressing similar receptor densities (B_{max} = 750–950 fmol/mg) were used in this study. 5-HT (EC_{50} = 1.5–2.0 nM) and sumatriptan (EC_{50} = 6–14 nM), a selective 5-HT$_{1D}$ agonist, produced dose-dependent inhibition of forskolin-stimulated cAMP accumulation in intact cells transfected with the 5-HT$_{1D\alpha}$ or 5-HT$_{1D\beta}$ receptor gene. The maximal inhibitory responses elicited by these agonists were slightly greater with the 5-HT$_{1D\alpha}$ receptor (~90%) than the 5-HT$_{1D\beta}$ receptor (~80%). 5-HT (EC_{50} = 1.7–2.4 nM) and sumatriptan (EC_{50} = 8–18 nM) also evoked dose-dependent elevations in intracellular calcium concentrations ($[Ca^{2+}]_i$), with EC_{50} values that were indistinguishable from those for inhibition of forskolin-stimulated cAMP accumulation. Cells expressing 5-HT$_{1D\beta}$ receptors displayed significantly larger 5-HT-induced increases in $[Ca^{2+}]_i$ than did cells expressing 5-HT$_{1D\alpha}$ receptors (206 nM versus 114 nM increase; $p < 0.01$). Dose-dependent elevations in inositol phosphates (IP) were also observed after application of 5-HT (EC_{50} = 29–54 nM) or sumatriptan (EC_{50} = 73–481 nM); the maximal increases in IP accumulation were modest (51–69%) for both 5-HT$_{1D}$ subtypes. In contrast to the cAMP and calcium responses, the concentration-response curves for IP accumulation were shifted to the right at least 10-fold. Methiothepin, a nonselective 5-HT$_1$ antagonist, competively antagonized the cAMP response, yielding an apparent dissociation constant (K_b) of 3–4 nM for the 5-HT$_{1D}$ receptors. Methiothepin (10 μM) significantly reduced the elevations in $[Ca^{2+}]_i$ (>90%) and IP (>75%) evoked by saturating concentrations (1 μM) of agonists. All three functional responses were significantly attenuated (>90%) by pretreatment with 100 ng/ml pertussis toxin. The sumatriptan-induced elevation of $[Ca^{2+}]_i$ via activation of the 5-HT$_{1D}$ subtypes may provide a molecular mechanism of action by which sumatriptan could directly constrict cerebral blood vessels and alleviate migraine symptoms.

The use of transfected cell lines expressing a single cloned receptor has provided valuable insights into signal transduction mechanisms activated by that subtype. Murine fibroblasts (LM tk^-) have been used as a host cell line for the functional expression of a variety of cloned GPCRs, including 5-HT$_{1A}$ (1, 2), dopamine D_2 (3, 4), 5-HT$_{1D\alpha}$ (5), and 5-HT$_{1D\beta}$ (6). Expression of these cloned receptors has permitted detailed characterization of functional coupling to effector mechanisms mediated through G proteins. Intracellular signaling pathways that have been shown to be modulated by recombinant GPCRs are adenylate cyclase, phospholipase C, phospholipase A_2, phospholipase D, $[Ca^{2+}]_i$, calcium channels, and potassium channels, some of which are mediated through PTX-sensitive G proteins (7).

Molecular cloning has demonstrated that pharmacologically defined 5-HT$_{1D}$ receptors are encoded by two separate but closely related genes, designated 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$, which are members of the GPCR superfamily (5). These receptors display highly conserved transmembrane homology (75%) and similar binding properties and second messenger coupling (in-

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ABBREVIATIONS: GPCR, G protein-coupled receptor; 5-HT, 5-hydroxytryptamine; $[Ca^{2+}]_i$, intracellular free calcium concentration; CRC, concentration-response curve; EC_{50}, concentration of agonist required to produce 50% maximal response; E_{max}, maximal response; FSCA, forskolin-stimulated cAMP accumulation; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HBS, HEPES-buffered saline; IP_3, inositol-1,4,5-trisphosphate; IP, inositol phosphates; PGE_2, prostaglandin E_2; PTX, pertussis toxin; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; AM, acetoxy methyl ester.

hibition of adenylate cyclase) (5, 8). However, additional signal transduction pathways activated by these receptor subtypes have not yet been fully investigated, because of the lack of either model cell lines expressing endogenous 5-HT_{1D} subtypes or selective pharmacological agents. To investigate additional effector coupling mechanisms activated by the two cloned 5-HT_{1D} subtypes, stable cell lines were generated using LM (tk^-) fibroblasts as the host. We provide evidence that, in addition to inhibition of FSCA, activation of the 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors results in an increase in $[Ca^{+2}]_i$ and that both effects are mediated via a PTX-sensitive G protein. The elevation of $[Ca^{+2}]_i$, rather than the inhibition of FSCA, may be the major pathway leading to contraction of smooth muscles in blood vessels containing 5-HT_{1D} receptors.

Materials and Methods

Functional expression of 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors in LM (tk^-) cells. The entire coding region of the 5-$HT_{1D\alpha}$ or 5-$HT_{1D\beta}$ receptor gene was subcloned into the expression vector pSVL, and stable cell lines expressing the 5-$HT_{1D\alpha}$ (L-1Dα) and 5-$HT_{1D\beta}$ (L-1Dβ) subtypes were generated using the calcium phosphate method (5). Cells were grown under standard conditions (37°, 5% CO_2) in Dulbecco's modified Eagle medium (GIBCO, Grand Island, NY). Clonal cell lines were grown in suspension for radioligand binding assays or as monolayers for the functional assays.

