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Coupling of an endogenous 5-HT_{1B}-like receptor to increases in intracellular calcium through a pertussis toxin-sensitive mechanism in CHO-K1 cells

[1]John M. Dickenson & Stephen J. Hill

Department of Physiology and Pharmacology, Medical School, Queen's Medical Centre, Nottingham NG7 2UH

1 Chinese hamster ovary cells (CHO-K1) express an endogenous 5-hydroxytryptamine $(5\text{-HT})_{1B}$-like receptor that is negatively coupled to adenylyl cyclase through a pertussis toxin (PTX)-sensitive mechanism. Furthermore, the human adenosine A_1 receptor when expressed in CHO-K1 cells (CHO-A1) has been shown to mobilize intracellular Ca^{2+} through a PTX-sensitive mechanism. Therefore the aim of this investigation was to determine whether the endogenous 5-HT_{1B}-like receptor was able to stimulate increases in intracellular free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) in CHO-A1 cells.

2 In agreement with previous studies using CHO cells, 5-hydroxytryptamine (5-HT) elicited a concentration-dependent inhibition of forskolin-stimulated [^{3}H]-cyclic AMP production in CHO-A1 cells (p[EC_{50}] = 7.73 ± 0.13). 5-HT (1 μM) inhibited 47 ± 5% of the [^{3}H]-cyclic AMP accumulation induced by 3 μM forskolin. Forskolin stimulated [^{3}H]-cyclic AMP accumulation was also inhibited by the 5-HT_1 receptor agonists (p[EC_{50}] values) 5-carboxyamidotryptamine (5-CT; 8.07 ± 0.08), RU 24969 (8.12 ± 0.33) and sumatriptan (5.80 ± 0.31).

3 5-HT elicited a concentration-dependent increase in $[Ca^{2+}]_i$ in CHO-A1 cells (p[EC_{50}] = 8.07 ± 0.05). In the presence of 2 mM extracellular Ca^{2+}, 5-HT (1 μM) increased $[Ca^{2+}]_i$ from 174 ± 17 nM to 376 ± 22 nM. The 5-HT_1 receptor agonists (p[EC_{50}] values), 5-carboxyamidotryptamine (5-CT; 7.9 ± 0.02), RU 24969 (8.1 ± 0.07) and sumatriptan (5.9 ± 0.11) all elicited concentration-dependent increases in $[Ca^{2+}]_i$. Similar maximal increases in $[Ca^{2+}]_i$ were obtained with each agonist. The selective 5-HT_{1A} receptor agonist, 8-OH-DPAT (10 μM) did not stimulate increases in $[Ca^{2+}]_i$. 5-HT (100 μM) and 5-CT (10 μM) did not stimulate a measurable increase in [^{3}H]-inositol phosphate accumulation in CHO-A1 cells.

4 5-HT (1 μM)-mediated increases in $[Ca^{2+}]_i$ were insensitive to the 5-HT receptor antagonist, ritanserin (5-HT_2; 100 nM), ketanserin (5-HT_2; 100 nM), LY-278,584 (5-HT_3; 1 μM) and WAY 100635 (5-HT_{1A}; 1 μM). The response to 5-HT (100 nM) was antagonized by the non-selective 5-HT_1 antagonist, methiothepin (pK_b = 8.90 ± 0.09) and the 5-HT_{1D} antagonist GR 127935 (pK_b = 10.44 ± 0.06).

5 Pretreatment with PTX (200 ng ml^{-1} for 4 h) completely attenuated the Ca^{2+} response to 100 μM 5-HT.

6 In untransfected CHO-K1 cells, 5-HT (1 μM), RU 24969 (1 μM), and 5-CT (1 μM) elicited increases in $[Ca^{2+}]_i$ similar to those observed in CHO-A1 cells.

7 These data demonstrate that in CHO-K1 cells the endogenously expressed 5-HT_{1B}-like receptor couples to the phospholipase C/Ca^{2+} signalling pathway through a PTX-sensitive pathway, suggesting the involvement of G_i/G_o protein(s).

Keywords: 5-Hydroxytryptamine; 5-HT_{1B} receptor; intracellular calcium; chinese hamster ovary cells; cyclic AMP

Introduction

Members of the 5-hydroxytryptamine $(5\text{-HT})_1$ receptor family (5-HT_{1A}, 5-HT_{1B}, 5-$HT_{1D\alpha}$, 5-$HT_{1D\beta}$, 5-HT_{1E} and 5-HT_{1F}) are predominantly coupled to the inhibition of adenylyl cyclase through pertussis toxin (PTX)-sensitive G_i proteins (Boess & Martin, 1994; Hoyer *et al.*, 1994; Martin & Humphrey, 1994). Recent studies using cloned 5-HT_{1A}, 5-$HT_{1D\alpha}$, 5-$HT_{1D\beta}$ and 5-HT_{1F} receptors expressed at high concentrations in mammalian cell lines have revealed that these G_i coupled receptors are able to inhibit adenylyl cyclase and activate phospholipase C (Fargin *et al.*, 1989; Liu *et al.*, 1991; Boddeke *et al.*, 1992; Zgombick *et al.*, 1993; Adham *et al.*, 1993a). However, at high concentrations of receptor expression, the fidelity of receptor-effector coupling may be lost enabling receptors to couple to several different effector systems (Milligan, 1993). It is notable, however, that an endogenous 5-HT_1-like receptor has been shown to inhibit adenosine 3':5'-cyclic monophosphate (cyclic AMP) production and increase intracellular free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) in cells derived from bovine basilar artery (Ebersole *et al.*, 1992). Recent reports have demonstrated that Chinese hamster ovary cells (CHO) endogenously express a 5-HT_{1B}-like receptor that inhibits adenylyl cyclase activity through a PTX-sensitive mechanism (Berg *et al.*, 1994; Giles *et al.*, 1994). Despite 5-HT being a potent inhibitor of forskolin-stimulated cyclic AMP production in CHO cells, Giles *et al.* (1994) did not detect any specific binding of [^{3}H]-5-HT or [^{125}I]-cyanopindolol. These results would seen to suggest a very efficient 5-HT_{1B} receptor-effector coupling in CHO cells. Studies using CHO cells transfected with high concentrations of the human adenosine A_1 receptor (CHO-A1 cells) have revealed that the A_1 receptor can inhibit forskolin stimulated cyclic AMP production and stimulate the mobilization of intracellular Ca^{2+} through PTX-sensitive mechanisms (Townsend-Nicholson & Shine, 1992; Iredale *et al.*, 1994). Adenosine A_1 receptors, like 5-HT_{1B} receptors, are negatively coupled to adenylyl cyclase through a PTX-sensitive G_i protein (Stiles, 1992). The adenosine A_1 receptor-mediated increases in $[Ca^{2+}]_i$ in CHO-A1 cells may be due to overexpression of the receptor leading to inappropriate coupling to calcium mobilization via the action of either $G_{\alpha i}$ or $G_{\beta\gamma}$ subunits on phospholipase C (Camps *et al.*, 1992; Clapham & Neer, 1993). In this study we have in-

