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Further investigation into the signal transduction mechanism of the 5-HT_4-like receptor in the circular smooth muscle of human colon

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1 The nature of the receptor coupling mechanism of the 5-hydroxytryptamine$_4$ (5-HT_4) receptor in the circular smooth muscle of the human colon has been further investigated.

2 5-HT stimulated cyclic AMP generation and caused a relaxation in a concentration-dependent fashion, with EC_{50} values of 175.5 and 274.9 nM respectively. DAU 6236 increased cyclic AMP formation and caused a relaxant effect but was a partial agonist relative to 5-HT.

3 The 5-HT_4 receptor antagonist, GR 113808, inhibited cyclic AMP formation and relaxation induced by 5-HT with -log K_i values of 9.1 (cyclic AMP) and 8.9 (relaxation) and apparent pA_2 values of 9.2 (cyclic AMP) and 9.5 (relaxation).

4 Ondansetron and methysergide failed to inhibit cyclic AMP formation or the relaxation induced by 5-HT.

5 The phosphodiesterase inhibitor, IBMX, produced a concentration-dependent relaxation ($EC_{50}=30$ μM) and at 1 μM it enhanced the 5-HT-induced relaxation producing a leftward shift of the 5-HT concentration-effect curve with a concentration-ratio of 4.1. Rolipram caused a concentration-dependent relaxation ($EC_{50}=564.8$ nM) and at 200 nM caused a leftward shift of the concentration-effect curve to 5-HT with a concentration-ratio of 5.5.

6 Application of the adenylyl cyclase inhibitor, SQ 22536 (0.1 mM), and the protein kinase inhibitors, H7 (100 nM) and H89 (200 nM), inhibited the relaxant effect of 5-HT inducing a rightward shift of the concentration-effect curve with concentration-ratios of 10.1, 2.7 and 4.2 respectively.

7 Forskolin stimulated cyclic AMP production and caused a relaxation. The maximum relaxant effect of forskolin (6 μM, 13.8 ± 1.9 cm.s) was not significantly different from the maximum relaxant effect of 5-HT (10 μM, 12.7 ± 4.9 cm.s). However, the cyclic AMP levels stimulated by forskolin (6 μM, 49.3 ± 6.6 pmol mg^{-1}) were markedly greater than those stimulated by 5-HT (10 μM, 7.6 ± 2.0 pmol mg^{-1}).

8 In conclusion, these results indicate that the 5-HT_4 receptors of the circular smooth muscle of human colon mediate relaxation and inhibition of spontaneous contractions via activation of adenylyl cyclase, formation of cyclic AMP and activation of protein kinase A.

Keywords: 5-Hydroxytryptamine (serotonin); 5-HT_4 receptors; human colon; cyclic AMP; adenylyl cyclase inhibitor; protein kinase inhibitor; phosphodiesterase inhibitor; GR 113808

Introduction

5-Hydroxytryptamine (5-HT, serotonin) is a ubiquitous neurotransmitter possessing a number of functions in mammalian physiology via an interaction with multiple receptor subtypes. Through binding and functional pharmacological approaches four 5-HT receptor classes have been defined; namely 5-HT_1, 5-HT_2, 5-HT_3, and 5-HT_4 (Hoyer *et al.*, 1994). Recently molecular biological techniques have revealed the existence of the 5-ht_5, 5-ht_6, and 5-ht_7 receptor classes each without definitive functional correlates (hence lower case appellation, Hoyer *et al.*, 1994). The possible exception is the 5-ht_7 receptor for which a number of functional isolated tissue preparations have recently been identified which show pharmacological profiles similar to the cloned 5-ht_7 receptor (eg. Carter *et al.*, 1995; McLean & Coupar, 1995b). The classification of 5-HT receptors is currently based on functional and binding data, signal transduction mechanisms and molecular structure. With regard to signal transduction mechanisms 5-HT_1 receptors are negatively coupled to adenylyl cyclase via regulatory G-proteins, 5-HT_2 receptors are also G-protein linked and activate phosphoinositide metabolism and 5-HT_3 receptors are present on certain neurones and are linked to ligand gated cation-selective channels. 5-ht_6 and 5-ht_7 receptors are both positively linked to adenylyl cyclase.