Radioligand binding studies. Cells were grown in suspension and harvested at a density of approximately 1.5×10^6 cells/ml, and membranes were prepared by standard techniques (9). [^{3}H]5-HT saturation studies were performed as described previously (10). Briefly, membranes harvested from stable transfectants were incubated with eight concentrations of [^{3}H]5-HT (0.5–75 nM), in the absence or presence of 10 μM unlabeled 5-HT, for 30 min at 37° in the dark. The assay was terminated by vacuum filtration. Protein concentrations were determined by the method of Bradford (11), using bovine serum albumin as the standard.

cAMP assays. The cAMP assay was performed according to the method outlined previously, with minor modifications (10). Cells were plated in 96-well microtiter plates at a density of 5×10^3 cells/well and were allowed to grow for 96 hr. Cells (~95% confluent) were preincubated in Dulbecco's modified Eagle's medium containing 10 mM HEPES, 5 mM theophylline, and 10 μM pargyline. Methiothepin (100 nM) was also included during this preincubation in antagonist experiments. Cells were incubated with forskolin or PGE_2 (10 μM final concentration) and various concentrations of agonists for an additional 10 min (37°, 5% CO_2). Initial studies indicated that the EC_{50} values for the 5-HT-mediated inhibition of cAMP were similar when intracellular cAMP was elevated by forskolin or via activation of endogenous PGE_2 receptors. We chose forskolin as the stimulatory agent due the superior signal to noise ratio, compared with PGE_2 (>5-fold versus ~2-fold). The assay was terminated by removal of medium and addition of 0.1 M HCl. Intracellular cAMP was measured by radioimmunoassay, as described by the manufacturer (Advanced Magnetics, Cambridge, MA).

IP assays. IP accumulation was measured essentially as described by Forray and El-Fakahany (12). Briefly, harvested cells were centrifuged, suspended in fresh medium, and incubated with 5 μCi of *myo*-[^{3}H]inositol for 1 hr (37°, 5% CO_2). Cells were washed to remove unincorporated radioactivity, resuspended in medium containing 10 mM LiCl, and incubated with appropriate drugs for 1 hr (37°, 5% CO_2). In separate experiments, methiothepin was added 20 min before the addition of agonists. The reaction was terminated by addition of 50% trichloroacetic acid, and IP were isolated by ion exchange chromatography (13). Columns were washed sequentially with water, 60 mM ammonium formate, and 5 mM sodium borate, and total [^{3}H]IP were eluted with 1 M ammonium formate/0.1 M formic acid. Radioactivity in the final fraction (containing IP) was measured by liquid scintillation counting.

$[Ca^{2+}]_i$ measurements. $[Ca^{2+}]_i$ was measured as described previously (14, 15), with minor modifications. Cells were plated onto 35-mm glass coverslip dishes coated with poly-D-lysine (MatTek Corporation, Ashland, MA). For loading with fura-2/AM, cells were washed three times with HBS (in mM: NaCl, 150; KCl, 5; HEPES, 20; $CaCl_2$, 1; $MgCl_2$, 1; glucose, 10; pH 7.4 with 10 N NaOH) and then incubated for 15–20 min at room temperature with a solution containing 10 μM fura-2/AM, 0.025% pluronic F-127, and 2% calf serum in HBS. After loading, the cells were again washed three times with HBS and then 2 ml of HBS were added. Cells were allowed to "chase" for approximately 10 min before measurement.

$[Ca^{2+}]_i$ was measured using an inverted Leitz Fluovert microscope equipped for UV transmission, with a Nikon 40× oil-immersion objective. Illumination was provided by a 100-W mercury arc lamp (Osram). Excitation was alternated between 340 and 380 nm using a motorized filter wheel. The time between the 340- and 380-nm readings was <1 sec; a ratio pair was taken approximately every 2 sec. Fluorescence emission (510 nm) was recorded with a Leitz photometer interfaced to a data acquisition and control unit from Kramer Scientific (Yonkers, NY). Cells were placed on the microscope and basal $[Ca^{2+}]_i$ was measured for about 15 sec. Drug solutions were then added and measurements were continued for at least 5 min. All measurements were conducted at room temperature.

To determine $[Ca^{2+}]_i$, background readings (taken from a dish lacking cells) were subtracted from the experimental record. To convert values to $[Ca^{2+}]_i$, the background-corrected ratios were compared with calibration buffers using a K_d for fura of 224 nM, as reported by Grynkiewicz *et al.* (16) and confirmed under our conditions.

Data analysis. Nonlinear regression analysis was used to analyze [^{3}H]5-HT saturation data generated in binding assays, and CRCs were obtained in the functional assays. Binding data were analyzed using ACCUFIT (Lundon Software, Chagrin Falls, OH) to obtain K_d and B_{max} values, and functional data were analyzed using GRAPHpad software (San Diego, CA) to obtain EC_{50} and E_{max} values. The apparent K_b value of methiothepin was determined by the response to various concentrations of agonist in the presence and absence of 100 nM methiothepin, using the equation K_b = [antagonist]/(dose ratio − 1), where dose ratio is defined as the EC_{50} for agonist in the presence of methiothepin divided by the EC_{50} value for agonist in its absence (17). Student's *t* test was used to determine statistical significance between experimental conditions.

Materials. Compounds were obtained from the following sources: [^{3}H]5-HT (specific activity, 24.4 Ci/mmol), New England Nuclear (Boston, MA); *myo*-[^{3}H]inositol (specific activity, 91 Ci/mmol), Amersham (Arlington, IL); 5-HT and PTX, Sigma Chemical Co. (St. Louis, MO); methiothepin, Biomol Research Laboratories (Plymouth Meeting, PA); and fura-2/AM, Molecular Probes (Eugene, OR).

Results

Stable cell lines expressing similar levels of either human 5-$HT_{1D\alpha}$ or 5-$HT_{1D\beta}$ receptors were selected for the present study (Table 1). B_{max} values obtained from [^{3}H]5-HT saturation experiments ranged from 750 to 950 fmol/mg of protein for L-1Dα and L-1Dβ. The K_d values of [^{3}H]5-HT derived from saturation studies were similar for both clonal cell lines, aver-

TABLE 1

Expression levels of the 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors prepared from membranes of cells stably expressing either the 5- $HT_{1D\alpha}$ or 5-$HT_{1D\beta}$ receptor gene

Binding assays were conducted as described in Materials and Methods. K_d and B_{max} values were determined by nonlinear regression analysis. Values represent mean values ± standard errors from three determinations.