[1] Author for correspondence.

vestigated whether the endogenous 5-HT_{1B} receptor, which could be expressed at low levels in CHO cells (Giles *et al.*, 1994), is capable of stimulating increases in $[Ca^{2+}]_i$ in CHO-A1 cells.

Methods

Cell culture

Chinese hamster ovary cells (CHO-K1) transfected with the human adenosine A_1 receptor sequence (CHO-A1) were a generous gift from Dr Andrea Townsend-Nicholson and Professor John Shine, Garvan Institute, Sydney, Australia. Untransfected CHO-K1 cells were obtained from the European Collection of Animal Cell Cultures (Porton Down, Salisbury, Wiltshire). CHO-A1 and parent CHO-K1 cells were cultured in 75 cm^2 flasks in Dulbeco's modified Eagles Medium/Nutrient Mix F12 (1:1) supplemented with 2 mM-L-glutamine and 10% (v/v) foetal calf serum (FCS). Cells were maintained at 37°C in a humidified 10% CO_2 atmosphere until confluency and were subcultured (1:5 split ratio) using trypsin (0.05% w/v). Cells for [^{3}H]-cyclic AMP and [^{3}H]-inositol phosphate accumulation were grown in 24 well cluster dishes (Costar) and all experiments were performed on confluent monolayers.

Measurements of intracellular free calcium

All measurements of intracellular free calcium were performed on CHO-K1 cells transfected with the human adenosine A_1 receptor (CHO-A1), unless otherwise stated. $[Ca^{2+}]_i$ was measured by loading CHO-A1 cells (grown on glass coverslips) with the Ca^{2+}-sensitive dye fura-2. Cells were grown to confluence and then seeded at a split ratio of 1:10 onto 22 mm circular glass coverslips in DMEM/F-12 containing 10% FCS and grown for 24 h at 37°C in 10% CO_2. Coverslips with attached cells were initially washed once with physiological buffer (composition (mM): NaCl 145, glucose 10, KCl 5, $MgSO_4$ 1, HEPES 10, $CaCl_2$ 2, pH 7.4). Washed coverslips were then incubated at 37°C for 45 min with physiological buffer containing 5 mM fura-2/AM and 10% FCS. Fura-2 'loaded' coverslips were washed twice with physiological buffer and transferred to a metal holder which was then mounted in a heated chamber maintained at 37°C. A volume of 900 μl of physiological buffer was added to the chamber and agonist drugs were added in a further volume of 100 μl to ensure rapid mixing. All coverslips were left at room temperature until required and used within 60 min of the end of the loading period.

$[Ca^{2+}]_i$ was monitored by viewing fura-2 loaded cell monolayers through a Nikon fluorescent microscope (typically 15–20 cells in the field of view) and using the 'MagiCal' image analysis system from Applied Imaging. Image analysis was performed using MagiCal hardware and TARDIS software supplied by Applied Imaging International Ltd. (Hylton Park, Sunderland, Tyne & Wear). Fluorescent images were detected with a Nikon Diaphot epifluorescence microscope with a 10 × quartz objective lens and then relayed through an image intensifying charged coupled device camera (Photonic Science) to the TARDIS software where images underwent analogue to digital conversion. Images captured were 256 × 256 pixels in size and each frame was routinely averaged 8 times with analogue hardware averaging to reduce camera noise. Incident light of alternating 340 and 380 nm wavelength was supplied to the cells by a rotating filter wheel. The time between image pairs was 1.5 s. Once a sequence of images had been captured they were subjected to background subtraction. This was achieved by capturing 340 and 380 nm images from an area of the coverslip devoid of cells and these images were then subtracted pixel by pixel from each of the corresponding 340 or 380 nm experimental images using the TARDIS software. Background-corrected image pairs were then ratioed (340/380) on a pixel-by-pixel basis and calcium ion concentration was calculated with a 2-D look up table calculated according to the equation of Grynkiewicz *et al.* (1985):

$$[Ca^{2+}]_i = \frac{(R - R_{min})}{(R_{max} - R)} \times (S_{380,min}/S_{380,max}) \times K_d$$

where K_d is the affinity of fura-2 for Ca^{2+} (224 nM at 37°C) and $S_{380,min}/S_{380,max}$ is the ratio (β value) of the fluorescent values obtained at 380 nM in the absence and presence of saturating $[Ca^{2+}]_i$. R_{max} was calculated for calibration purposes by exposing the cells to 40 μM ionomycin in the presence of 20 mM $CaCl_2$. R_{min} was determined from a separate coverslip by chelation of free intracellular calcium ions with 15 mM EGTA (20 μl of 1.0 M NaOH was added to compensate for the decrease in pH) in the presence of 40 μM ionomycin. Increases in $[Ca^{2+}]_i$ represent the mean of all (typically 15–20) cells in the microscopic field of view.