5-HT_4 receptors are also positively linked to adenylyl cyclase in numerous tissues such as human frontal cortex (Monferini *et al.*, 1993), human right atrium (Kaumann *et al.*, 1990), human adrenocortical tissue (Lefebvre *et al.*, 1992) and a number of tissues from laboratory animals such as mouse embryo colliculi neurones (Dumuis *et al.*, 1998;1989), rat oesophagus (Ford *et al.*, 1992), piglet left atrium (Kaumann *et al.*, 1991) and guinea-pig hippocampus (Bockaert *et al.*, 1990).

Recently a 5-HT_4-like receptor has been identified and characterized pharmacologically in the human colon (Tam *et al.*, 1994; 1995; McLean *et al.*, 1995; McLean & Coupar, 1995a). Activation of this receptor induces relaxation and inhibition of the spontaneous contractions of the circular smooth muscle. Until recently the signal transduction mechanism mediating the 5-HT_4 receptor-induced action in this tissue was unknown (Hoyer *et al.*, 1994). However, we have shown in a preliminary account that the second messenger is possibly cyclic AMP (McLean & Coupar, 1996).

The present study was undertaken to investigate further the signal transduction mechanisms of the 5-HT_4 receptor in human colon. The criteria for an agonist-mediated response to be

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defined as mediated through cyclic AMP are: (i) that the agonist activates adenylyl cyclase, (ii) that an elevation of cyclic AMP is observed, and (iii) the response is potentiated by phosphodiesterase inhibitors (Sutherland, 1972). The study also aimed to determine if the 5-HT$_4$ receptor-induced cyclic AMP formation mediates relaxation and inhibition of spontaneous activity via activation of protein kinase.

A preliminary account of this work was presented at the Australasian Society of Clinical and Experimental Pharmacologists and Toxicologists meeting, Adelaide, Australia, December 1995 (McLean & Coupar, 1995c).

Methods

Specimens of human colon were obtained from patients undergoing surgical resection for colonic or rectal cancer. The specimens were placed into cold Krebs-Henseleit solution of the following composition (mM): NaCl 118, KCl 4.7, $NaHCO_3$ 25, KH_2PO_4 1.2, $CaCl_2$ 2.5, $MgSO_4$ 1.2 D-(+)-glucose 11, as soon as possible after resection. The mucosa was cut away from the muscle layers, and the intertaenial muscle was cut along the circular axis to produce strips 3–5 cm in length and 3–5 mm in width. These were mounted in Krebs-Henseleit solution, warmed at 37°C and equilibrated under a tension of 0.75 g for 45 min. Ugo Basile transducers connected to a MacLab/4e (ADInstruments) were used to measure isotonic changes in length. Within 30 min most tissues showed regular spontaneous contractions returning to a relatively stable baseline.

Incubation of muscle strips for cyclic AMP production

Adenosine 3′:5′-cyclic monophosphate (cyclic AMP) production was stimulated by the application of a single concentration of adenylyl cyclase activator or 5-HT receptor agonist either in the absence or presence of antagonist(s). The tissues were snap frozen in liquid nitrogen after the maximum relaxant response was observed. The frozen tissue was pulverized in an ice cold mortar and pestle, and homogenized in 1.5 ml cold 6% trichloroacetic acid (TCA) with a Polytron (PT1200) at top speed for 1 min.

Extraction and determination of cyclic AMP in tissue extracts

The homogenate was centrifuged and each 1 ml of the supernatant was treated with 3 ml 0.5 M tri-n-octylamine dissolved in 1,1,2 trichloro-trifluoroethane to remove the TCA. The cyclic AMP content of the neutralized aqueous phase was determined by radioimmunoassay following the methods of Marley *et al.* (1991). Cyclic AMP levels were normalized to account for tissue protein content using the Bradford (1976) protein assay with bovine serum albumin as the protein standard.