Binding parameters	5-$HT_{1D\alpha}$	5-$HT_{1D\beta}$
K_d (nM)	6.6 ± 0.7	6.2 ± 1.3
B_{max} (fmol/mg of protein)	946 ± 210	773 ± 79

aging 6 nM. The K_d value for [^{3}H]5-HT obtained in saturation studies matched previously obtained K_i values for 5-HT (4 nM) derived from competition studies (5). The affinity of sumatriptan for the 5-HT$_{1D\alpha}$ receptor (K_i = 3.4 nM) was approximately 2-fold higher than that for the 5-HT$_{1D\beta}$ receptor (K_i = 7.7 nM) (5). No specific [^{3}H]5-HT binding to membranes prepared from untransfected LM (tk^-) cells was observed. Additionally, no agonist-induced modulation of intracellular cAMP levels, elevation of $[Ca^{2+}]_i$, or stimulation of IP accumulation was detected in untransfected host cells.

As shown previously, both the cloned human 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptors stably expressed in LM (tk^-) cells are negatively coupled to adenylate cyclase (5, 6). Both 5-HT and sumatriptan, a 5-HT$_{1D}$ agonist (18), produced dose-dependent inhibition of FSCA (Fig. 1). Response parameters (EC_{50} and E_{max} values) for both compounds are summarized in Tables 2 and 3. The EC_{50} values for both agonists closely matched their K_i values obtained in binding studies. The maximal inhibitions produced by 5-HT and sumatriptan were slightly greater with L-1Dβ (91% and 86%, respectively) than with L-1Dα (81% and 77%, respectively). However, no reduction of basal cAMP levels was observed in transfected LM (tk^-) cells stably expressing the 5-HT$_{1D\alpha}$ or 5-HT$_{1D\beta}$ receptors, possibly due to the low basal levels of intracellular cAMP (~70 fmol/ml/min).

To determine whether the 5HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptors could couple to additional signaling pathways, we used fura-2 microspectrofluorometry to monitor changes in $[Ca^{2+}]_i$ in stably transfected LM (tk^-) cells. Basal $[Ca^{2+}]_i$ values were similar in untransfected LM (tk^-) cells (113 ± 9 nM, three dishes), L-1Dα (91 ± 17 nM, 77 dishes), and L-1Dβ (99 ± 16 nM, 80 dishes).

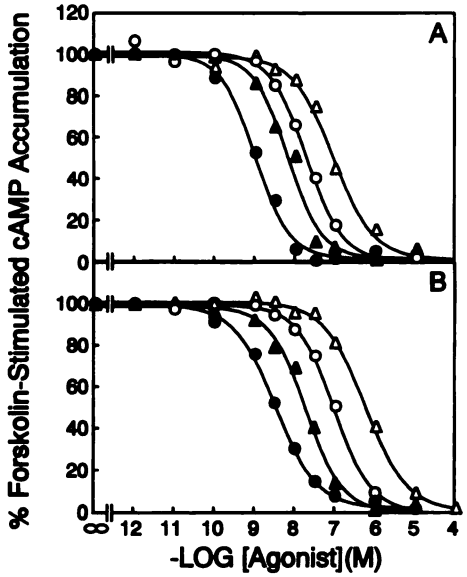

Fig. 1. Representative CRC for the inhibition of FSCA by 5-HT and sumatriptan in the absence and presence of 100 nM methiothepin in intact cells stably expressing cloned human 5-HT$_{1D\alpha}$ (A) or 5-HT$_{1D\beta}$ (B) receptors. Data were normalized to 100%, relative to the maximal inhibition obtained with the individual agonists at each subtype. Each data point represents a single determination. *Point preceding the break in the curve*, FSCA in the absence of drugs. Basal levels of cAMP averaged 0.0744 ± 0.0164 and 0.0692 ± 0.018 pmol/ml/min for L-1Dα and L-1Dβ, respectively (three experiments). Forskolin (10 μM) stimulated cAMP production 4.7 ± 0.7- and 6.9 ± 1.0-fold for L-1Dα and L-1Dβ, respectively (three experiments). Response parameters are summarized in Tables 2 and 3. ●, 5-HT; ▲, sumatriptan; ○, 5-HT plus methiothepin; △, sumatriptan plus methiothepin.

TABLE 2

Response parameters of 5-HT and sumatriptan for functional coupling to signal transduction pathways by the cloned human 5-HT$_{1D\alpha}$ receptors stably expressed in murine fibroblasts (LM (tk^-))

Assays were conducted as described in Materials and Methods. All functional data were analyzed by nonlinear regression analysis to obtain EC_{50} and E_{max} values. Values represent mean values ± standard errors from three to five determinations.

Functional assay	5-HT		Sumatriptan	
	EC_{50}	E_{max}	EC_{50}	E_{max}
	nM		nM	
cAMP inhibition	1.5 ± 0.7	81 ± 6%[a]	6.1 ± 1.4	77 ± 4%
$[Ca^{2+}]_i$ elevation	2.4 ± 0.9	114 ± 15 nM[b]	8.3 ± 2.1	93 ± 8 nM
IP stimulation	29 ± 9	69 ± 11%[c]	73 ± 24	62 ± 8%

[a] E_{max} values represent the maximal percentage inhibition of forskolin-stimulated cAMP responses.
[b] E_{max} values represent the maximal change in $[Ca^{2+}]_i$ (peak minus basal).
[c] E_{max} values represent percentage increase over basal.

TABLE 3

Response parameters of 5-HT and sumatriptan for functional coupling to signal transduction pathways by the cloned human 5-HT$_{1D\beta}$ receptors stably expressed in murine fibroblasts (LM (tk^-))

Assays were conducted as described in Materials and Methods. All functional data were analyzed by nonlinear regression analysis to obtain EC_{50} and E_{max} values. Values represent mean values ± standard errors from three to five determinations.

Functional assay	5-HT		Sumatriptan	
	EC_{50}	E_{max}	EC_{50}	E_{max}
	nM		nM	
cAMP inhibition	2.0 ± 0.6	91 ± 4%[a]	14 ± 3	86 ± 4%
$[Ca^{2+}]_i$ elevation	1.7 ± 1.0	206 ± 32 nM[b]	18 ± 7	142 ± 33 nM
IP stimulation	54 ± 10	51 ± 13%[c]	481 ± 60	55 ± 12%

[a] E_{max} values represent the maximal percentage inhibition of forskolin-stimulated cAMP responses.
[b] E_{max} values represent the maximal change in $[Ca^{2+}]_i$ (peak minus basal).
[c] E_{max} values represent percentage increase over basal.