Measurement of [^{3}H]-cyclic AMP accumulation

Confluent CHO-A1 cell monolayers were incubated for 2 h at 37°C with 500 μl of Hanks/HEPES buffer (pH 7.4) containing [^{3}H]-adenine (37 kBq/well). The cells were washed once and then incubated in 1 ml/well Hanks/HEPES buffer containing the cyclic AMP phosphodiesterase inhibitor, rolipram (100 μM) for 15 min at 37°C. Agonists were added (in 10 μl of medium) 5 min prior to incubation with 3 μM forskolin (10 min). Incubations were terminated by the addition of 50 μl concentrated HCl. [^{3}H]-cyclic AMP was isolated by sequential Dowex-alumina chromatography as previously described (Donaldson *et al.*, 1988). After elution, the levels of [^{3}H]-cyclic AMP were determined by liquid scintillation counting (scintillator plus, Packard).

Measurement of [^{3}H]-inositol phosphate accumulation

[^{3}H]-inositol phosphate accumulation was measured by prelabelling cell monolayers with [^{3}H]-myo-inositol as described previously (Megson *et al.*, 1995).

Data analysis

Agonist concentration-response curves were fitted to a logistic equation by the non-linear regression programme Inplot4 (GraphPad Software, San Diego) as described previously (Ruck *et al.*, 1990). The equation fitted was:

$$\text{Response} = \frac{E_{max} \times A^{n_H}}{(EC_{50})^{n_H} + A^{n_H}}$$

where E_{max} is the maximum agonist response, A is the agonist concentration and n_H is the Hill coefficient. Antagonist dissociation constants (K_D) were estimated by a modification of the method of Lazareno & Roberts (1987). Briefly, a concentration-response curve to 5-HT was generated and a concentration of 10^{-7} M (C) was chosen which gave a response greater than 50% of the maximal response. The concentration of antagonist required to reduce the response of this concentration (C) of agonist by 50% was then determined (IC_{50}). The agonist concentration curve was fitted to a logistic equation as described above and a concentration of the agonist identified (C^1) which yielded a response equivalent to 50% of that produced by concentration C (in the absence of antagonist). The apparent K_D was then determined from the following relationship:

$$C/C^1 = IC_{50}/K_D + 1$$

values in the text are expressed as $-\log(K_D)$ or pK_b.

p[EC_{50}] ($-\log EC_{50}$; concentrations of drug producing 50% of the maximal stimulation) values were obtained by computer assisted curve fitting by use of the computer programme Inplot 4 as described above. Calcium data were imported into

the graphics programme Sigma-Plot (Jandel, California, U.S.A.). Statistical significance was determined by Student's unpaired t test ($P<0.05$ was considered statistically significant). All data are presented as mean ± s.e.mean.

Chemicals

[2,8-^{3}H]-adenine (888 GBq mmol^{-1}) and [2-^{3}H]-*myo*-inositol (758 GBq mmol^{-1}) were supplied by New England Nuclear (Stevenage, U.K.). 5-Hydroxytryptamine, ATP, N^6-cyclopentyladenosine and forskolin were obtained from Sigma Chemical Co. (Poole, Dorset). Ketanserin, ritanserin, methiothepin, 5-carboxyamidotryptamine, 8-OH-DPAT (8-hydroxy-2-(di-n-propylamino)-tetralin) and LY-278,584 (1-methyl-N-(8-methyl-8-azibicyclo [3.2.1] -oct-3-yl)-1H-indazole-3-carboxamide) were obtained from Research Biochemicals International. Rolipram was from Schering A.G. (Berlin, Germany). The kind gifts of sumatriptan and GR 127935 (N-[4-methoxy-3-(4-methyl-1-piperazinyl)phenyl]-2-methyl-4-(5-methyl-1,2, 4-oxadiazol-3-yl) [1,1,biphenyl]-4-carboxamide) from Glaxo Group Research Ltd, RU 24969 (5-methoxy-3-(1,2,3,6-tetrahydro-4-pyrindinyl)-1H indole) from Roussel-UCLAF (Paris, France), and WAY 100635 (N-[2-[4-2-methyoxyphenyl)-1-piperazinyl]ethyl]-*N*-(2-pyridinyl) cyclohexanecarboxamide (Wyeth Research Ltd) are gratefully acknowledged. Fura-2/AM and ionomycin were from Calbiochem/Novobiochem (Nottingham). Pertussis toxin was obtained from Porton Products Ltd. Dulbeco's modified Eagles Medium/Nutrient Mix F-12 (1:1) and foetal calf serum were from Sigma Chemical Co. (Poole, Dorset). All other chemicals were of analytical grade.

Results

Effects of 5-HT$_1$ receptor agonists on [^{3}H]-cyclic AMP accumulation in CHO-A1 cells

In agreement with previous studies using CHO cells (Berg *et al.*, 1994; Giles *et al.*, 1994) 5-HT elicited a concentration-dependent inhibition of forskolin-stimulated [^{3}H]-cyclic AMP production in CHO-A1 cells (p[EC$_{50}$] = 7.73 ± 0.13; $n=5$; Figure 1). 5-HT (1 μM) inhibited 48 ± 6% ($n=4$) of the cyclic AMP accumulation induced by 3 μM forskolin. Forskolin-stimulated cyclic AMP accumulation was also inhibited by the selective 5-HT$_1$ receptor agonists, 5-carboxyamidotryptamine (5-CT), RU 24969 and sumatriptan. The effects of the various 5-HT$_1$ receptor agonists on forskolin-stimulated cyclic AMP production are summarized in Table 1.

Effects of 5-HT$_1$ receptor agonists on $[Ca^{2+}]_i$ in CHO-A1 cells

5-HT elicited a concentration-dependent increase in $[Ca^{2+}]_i$ in Chinese hamster ovary cells (p[EC$_{50}$] = 8.07 ± 0.05; $n=3$; Figure 2). In the presence of 2 mM extracellular Ca^{2+}, 5-HT (1 μM) increased $[Ca^{2+}]_i$ from 174 nM to 376 nM ($n=12$). Si-

Table 1 Effects of various 5-HT$_1$ receptor agonists on forskolin stimulated [^{3}H]-cyclic AMP accumulation in CHO-A1 cells

Agonist	*p[EC]$_{50}$*	*Maximum % inhibition*	n
5-HT	7.73 ± 0.13	47 ± 5	5
5-CT	8.07 ± 0.08	50 ± 7	4
RU 24969	8.12 ± 0.33	42 ± 5	3
Sumatriptan	5.80 ± 0.31	38 ± 3	5

CHO-A1 cells were prestimulated for 5 min with the various agonists (in absence of forskolin). Following this, cells were stimulated with 3 μM forskolin for 10 min in the continued presence of agonist. Agonist p[EC$_{50}$] values (mean ± s.e. mean) were determined from individual concentration response curves obtained by computer assisted curve fitting by use of the computer programme Inplot4 (GraphPAD). The percentage inhibition values represent the maximum inhibition of the response to 3 μM forskolin (in the absence of agonist and after subtracting basal [^{3}H]-cyclic AMP accumulation) obtained with maximal concentrations of 5-HT (1 μM), 5-CT (1 μM), RU24969 (1 μM) and sumatriptan (100 μM). Values represent means ± s.e.mean of (n) individual experiments.