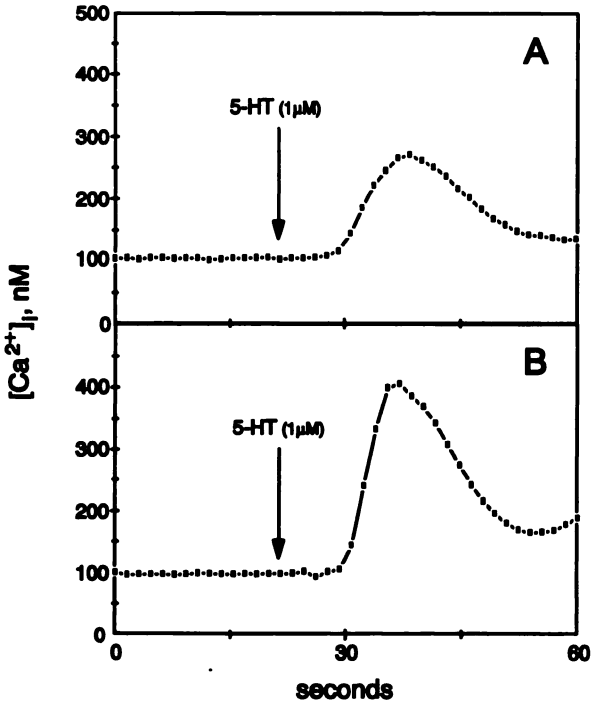

Fig. 2. Representative traces for the elevation of $[Ca^{2+}]_i$ by 5-HT in intact cells stably expressing cloned human 5-HT$_{1D\alpha}$ (A) and 5-HT$_{1D\beta}$ (B) receptors. 5-HT was added at the concentration indicated (*arrow*).

As shown in Fig. 2, 1 μM 5-HT evoked rapid elevations in $[Ca^{2+}]_i$ in both stable cell lines. This finding is consistent with preliminary results of Albert *et al.* (19), who recently demonstrated that the cloned human 5-$HT_{1D\beta}$ receptor can elevate $[Ca^{2+}]_i$ in murine fibroblasts and that the magnitude of the calcium response is dependent upon the host cell. In contrast, a calcium response was not observed by Levy *et al.* (6) after stable transfection of the 5-$HT_{1D\beta}$ receptor gene into LM (tk^-) cells, even though expression levels were comparable to those of the present study. The reason for this discrepancy is not known at the present time but may relate to clonal variation in the host cell line.

The 5-HT-evoked calcium responses consisted of an initial peak followed by a prolonged plateau phase (Fig. 2). The plateau phase varied considerably between dishes but was generally small; the nature of these two phases is described below. In some cases, oscillations were observed during the plateau phase but were not further characterized. The magnitude of the peak calcium response was dose dependent; representative CRCs for L-1Dα and L-1Dβ are shown in Fig. 3, A and B, respectively, and the pooled results are tabulated in Tables 2 and 3. Sumatriptan also increased $[Ca^{2+}]_i$ in L-1Dα and L-1Dβ; however, the peak response to 1 μM sumatriptan was consistently smaller (20–30%) than that to 1 μM 5-HT (Tables 2 and 3). The EC_{50} values of both agonists to elicit elevations in $[Ca^{2+}]_i$ closely matched their EC_{50} values for inhibition of cAMP accumulation for both subtypes. The maximal responses to saturating concentrations (1 μM) of 5-HT and sumatriptan in L-1Dβ were consistently larger (81% and 53%, respectively) than responses elicited by these agonists in L-1Dα.

Increases in $[Ca^{2+}]_i$ can result from calcium release from intracellular stores, calcium influx from the extracellular medium, or both. To determine the source of the elevated $[Ca^{2+}]_i$ resulting from stimulation of 5-HT_{1D} receptors, we compared the responses to 1 μM 5-HT in normal HBS (containing 1 mM Ca^{2+}) and in Ca^{2+}-free HBS (containing 1 mM EGTA and no added Ca^{2+}). The peak calcium response in EGTA-containing medium was 128 ± 24 and 111 ± 10% (four experiments) of control values in L-1Dα and L-1Dβ, respectively, indicating that the peak response was the result of release of calcium from intracellular stores. The effect of EGTA on the plateau phase was variable and was not studied further.

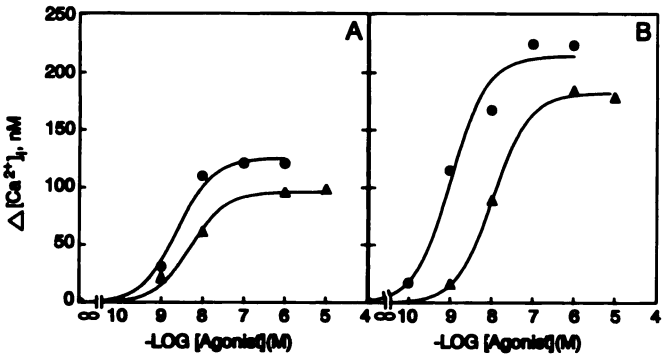

Fig. 3. CRCs for the elevation of $[Ca^{2+}]_i$ by 5-HT and sumatriptan in intact cells stably expressing cloned human 5-$HT_{1D\alpha}$ (A) and 5-$HT_{1D\beta}$ (B) receptors. Data represent the peak increase above basal levels of $[Ca^{2+}]_i$ versus the concentration of agonist. Each data point represents the mean of triplicate determinations obtained from separate experiments; standard deviations averaged <7% of the mean. *Point preceding the break in the curve*, elevation of $[Ca^{2+}]_i$ in the absence of drugs. Basal levels of $[Ca^{2+}]_i$ averaged 91 ± 17 nM (77 dishes) and 99 ± 16 nM (80 dishes) for L-1Dα and L-1Dβ, respectively. Response parameters are summarized in Tables 2 and 3. ●, 5-HT; ▲, sumatriptan.

The ability of the cloned human 5-HT_{1D} subtypes to activate phospholipase C was also investigated. 5-HT and sumatriptan increased the production of IP in a dose-dependent manner (Fig. 4). The maximal stimulation was only 50–70% above basal levels in both cell lines. It should be noted that the basal levels of IP were approximately 10-fold higher in L-1Dα (11,548 ± 2,160 cpm; three experiments) than in L-1Dβ (993 ± 141 cpm; three experiments); the basal levels of IP in L-1Dβ were similar to those in untransfected LM (tk^-) cells (1111 ± 155 cpm, two experiments). Thus, the absolute increase in IP accumulation produced by agonists was actually 12-fold greater with L-1Dα than L-1Dβ, even though L-1Dβ produced a larger calcium response (Fig. 2; Tables 2 and 3). The reason for this differential precursor incorporation for L-1Dα is not understood. However, differences in basal IP levels have been observed previously in our laboratory with various clonal lines stably expressing recombinant human α_1-adrenergic receptors, using the same LM (tk^-) cells as transfection host.[1] Interestingly, the EC_{50} values for 5-HT and sumatriptan for increasing IP accumulation were >1 order of magnitude greater (i.e., lower affinity) than the EC_{50} values for cAMP and calcium responses (Tables 2 and 3).

The ability of methiothepin, a nonselective 5-HT_1 antagonist, to block these three functional responses was investigated. Methiothepin competively antagonized, in a surmountable manner, the inhibitory cAMP response to both 5-HT and sumatriptan (Figs. 1 and 5). The affinities of methiothepin for the 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ subtypes were similar. The apparent K_b values for methiothepin averaged 3–4 nM and were inde-

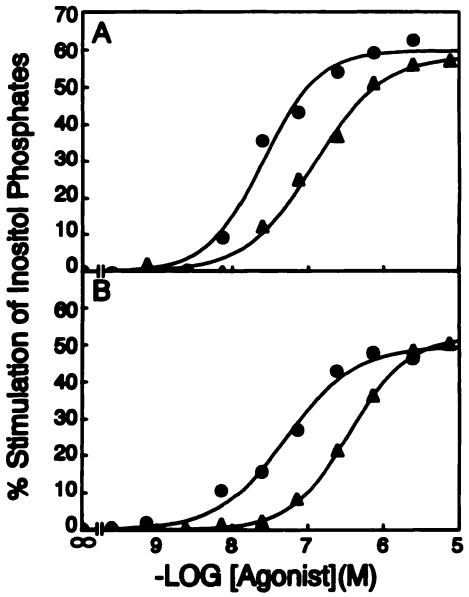

Fig. 4. Representative CRC for the stimulation of IP accumulation by 5-HT and sumatriptan in intact cells stably expressing the cloned human 5-$HT_{1D\alpha}$ (A) or 5-$HT_{1D\beta}$ (B) receptors. Data represent the percentage increase above basal levels of IP versus the concentration of agonist. Each data point represents the mean of duplicate determinations and standard deviations averaged <5% of the mean. *Point preceding the break in the curve*, elevation of IP accumulation in the absence of drugs. Basal levels of IP averaged 11,548 ± 2,160 cpm and 993 ± 141 cpm for L-1Dα and L-1Dβ, respectively (three experiments). Response parameters are summarized in Tables 2 and 3. ●, 5-HT; ▲, sumatriptan.

[1] C. Forray, unpublished observations.