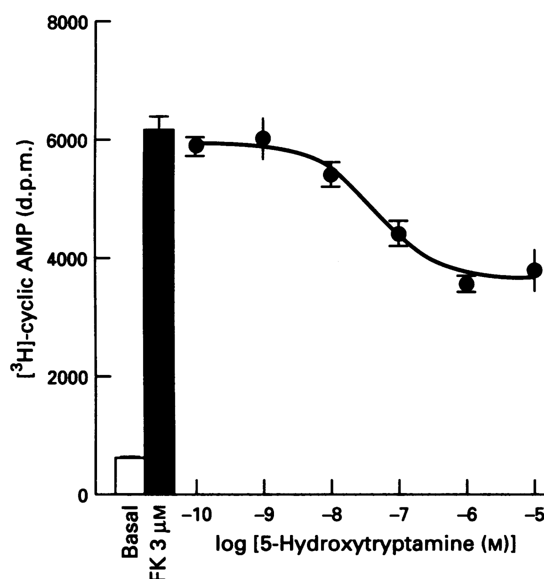

Figure 1 Effect of 5-hydroxytryptamine (5-HT) on forskolin stimulated [^{3}H]-cyclic AMP accumulation in CHO-A1 cells. Cells were initially pre-stimulated for 5 min with the various 5-HT concentrations. Following this, cells were stimulated with 3 μM forskolin for 10 min in the continued presence of 5-HT. The histograms show the basal and 3 μM forskolin-stimulated [^{3}H]-cyclic AMP accumulation. Values represent mean ± s.e.mean of triplicate determinations in a single experiment. Similar data were obtained in four other experiments.

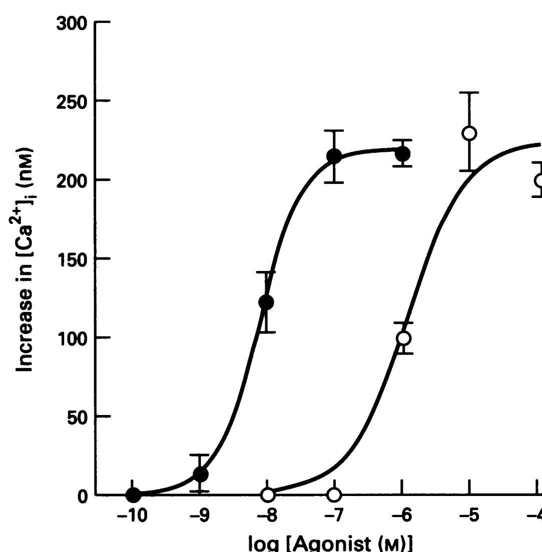

Figure 2 Effect of 5-HT and sumatriptan on $[Ca^{2+}]_i$ in fura-2 loaded CHO-A1 cells. Fura-2 loaded coverslips were stimulated with varying concentrations of 5-HT (●) or sumatriptan (○). Experiments were performed in the presence of 2 mM extracellular Ca^{2+}. The data represent the increase in $[Ca^{2+}]_i$ (nM) after substracting basal $[Ca^{2+}]_i$. Data for each concentration of agonist represent the combined mean ± s.e.mean from six coverslips measured in three separate experiments (two coverslips in each separate experiment).