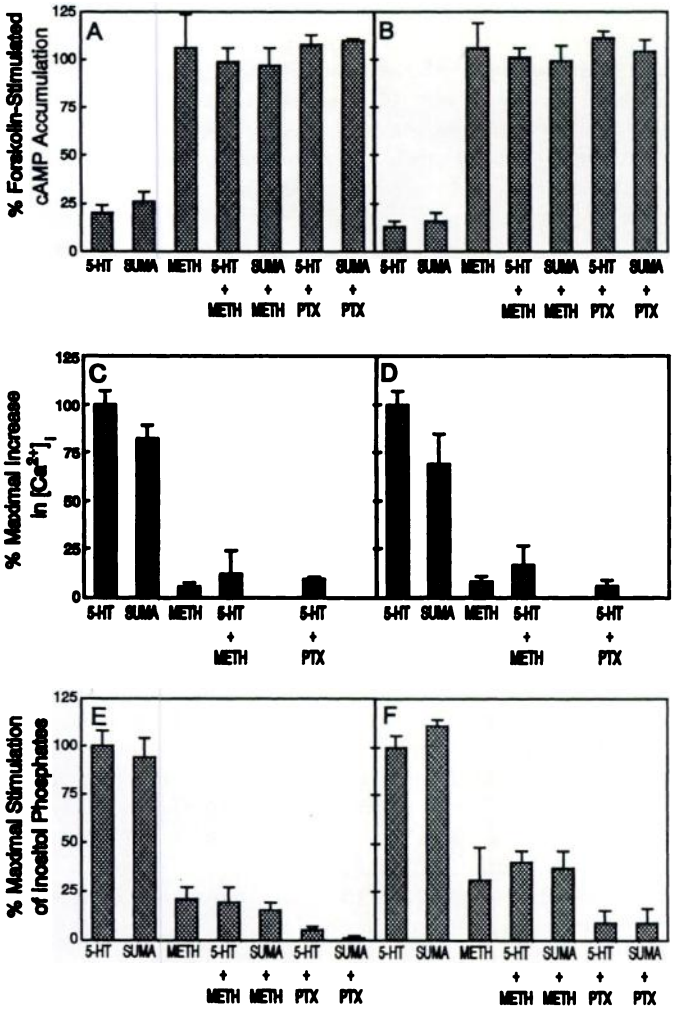

Fig. 5. Effect of methiothepin (*METH*) and PTX pretreatment on the inhibition of FSCA, elevation of $[Ca^{2+}]_i$, and stimulation of IP accumulation elicited by 5-HT and sumatriptan (*SUMA*) in intact cells stably expressing the cloned human 5-HT$_{1D\alpha}$ (A, C, and E) or 5-HT$_{1D\beta}$ (B, D, and F) receptors. Methiothepin (10 μM; 20-min preincubation) or PTX (100 ng/ml; 12–18-hr preincubation) was added before the addition of saturating concentrations (1 μM) of 5-HT and sumatriptan. Basal levels of cAMP, $[Ca^{2+}]_i$, and IP in the absence of PTX for L-1Dα and L-1Dβ were the same as those reported in the legends to Figs. 1, 3, and 4, respectively. PTX did not significantly alter basal levels of cAMP (0.0820 ± 0.0242 and 0.0824 ± 0.0240 pmol/ml/min), $[Ca^{2+}]_i$ (104 ± 17 and 121 ± 11 nM), or IP (10, 298 ± 546 and 1221 ± 346 cpm) for L-1Dα and L-1Dβ, respectively (three experiments). Data represent mean ± standard error values from three determinations.

pendent of the agonist used (data not shown). Methiothepin displayed higher affinity at both clones in this functional assay than would be predicted from its K_i values for inhibiting the binding of [^{3}H]5-HT (K_i = 11 nM and 25 nM for 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$, respectively) (5). The reason for this is not understood but may reflect differences in assay protocols (membranes versus whole cells) and the extreme lipophilicity of methiothepin (20). Methiothepin also antagonized the peak calcium and IP responses resulting from activation of the 5-HT$_{1D}$ subtypes. In contrast to its lack of intrinsic activity in the cAMP assay (i.e., a silent antagonist), methiothepin behaved as a weak partial agonist when tested at 10 μM in the calcium (108 ± 5% of basal levels, nine dishes, and 111 ± 3% of basal levels, eight dishes, for L-1Dα and L-1Dβ, respectively; $p <$ 0.01) and IP (~25% of the 5-HT response; $p < 0.01$) assays (Fig. 5). Methiothepin blocked the elevation of $[Ca^{2+}]_i$ and the production of IP elicited by 5-HT and sumatriptan to a level obtained with methiothepin alone, consistent with antagonism predicted for a partial agonist.

To gain insight into which G proteins mediate the inhibition of FSCA, elevation of $[Ca^{2+}]_i$, and stimulation of IP hydrolysis by the cloned human 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ receptors, we examined the sensitivity of these receptor-mediated responses to PTX. PTX ADP-ribosylates a cysteine residue of the α subunit of GDP-bound heterotrimeric G_i/G_o isoforms, uncoupling the G protein from the receptor and thereby preventing receptor interactions with effector systems (21). In contrast, members of the G_q/G_{11} family are resistant to this toxin (21). Pretreatment (12–18 hr) of cells with 100 ng/ml PTX significantly reduced (>90%) the maximal responses for cAMP inhibition, $[Ca^{2+}]_i$ elevation, and IP accumulation elicited by saturating concentrations of 5-HT and sumatriptan in both L-1Dα and L-1Dβ (Fig. 5). However, PTX pretreatment did not significantly affect basal levels of these intracellular second messengers (data not shown). Because PTX had no effect on the high basal IP levels observed with L-1Dα, it appears that the 5-HT$_{1D\alpha}$ receptor does not exhibit significant agonist-independent activity but, rather, the high basal levels may be a property of this stable clonal line. This hypothesis is supported by our observation that basal $[Ca^{2+}]_i$ is similar in both transfected and untransfected cells. These results indicate that all three functional responses are likely to be mediated by PTX-sensitive G_i and/or G_o proteins.

Discussion

We and others demonstrated previously that, when expressed in appropriate host cell lines, both 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ subtypes negatively couple to adenylate cyclase (Fig. 1) (5, 6, 22, 23, 44), as expected for members of the 5-HT$_{1D}$ receptor subfamily (8). The two well established physiological responses associated with activation of 5-HT$_{1D}$-like receptors are contraction of vascular smooth muscle and inhibition of 5-HT release (24); however, the relationship between these physiological responses and the reduction of intracellular cAMP levels is not fully understood (25). In the case of muscle contraction, it is well established that elevation of $[Ca^{2+}]_i$ is the primary trigger. In the present study we demonstrate that both cloned human receptor subtypes can elevate $[Ca^{2+}]_i$ (Figs. 2 and 3) and stimulate phospholipase C (Fig. 4), thereby providing a signaling mechanism by which endogenous 5-HT can produce muscle contraction via activation of 5-HT$_{1D}$ receptors.

The elevation of $[Ca^{2+}]_i$ can result from the release of calcium from intracellular stores and/or influx from the extracellular medium. In the dog saphenous vein, a widely used model of 5-HT$_{1D}$-like receptors, sumatriptan-induced contraction has been shown to be mostly dependent upon extracellular calcium (26), which enters the cell through both voltage-dependent and -independent calcium channels (25). In transfected murine fibroblasts, the elevation of $[Ca^{2+}]_i$ after activation of the cloned human 5-HT$_{1D}$ subtypes is primarily the result of release from intracellular stores, with only a small component arising from influx. However, because LM (*tk*$^-$) cells appear to lack voltage-sensitive calcium channels,[2] it remains to be determined

[2] L. Borden, unpublished observations.

whether the cloned human subtypes can activate such channels. Additionally, we find that the magnitude of calcium entry after activation of cloned human α_1-adrenergic receptors is dependent on the transfectant host,[2] suggesting that fibroblast-like cells differ in their expression of endogenous calcium channels. Nonetheless, the results demonstrate clearly the ability of both subtypes to potently alter $[Ca^{2+}]_i$.

Serotonin has been shown to play an important role in the pathophysiology of migraine, which is believed to result from excessive dilation of cerebral blood vessels (27). Sumatriptan, a "5-HT_{1D}-selective" agonist (18), has been shown to be effective in the treatment of migraine (28). The molecular mechanism whereby sumatriptan exerts its therapeutic effects is not known at present; however, both neurogenic (29) and vascular (30) hypotheses have been forwarded. Sumatriptan has been shown to block dilation of the cerebral vasculature through activation of 5-HT_{1D}-like receptors that mediate constriction of arteriovenous anastomoses (31), presumably via a calcium-dependent mechanism. The demonstration that both endogenous (32) (see below) and transfected (present study) 5-HT_{1D} subtypes can elevate $[Ca^{2+}]_i$ provides a molecular basis whereby sumatriptan can directly constrict the cerebral vasculature to alleviate migraine symptoms.

It is important to note that the ability of 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ subtypes to both inhibit cAMP and elevate $[Ca^{2+}]_i$ is not restricted to transfected systems but appears to be characteristic of a number of endogenous G_i-linked receptors. Specifically, Ebersole *et al.* (32) have recently demonstrated that an endogenous 5-HT_1-like receptor can inhibit cAMP production and increase $[Ca^{2+}]_i$ in cells derived from bovine basilar artery. The pharmacological profile of the receptor(s) mediating these responses resembles those of the cloned 5-HT_{1D} subtypes. Due to the closely related pharmacological profiles of these subtypes (5) and the lack of selective agents (18), the subtype mediating these responses has not been determined, nor is it known whether these responses are evoked within the same cell. Additionally, sumatriptan has been shown to cause contraction via a 5-HT_1-like receptor exhibiting a 5-HT_{1D} pharmacological profile in a variety of isolated vascular preparations (33, 34). Moreover, endogenous α_2-adrenergic receptors, another subfamily of inhibitory GPCRs, have been shown to elicit vascular smooth muscle contraction primarily via an elevation of $[Ca^{2+}]_i$ through pathways that are both dependent and independent of external calcium (35). These data obtained in isolated tissue preparations further support our hypothesis that the 5-HT_{1D}-mediated increases in $[Ca^{2+}]_i$ may represent a relevant physiological response. In contrast, the role of cAMP inhibition in smooth muscle contraction is not fully understood at present (25, 26, 35) (see below).

In addition to muscle contraction, the other well established role of 5-HT_{1D} receptors is their ability to inhibit release of 5-HT from synaptic terminals (presynaptic inhibition) (18, 24). As with muscle contraction, there is no clearly defined role for decreased intracellular cAMP levels in this process (25). Likewise, the elevation of $[Ca^{2+}]_i$ by the recombinant 5-HT_{1D} subtypes cannot account for the inhibition of 5-HT release by these receptors, which requires a reduction of $[Ca^{2+}]_i$. Presynaptic inhibition of transmitter release is associated with a reduction of calcium entry via voltage-gated channels, which may be mediated by activation of K^+ channels and/or direct inhibition of voltage-gated Ca^{2+} channels (25, 36). Modulation of ionic conductances has yet to be demonstrated for 5-HT_{1D} receptors; however, correlative electrophysiological responses have been observed with the related 5-HT_{1A} receptor in several preparations (37). Thus, G_i-coupled receptors can activate a variety of signaling pathways and elicit divergent physiological responses, depending on the cell type and, possibly, subcellular distribution (e.g., cell body versus terminal).

Although agonist-mediated inhibition of FSCA is not physiological, it nonetheless provides a useful tool for evaluating the functional activation of inhibitory GPCRs. Importantly, the EC_{50} values of 5-HT for cAMP inhibition were indistinguishable in L-1Dα and L-1Dβ when either forskolin or PGE_2 (via activation of endogenous PGE_2 receptors) was used as the stimulatory agent, despite the different mechanisms of adenylate cyclase activation (see Materials and Methods). Furthermore, similar pharmacological profiles were also obtained for the elevation of $[Ca^{2+}]_i$ with both 5-HT_{1D} subtypes (Tables 2 and 3). Therefore, it appears that the inhibition of FSCA by 5-HT and related compounds can be used as a valid model for investigating 5-HT_{1D} receptor function.

Multiple effector coupling has been observed for a number of cloned GPCRs in heterologous expression systems (Ref. 38 and references cited therein); in some cases activation of these signal transduction pathways requires different concentrations of agonist. For example, Fargin *et al.* (39) demonstrated that the 5-HT_{1A} receptor transfected into HeLa cells coupled to the inhibition of adenylate cyclase and stimulation of phospholipase C; however, the concentration of 5-HT required for activation of phospholipase C was 100-fold higher than that required for inhibition of adenylate cyclase. It has often been assumed that the higher affinity response represents the physiologically relevant effector system, whereas the lower affinity response is the result of promiscuous coupling. It is thus important to note that, for both human 5-HT_{1D} receptors, the EC_{50} values for elevation of $[Ca^{2+}]_i$ closely match those for inhibition of FSCA (Tables 2 and 3), an accepted signaling pathway for the 5-HT_{1D} subtypes (8). A similar correspondence between EC_{50} values for cAMP inhibition and $[Ca^{2+}]_i$ elevation was observed previously with the 5-HT_{1A} (2) and dopamine D_2 (4) receptors transfected in murine fibroblasts. Moreover, the magnitude of the 5-HT-induced calcium response observed with the 5-$HT_{1D\beta}$ subtype (E_{max} = 200 nM elevation; Table 3) is quantitatively similar to the peak calcium response obtained with a phospholipase C-coupled α_1-adrenergic receptor transfected in the same host cell line.[3]

Regarding the dual coupling, it is possible that alterations in calcium and cAMP are coupled, rather than independent. Recently, Garritsen *et al.* (40) demonstrated that bradykinin-induced elevations in $[Ca^{2+}]_i$ directly inhibit adenylate cyclase activity in PC12 cells, presumably by acting at the catalytic subunit of this enzyme. We are currently investigating whether the increase in $[Ca^{2+}]_i$ evoked by the 5-HT_{1D} receptors is causally related to inhibition of cAMP levels.