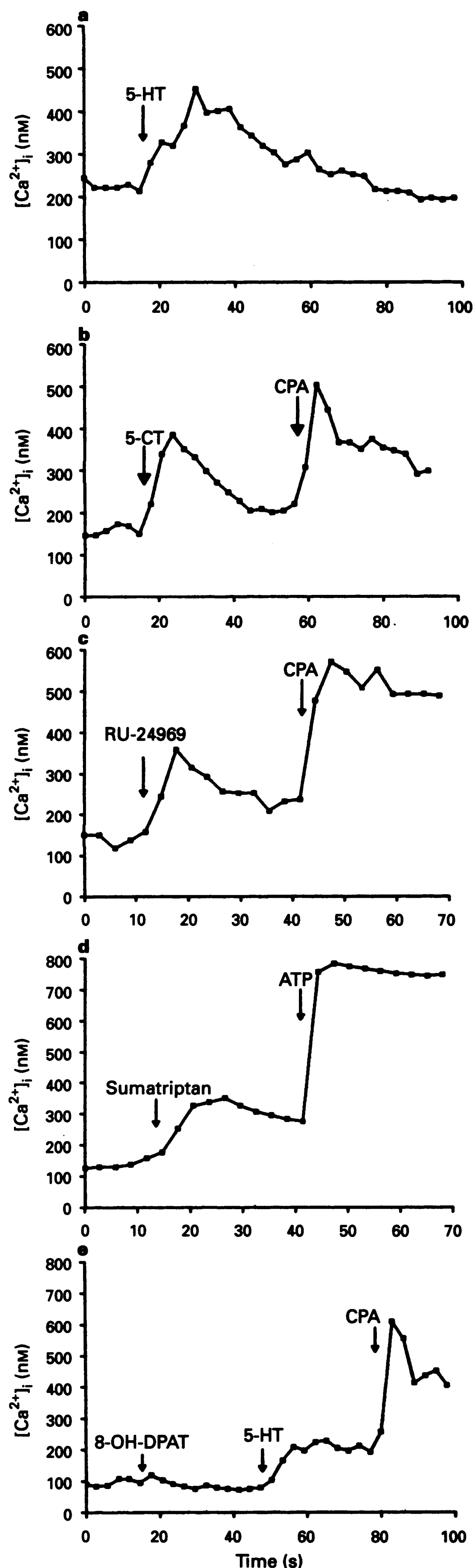

Figure 3 The effect of various 5-HT$_1$ receptor agonists on $[Ca^{2+}]_i$ in CHO-A1 cells. Fura-2 loaded coverslips were stimulated with (a) 5-HT (1 μM), (b) 5-CT (1 μM), (c) RU 24969 (1 μM), (d) sumatriptan (100 μM) and (e) 8-OH-DPAT (10 μM) in the presence of 2 mM $CaCl_2$. ATP (100 μM) and the selective adenosine A_1 receptor agonist, N^6-cyclopentyladenosine (CPA; 100 nM) were added at the end of some experiments for comparison. Increases in $[Ca^{2+}]_i$ represent the mean of all (typically 15–20) cells in the microscopic field of view.

milar concentration-dependent increases in $[Ca^{2+}]_i$ were obtained with the selective 5-HT$_1$ receptor agonists, 5-CT, RU 24969 and sumatriptan. Representative Ca^{2+} profiles for each agonist are shown in Figure 3. The effects of the various 5-HT$_1$ receptor agonists on $[Ca^{2+}]_i$ are summarized in Table 2. 5-HT (1 μM) also elicited calcium responses in the absence of extracellular calcium. In nominally Ca^{2+}-free buffer containing 0.1 mM EGTA, 1 μM 5-HT increased $[Ca^{2+}]_i$ from 105 ± 9 nM to 251 ± 28 nM ($n=9$). The selective 5-HT$_{1A}$ receptor agonist, 8-OH-DPAT (10 μM; 1000 times higher than its published affinity for the 5-HT$_{1A}$ receptor, Hoyer, 1988) did not stimulate increases in $[Ca^{2+}]_i$ (Figure 3e, Table 2). The cells used in these experiments did respond to 1 μM 5-HT ($[Ca^{2+}]_i$ increased from 116 ± 16 nM to 267 ± 32 nM ($n=4$); see Figure 3e). The effect of 5-HT and 5-CT on [^{3}H]-inositol phosphate accumulation was determined in CHO-A1 cell monolayers prelabelled with [^{3}H-*myo*]-inositol. 5-HT (100 μM; $n=3$) and 5-CT (10 μM; $n=6$) failed to stimulate a measurable increase in total [^{3}H]-inositol phosphate accumulation in CHO-A1 cells (data not shown). These data are in agreement with Berg *et al.* (1994) who were also unable to demonstrate inositol phosphate accumulation in CHO cells in response to 5-HT or 5-CT. It is generally accepted that the inhibition of adenylyl cyclase occurs through the activation of pertussis toxin (PTX)-sensitive G_i proteins, whereas the activation of phospholipase C (and hence increases in $[Ca^{2+}]_i$) is mediated through PTX-insensitive G_q/G_{11} proteins (Offermanns & Schultz, 1994). PTX pretreatment (200 ng ml^{-1} for 4 h) completely abolished 5-HT and N^6-cyclopentyladenosine (selective adenosine A_1 receptor agonist)-mediated increases in $[Ca^{2+}]_i$ (compare Figures 4a and b). These results indicate that 5-HT stimulated increases in $[Ca^{2+}]_i$ are mediated by PTX-sensitive G_i/G_o proteins.

Finally, in untransfected CHO-K1 cells, 5-HT (1 μM; 251 ± 20 nM to 469 ± 43 nM; $n=3$), RU 24969 (1 μM; 205 ± 20 nM to 406 ± 23 nM; $n=3$) and 5-CT (1 μM; 174 ± 13 nM to 444 ± 33 nM; $n=3$) all stimulated increases in $[Ca^{2+}]_i$.

Effects of various 5-HT receptor antagonists on 5-HT mediated increases in $[Ca^{2+}]_i$

5-HT-induced increases in $[Ca^{2+}]_i$ may be mediated through the activation of 5-HT$_2$ receptor subtypes (5-HT$_{2A}$, 5-HT$_{2B}$ and 5-HT$_{2C}$) which are coupled to inositol phospholipid hydrolysis and Ca^{2+} mobilization (Hoyer *et al.*, 1994). The 5-HT$_2$ receptor antagonists, ketanserin (to block 5-HT$_{2A}$) and ritanserin (to block 5-HT$_{2B}$ and 5-HT$_{2C}$; Wainscott *et al.*, 1993; Hoyer *et al.*, 1994) had no effect on 5-HT (1 μM)-mediated increases in $[Ca^{2+}]_i$ (Table 3). Alternatively, 5-HT may be activating 5-HT$_3$ receptors which contain an integral non-selective ion channel pore (Derkach *et al.*, 1989). The Ca^{2+} responses elicited by 5-HT were insensitive to the potent 5-HT$_3$ receptor antagonist, LY-278,584 (Wong *et al.*, 1989; Table 2). The selective 5-HT$_{1A}$ receptor antagonist, WAY 100635 (Fletcher *et al.*, 1994) had no effect on 5-HT Ca^{2+} responses. The data in Table 3 summarize the effects of the various 5-HT receptor antagonists.

In contrast, the non-selective 5-HT$_1$ antagonist, methiothepin, caused a concentration-dependent inhibition of 5-HT (100 nM)-mediated increases in $[Ca^{2+}]_i$ with a pK_b of 8.9 ± 0.09 ($n=3$; Figure 5). 5-HT (100 nM)-stimulated increases in $[Ca^{2+}]_i$ were also antagonized by the 5-HT$_{1D}$ receptor antagonists, GR 127935 (p$K_b = 10.44 \pm 0.06$; $n=3$, Figure 5).