As mentioned above, the peak calcium increase that occurs in response to activation of 5-$HT_{1D\alpha}$ and 5-$HT_{1D\beta}$ receptors is primarily the result of release of calcium from intracellular stores, as demonstrated by its maintenance in calcium-free medium. Because IP_3 is the most well characterized mediator of calcium release, we examined the ability of 5-HT and su-

[3] L. Borden and C. Forray, unpublished observations.

matriptan to stimulate production of IP in LM (tk^-) cells expressing the 5-HT$_{1D\alpha}$ or 5-HT$_{1D\beta}$ receptor gene. Surprisingly, the response was modest, amounting to a maximal increase of only 50–70% above basal levels (Tables 2 and 3), similar to the maximal IP responses observed with transfected 5-HT$_{1A}$ (2) and dopamine D$_2$ (4) receptors in the same cell host. Furthermore, the EC$_{50}$ values for the response were nearly 10-fold higher (i.e., lower affinity) than those for the increase in $[Ca^{2+}]_i$ (Tables 2 and 3). These data are in contrast to results reported for the 5-HT$_{1A}$ (2) and dopamine D$_2$ (4) receptors, for which the EC$_{50}$ values for elevation of $[Ca^{2+}]_i$ and IP accumulation were closely matched. A poor correspondence between IP production and elevations in $[Ca^{2+}]_i$ has been observed previously in several systems (see Ref. 14 and references cited therein). A possible explanation for this phenomenon is that there exists a receptor reserve for IP$_3$ receptors, such that a low concentration of IP$_3$ is adequate to discharge calcium from intracellular stores. Alternatively, it is possible that IP$_3$ reaches high concentrations in localized regions of the cell, which would not be detectable by the whole-cell methods used in the present study. A less likely although formal possibility is that IP$_3$ is not the mediator of calcium release in the system under study. Recently, cADP-ribose has been suggested as a novel mediator of calcium release (for review, see Ref. 41). It is not yet known whether this intracellular messenger is involved in the responses mediated by 5-HT$_{1D}$ receptors.

Second messenger responses of GPCRs are mediated by different classes of G proteins (21). The classical dogma is that inhibition of adenylate cyclase occurs via activation of PTX-sensitive G$_i$ and/or G$_o$ proteins, whereas production of IP (via activation of phospholipase C) and the resultant increase in $[Ca^{2+}]_i$ are mediated by PTX-insensitive members of the G$_q$/G$_{11}$ class. Unexpectedly, we found that cAMP inhibition, IP production, and increases in $[Ca^{2+}]_i$ were all sensitive to PTX (Fig. 5), suggesting that inhibitory G$_i$ and/or G$_o$ proteins activate these signaling pathways. PTX-sensitive calcium and IP responses have also been observed in aortic smooth muscle cells in response to thrombin (42) and in LM (tk^-) fibroblasts transfected with 5-HT$_{1A}$ (2) or dopamine D$_2$ (4) receptors. Fargin *et al.* (43) demonstrated that the 5-HT$_{1A}$ receptor transfected into HeLa cells coupled to both cAMP inhibition and enhanced IP accumulation, and they suggested that both effects were mediated by G$_{i\alpha 3}$. Immunocytochemistry revealed that LM (tk^-) cells express G$_{i\alpha 1}$, G$_{i\alpha 2}$, and G$_{i\alpha 3}$ (44). Thus, we are presently unable to determine whether inhibition of cAMP and increases in $[Ca^{2+}]_i$ result from the activation of a single or multiple G proteins. Experiments are currently in progress to determine the specificity of G protein coupling involved in these receptor-mediated signaling pathways.

Although PTX-sensitive increases in IP and/or $[Ca^{2+}]_i$ have been observed in a variety of systems (see above), the underlying mechanism remains obscure. Although there is no conclusive evidence that G$_{i\alpha}$ isoforms can directly activate phospholipase C (21), it has recently been shown that $\beta\gamma$ subunits can activate certain isozymes of phospholipase C (45, 46). Because the $\beta\gamma$ units are common to many G proteins, this could provide a mechanism whereby activation of G$_i$ could activate phospholipase C indirectly via dissociation of the activated α_i and $\beta\gamma$ subunits. Regardless of which G protein subunit is involved, an additional complication that must be considered is the multiplicity of phospholipase C isozymes (reviewed in Ref. 47).

The existence of two closely related 5-HT$_{1D}$ subtypes in the human genome has raised much speculation (5, 8). Possible explanations for the expression of two closely related subtypes include differential tissue distribution, specificity in G protein coupling, and activation of different signal transduction mechanisms. Although the functional responses mediated by the two cloned subtypes are qualitatively similar, they exhibit a quantitative difference; when murine fibroblasts are used as the transfection host, the maximal elevation of $[Ca^{2+}]_i$ is significantly larger with the 5-HT$_{1D\beta}$ receptor than with the 5-HT$_{1D\alpha}$ subtype. In contrast, the maximal increase in IP accumulation is similar between the two 5-HT$_{1D}$ subtypes, as is the maximal inhibition of cAMP levels (Tables 2 and 3). These results further emphasize the dissociation between the increases in $[Ca^{2+}]_i$ and IP accumulation in this heterologous expression system. Despite the differences in the magnitude of the calcium responses, the EC$_{50}$ values for each of the three functional responses mediated by the two receptors are similar (Tables 2 and 3). It is important to note that the levels of expression of the 5-HT$_{1D\alpha}$ receptor are slightly higher than those of the 5-HT$_{1D\beta}$ subtype (Table 1); thus, the actual difference in magnitude of the calcium responses is probably greater than that observed. The two most likely explanations for these results are that the 5-HT$_{1D\beta}$ subtype couples 1) more effectively to the relevant G protein(s) and/or 2) to a larger pool of G protein isoforms, compared with the 5-HT$_{1D\alpha}$ receptor. Irrespective of the molecular mechanism(s) involved, the results indicate that activation of the two receptor subtypes may result in either quantitatively similar or different cellular responses, depending upon the second messenger system.

In conclusion, we have demonstrated that the cloned human 5-HT$_{1D\alpha}$ and 5-HT$_{1D\beta}$ subtypes can both elevate $[Ca^{2+}]_i$ and decrease stimulated cAMP and that both responses occur at similar low concentrations of agonist. The ability of these receptors to elevate $[Ca^{2+}]_i$ may provide a primary mechanism by which 5-HT, sumatriptan, and related compounds may contract smooth muscle in blood vessels and various organs. These findings may aid in the development of novel therapeutic agents useful in the treatment of migraine and other pathophysiological conditions associated with alterations in 5-HT$_{1D}$ receptor function.

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