Table 2 Effect of various 5-HT_1 receptor agonists on $[Ca^{2+}]_i$ in CHO-A1 cells

Agonist	*p[EC₅₀]*	*Basal $[Ca^{2+}]_i$* (nM)	*Stimulated $[Ca^{2+}]_i$* (nM)	*n*
5-HT	8.07±0.05	174±17	376±22*	12
5-CT	7.90±0.02	181±13	403±33*	5
RU 24969	8.10±0.07	143±17	348±39*	3
Sumatriptan	5.90±0.11	153±11	351±19*	3
8-OH-DPAT		123±8	126±9	4

Agonist $p[EC_{50}]$ values (mean±s.e.mean) were determined from three separate concentration-response curves obtained by computer assisted curve fitting by use of the computer programme InPlot (GraphPAD). The basal $[Ca^{2+}]_i$ and maximum stimulated increases in $[Ca^{2+}]_i$ represent the responses obtained with maximal concentrations of 5-HT (1 μM), 5-CT (1 μM), RU 24969 (1 μM), sumatriptan (100 μM) and 8-OH-DPAT (10 μM). Increases in $[Ca^{2+}]_i$ represent the mean of all (typically 15–20) cells in the microscopic field of view. Values represent means±s.e.mean of (*n*) individual coverslips. *Statistically significant ($P<0.05$) difference from basal $[Ca^{2+}]_i$.

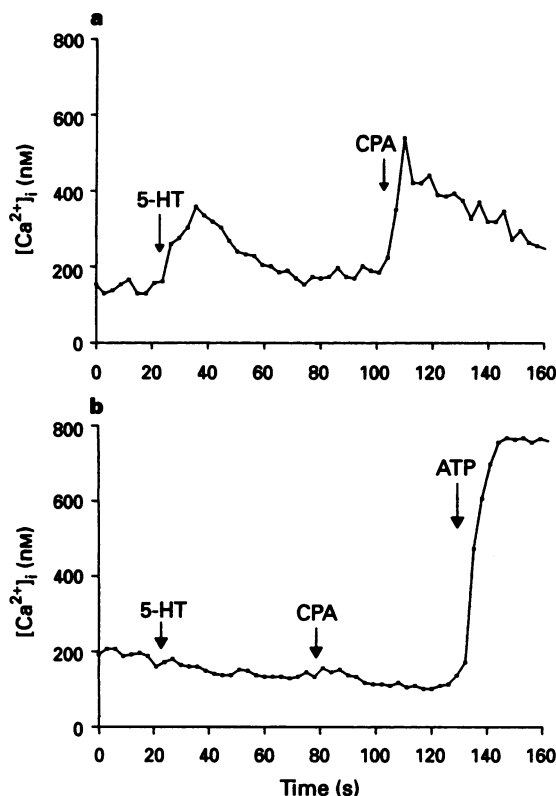

Figure 4 Effect of pertussis toxin on 5-HT-mediated increases in $[Ca^{2+}]_i$ in CHO-A1 cells. Representative profiles for 5-HT (1 μM)-mediated increases in $[Ca^{2+}]_i$ in (a) control cells and (b) cells pretreated with pertussis toxin (200 ng ml^{-1}) for 4 h. Increases in $[Ca^{2+}]_i$ represent the mean of all (typically 15–20) cells in the microscopic field of view. The application of ATP (100 μM) confirmed cell viability in PTX-treated coverslips. 5-HT (1 μM), N^6-cyclopentyladenosine (CPA; 100 nM) and ATP (100 μM) were added where indicated. Similar results were obtained in five other experiments.

Table 3 Effects of various 5-HT antagonists on 5-HT-mediated increases in $[Ca^{2+}]_i$ in CHO-A1 cells

	Basal $[Ca^{2+}]_i$ (*nM*)	*Stimulated $[Ca^{2+}]_i$* (nM)	n
Control (1 μM 5-HT)	174±17	376±22	12
Ritanserin (100 nM)	195±30	392±35	8
Ketanserin (100 nM)	155±11	357±20	9
LY-278,584 (1 μM)	185±18	396±22	7
WAY 100635 (1 μM)	148±10	334±24	7

Coverslips were preincubated for 15 min with the 5-HT receptor antagonists, ritanserin (5-HT_2), ketanserin (5-HT_2), LY-278,584 (5-HT_3) and WAY 100635 (5-HT_{1A}) before stimulation with 1 μM 5-HT. The control response (1 μM 5-HT) was obtained by stimulating cells that had not been preincubated with the various antagonists. Increases in $[Ca^{2+}]_i$ represent the mean of all (typically 15–20) cells in the microscopic field of view. Values represent means±s.e.mean of (*n*) individual coverlips.

Discussion

Recent reports have demonstrated that Chinese hamster ovary cells endogenously express a 5-HT_{1B}-like receptor that inhibits adenylate cyclase activity through a pertussis toxin (PTX)-sensitive mechanism (Bert *et al.*, 1994; Giles *et al.*, 1994). In agreement with the studies of Berg *et al.* (1994) and Giles *et al.* (1994), 5-HT elicited a concentration-dependent inhibition of forskolin-stimulated cyclic AMP accumulation in CHO-A1 cells ($p[EC_{50}]=7.7$). Furthermore, we have previously reported that the human adenosine A_1 receptor expressed in CHO cells (CHO-A1) stimulates calcium mobilization through a PTX-sensitive mechanism (Iredale *et al.*, 1994). In this study we have shown that 5-HT also stimulates a rapid and PTX-sensitive increase in $[Ca^{2+}]_i$ in fura-2 loaded CHO-A1 cells. The PTX-sensitivity of the 5-HT calcium response suggests that a 5-HT_1 receptor subtype is involved. 5-HT_1 receptors are negatively coupled to adenylate cyclase through a PTX-sensitive G_i protein (Boess & Martin, 1994; Hoyer *et al.*, 1994; Martin & Humphrey, 1994).

To date seven members of the 5-HT_1 receptor family have been identified, namely 5-HT_{1A}, 5-HT_{1B}, 5-$HT_{1D\alpha}$, 5-$HT_{1D\beta}$, 5-HT_{1E} and 5-HT_{1F} (Boess & Martin, 1994; Hoyer *et al.*, 1994 ; Martin & Humphrey, 1994). The 5-HT_{1B} receptor is found in certain rodent species including mouse (Maroteaux *et al.*, 1992), rat (Adham *et al.*, 1992) and hamster (Seuwen *et al.*, 1988) and is considered to be the rodent homologue of the 5-$HT_{1D\beta}$ receptor that is found in man (Adham *et al.*, 1992) and most other mammalian species. In addition, the 5-HT_{1B} receptor has been extensively studied in opossum (non-rodent) kidney cells (Cerutis *et al.*, 1994; Pauwels & Palmier, 1994).

The involvement of a 5-HT_1 receptor subtype mediating the increase in $[Ca^{2+}]_i$ was initially confirmed by the potency of the 5-HT_1 receptor selective agonist, 5-CT ($p[EC_{50}]=7.9$; Hoyer *et al*, 1994). However, 5-CT does not discriminate between 5-HT_{1A}, 5-HT_{1B}, and 5-HT_{1D} receptor subtypes (Hoyer *et al.*, 1994). 5-HT_{1E} and 5-HT_{1F} receptors are unlikely to be involved because these subtypes have a comparatively low affinity for 5-CT (McAllister *et al.*, 1992; Adham *et al.*, 1993b). RU 24969 is a potent 5-HT_{1B} agonist and shows some selectivity between 5-HT_{1B} and 5-HT_{1D} receptors (Middlemiss & Tricklebank, 1992; Pauwels & Palmier, 1994; Hoyer *et al.*, 1994). For example, RU 24969 stimulates 5-HT_{1B} and 5-HT_{1D} receptor-mediated inhibition of forskolin stimulated cyclic AMP accumulation with $p[EC_{50}]$ values of 8.52 and 6.80 respectively (Hoyer *et al.*, 1994). In this study the $p[EC_{50}]$ value of 8.10 for RU 24969 stimulated increases in $[Ca^{2+}]_i$ in CHO-A1 cells is indicative of the 5-HT_{1B} receptor.

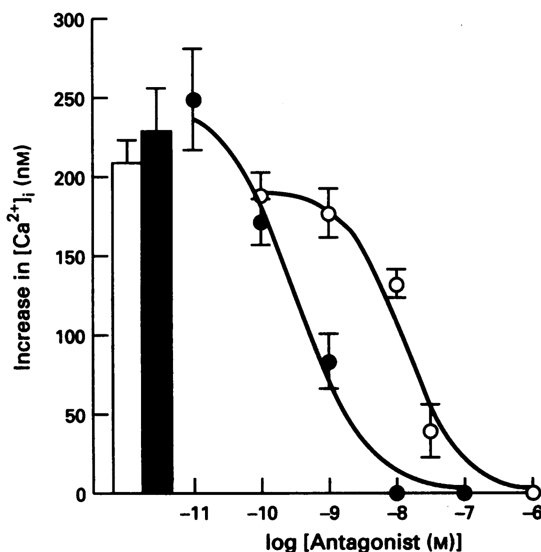

Figure 5 Inhibition of 5-HT-mediated increases in $[Ca^{2+}]_i$ by methiothepin and GR 127935. Fura-2-loaded coverslips were preincubated for 15 min with various concentrations of the non-selective 5-HT_1 receptor antagonist, methiothepin (○) and the 5-HT_{1D} receptor antagonist, GR 127935 (●) before stimulating with 100 nM 5-HT. Experiments were performed in the presence of 2 mM extracellular Ca^{2+}. The data represent the increase in $[Ca^{2+}]_i$ (nM) after subtracting basal $[Ca^{2+}]_i$. Control responses to 100 nM 5-HT in the absence of methiothepin (open column) and GR 127935 (solid column) are shown. Data for each concentration of antagonist represent the combined mean ± s.e.mean from six coverslips measured in three separate experiments (two coverslips in each separate experiment).

The agonist, sumatriptan, has been shown to possess limited selectivity for 5-HT_{1D} over 5-HT_{1B} receptors (Hoyer *et al.*, 1994). Sumatriptan EC_{50} values (determined from inhibition of forskolin stimulated cyclic AMP accumulation) obtained for the 5-HT_{1D} receptor are in the range 10–100 nM (Zgombick *et al.*, 1993; Hoyer *et al.*, 1994). The comparatively low potency for sumatriptan-stimulated increases in $[Ca^{2+}]_i$ (p[EC_{50}] = 5.9 (1200 nM)) obtained in the present study suggests that 5-HT_{1B} receptor subtype is involved. In summary, the rank order of agonist potencies (RU 24969 ≥ 5-HT > 5-CT > sumatriptan) is characteristic of the 5-HT_{1B} receptor (Hoyer *et al.*, 1994; Berg *et al.*, 1994; Pauwels & Palmier, 1994; Schoeffter *et al.*, 1995). The pharmacological characterization of 5-HT_{1B} receptors is hampered by the lack of potent and selective antagonists. However, 5-HT-induced Ca^{2+} responses were inhibited in a concentration-dependent manner by the non-selective 5-HT_1 receptor antagonist, methiothepin (Pauwels & Palmier, 1994). The antagonist affinity of methiothepin reported in this study (pK_b = 8.9) is comparable to the pK_i (8.31) obtained for methiothepin in CHO cells transfected with the opossum 5-HT_{1B} receptor (Cerutis *et al.*, 1994). However, the pK_b for methiothepin obtained in this study is about 1 order of magnitude higher than its affinity for rat and mouse 5-HT_{1B} receptors (Boess & Martin, 1994). The high affinity of methiothepin for the 5-HT_{1B}-like receptor in CHO cells may reflect species differences (rat and mouse versus hamster) between the 5-HT_{1B} receptor. Interestingly, the 5-HT_{1D} receptor antagonist, GR 127935 (Skingle *et al.*, 1993) potently inhibited 5-HT-induced Ca^{2+} responses (pK_b = 10.44). This high affinity is similar to the pK_i value (9.9) reported for the inhibition of [^{3}H]-5-HT binding to 5-$HT_{1D\beta}$ receptors in HeLa cells (Skingle *et al.*, 1993). These data suggest that GR 127935 may also be a potent antagonist of the 5-HT_{1B} receptor, which is considered to be the rodent homologue of the 5-$HT_{1D\beta}$ receptor.

The ability to inhibit adenylyl cyclase and elevate $[Ca^{2+}]_i$ is characteristic of a number of transfected and endogenous G_i-linked receptors. For example, adenosine A_1, muscarinic M_2 and α_2-adrenoceptors expressed at high concentrations in transfected CHO cells are able to inhibit adenylyl cyclase (the normal effector system for these receptors) and activate phospholipase C (Felder *et al.*, 1991; Dell'Acqua *et al.*, 1993; Iredale *et al.*, 1994). In DDT$_1$MF-2 cells, activation of the endogenously expressed adenosine A_1 receptor results in both the inhibition of adenylyl cyclase and the activation of phospholipase C through PTX-sensitive pathways (Ramkumar *et al.*, 1990; White *et al.*, 1992; Dickenson & Hill, 1993). Furthermore, Ramkumar *et al.* (1990) have shown that the endogenous adenosine A_1 receptor in DDT$_1$MF-2 cells is expressed at high levels (0.8–0.9 pmol mg^{-1} of protein). Recent findings suggest that the activation of phospholipase C through receptors coupled to PTX-sensitive G_i proteins may be mediated by G_i protein $\beta\gamma$ subunits (Camps *et al.*, 1992; Clapham & Neer, 1993). Hence, α and $\beta\gamma$ subunits released from G proteins sensitive to PTX (G_{i1}, G_{i2}, G_{i3}, G_{o1} and G_{o2}) may independently inhibit adenylyl cyclase and stimulate phospholipase C. Western blot analysis has revealed that CHO cells express $G_{i\alpha2}$, $G_{i\alpha3}$ and $G_{o\alpha2}$ subunits (Prather *et al.*, 1994). Experiments are currently in progress to determine whether the inhibition of cyclic AMP and increases in $[Ca^{2+}]_i$ (mediated by adenosine A_1 and 5-HT_{1B} receptors in CHO cells) are occurring through the same or different PTX-sensitive G protein(s).

The increases in $[Ca^{2+}]_i$ observed in this study suggest coupling of the 5-HT_{1B} receptor to phospholipase C in CHO-A1 cells. However, both 5-HT and 5-CT failed to stimulate a measurable increase in total [^{3}H]-inositol phosphate accumulation in CHO-A1 cells. These data are in agreement with Berg *et al.* (1994) who were also unable to demonstrate inositol phosphate accumulation in CHO cells in response to 5-HT or 5-CT. This apparent discrepancy may be due to 5-HT_{1B} receptors stimulating small increases in Ins(1,4,5)P_3, which although sufficient to increase $[Ca^{2+}]_i$, are too small to contribute to the pool of total [^{3}H]-inositol phosphates measured in the presence of LiCl.

5-HT-mediated increases in $[Ca^{2+}]_i$ have been observed using cloned 5-HT_{1A}, 5-$HT_{1D\alpha}$, 5-$HT_{1D\beta}$ and 5-HT_{1F} receptors expressed to high levels in mammalian cell lines (Liu & Albert, 1991; Boddeke *et al.*, 1992; Zgombick *et al.*, 1993; Adham *et al.*, 1993a). Furthermore, Liu & Albert (1991) and Zgombick *et al.* (1993) showed that the 5-HT-mediated increases in $[Ca^{2+}]_i$ and inhibition of cyclic AMP production were sensitive to PTX, suggesting the involvement of G_i/G_o proteins in both signalling pathways. The p[EC_{50}] values for 5-HT_{1A} (Liu & Albert, 1991) and 5-HT_{1D} receptor (Zgombick *et al.* 1993)-mediated inhibition of cyclic AMP production and stimulation of calcium mobilization were similar, suggesting that 5-HT_{1A} and 5-HT_{1D} receptors couple efficiently to both second messenger systems.

The EC_{50} value of 5-HT-mediated increases in $[Ca^{2+}]_i$ (8 nM) obtained in this study is similar to the p[EC_{50}] value for 5-HT-mediated inhibition of forskolin-stimulated cyclic AMP production in CHO cells (Berg *et al.*, 1994; Giles *et al.*, 1994). In contrast, the EC_{50} values for N^6-cyclopentyladenosine (CPA; selective adenosine A_1 receptor agonist) stimulated calcium mobilization and inhibition of forskolin stimulated cyclic AMP production in CHO-A1 cells are 15 nM and 3 nM respectively (Iredale *et al.*, 1994; Megson *et al.*, 1995). The higher concentration of CPA required to stimulate calcium mobilization may reflect a difference in the coupling of the adenosine A_1 receptor to the two different signalling pathways. It is important to note that the transfected adenosine A_1 receptor is expressed at high levels, whereas the endogenous 5-HT_{1B} receptor is expressed at low levels in CHO cells (Giles *et al.*, 1994, did not detect any specific [^{3}H]-5-HT or [^{125}I]-cyanopindolol binding to CHO cells). Hence, the 5-HT-mediated increased in $[Ca^{2+}]_i$ observed in CHO-A1 cells are not a consequence of 5-HT_{1B} receptor overexpression. Furthermore, the low 5-HT_{1B} receptor number and the high potency of 5-HT in mediating increases in $[Ca^{2+}]_i$ (p[EC_{50}] = 8.07) and inhibiting cyclic AMP production (p[EC_{50}] = 7.73) would seem to suggest a very efficient coupling of the 5-HT_{1B} receptor to these two second messenger pathways.

Endogenous 5-HT$_1$-like receptors have been shown to inhibit cyclic AMP production and increase [Ca^{2+}]$_i$ in cells derived from bovine basilar artery (Ebersole *et al.*, 1992). Furthermore, sumatriptan has been shown to cause contraction, via a 5-HT$_{1D}$-like receptor, in a range of isolated vascular smooth muscle preparations presumably through increases in [Ca^{2+}]$_i$ (Parsons *et al.*, 1992; Choppin & O'Connor, 1995). To our knowledge this is the first paper describing the coupling of an endogenous 5-HT$_{1B}$ receptor to increases in [Ca^{2+}]$_i$ through a PTX-sensitive mechanism.

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