Influence of enzyme inhibitors

In other tissues cumulative concentration-effect curves to the 5-HT-induced relaxation were determined on each strip after 45 min using a 2 min exposure time. At least four separate pieces of colon were used from each specimen to measure the effect of the enzyme inhibitors. One was used to determine the control EC_{50} value to 5-HT while the other tissues were incubated with the inhibitors for 30 min before determination of the concentration-effect curve to 5-HT.

Data analysis

Concentration-effect curves for 5-HT and concentration-inhibition curves for GR 113808 were expressed as either the percentage cyclic AMP stimulation above basal levels or as the amount of cyclic AMP produced in pmol mg^{-1}. The cyclic AMP assay procedure was performed in duplicate. The relaxant response to 5-HT was expressed as a percentage of the maximum relaxation induced by 5-HT, and also as an absolute value expressed in cm.s calculated from the integral of the area under the trace using 1 min as the time interval. EC_{50} values were calculated graphically from the 50% response level and expressed as geometric mean with 95% confidence limits in parentheses. All other data are given as arithmetic mean ± s.e. mean or geometric mean with 95% confidence limits. Concentration-ratios were calculated as the ratio of EC_{50} values between the control concentration-effect curve and the curve in the presence of the enzyme inhibitor. The number of observations is indicated by n.

Concentration-effect/inhibition curves were analysed by fitting a four parameter logistic equation to the data to obtain location and slope parameters. The equation is:

$$y = a + b/\{1 + 10^{-d(c+\log[A])}\}$$

where A is the agonist concentration, a is the basal value, b is the vertical range, c is the pEC_{50}, and d is the mid-point slope (Lew, 1995). The data points were fitted to the equation by a non-linear curve fitting analysis programme (Graph Pad Prism, Graph Pad Software Inc., San Diego, U.S.A.).

The K_i estimate for GR 113808 was calculated from an adaptation of the Cheng & Prusoff (1973) equation (assuming the agonist Hill slope is 1):

$$K_i = IC_{50}/(1 + [A]/EC_{50}),$$

where [A] is the concentration of 5-HT used and IC_{50} is the concentration of GR 113808 yielding a response equivalent to 50% of that achieved by 5-HT alone (Leff & Dougall, 1993). An apparent pA_2 estimate was calculated from the Furchgott (1972) relationship:

$$pA_2 = \log(CR - 1) - \log[B],$$

where CR is the concentration-ratio of agonist used in the presence and absence of antagonist (B) and is expressed as pA_2 ± s.e. mean. The concentration-ratios required for the above analysis were determined from EC_{50} values in the presence and absence of the antagonist.

The significance of differences between values was determined by use of Student's unpaired t test and for multiple comparisons, Dunnett's t-test. The differences between curves were determined using two-way ANOVA. The criterion for statistical significance was set at $P < 0.05$.

Materials

Adenosine 3′:5′-cyclic monophosphate (cyclic AMP), 1-(5-isoquinolinylsulphonyl)-2-methylpiperazine (H7) and 5-hydroxytryptamine creatinine sulphate; Sigma Chemical Company (Castle Hill, Australia), GR 113808 ({1-[2-(methyl-sulphonylamino)ethyl]-4-piperidinyl}methyl 1-methyl-1H-indole-3-carboxylate) and ondansetron hydrochloride; Glaxo (Melbourne, Australia), DAU 6236 (endo-8-methyl-8-azabicyclo [3.2.1] oct-3-yl 2,3-dihydro-3-ethyl-2-oxo-1H-benzimidazole-1-carboxylate hydrochloride); Boehringer-Ingelheim, (Milan, Italy), {N-[2-((3-(4-Bromophenyl)-2-propenyl) amino) ethyl]-5-isoquinoline sulphonamide, HCl} (H89, Calbiochem, Australia), were dissolved in distilled water. Methysergide hydrogen maleate (Sandoz, Basle, Switzerland) was dissolved in 90% ethanol and diluted with (+)-tartaric acid 0.1% in distilled water. 3-Isobutyl-1-methyl-xanthine (IBMX, Sigma, Australia), SQ 22536 (9-(tetrahydro-2-furyl) adenine, (Bristol-Myers Squibb, Melbourne, Australia), forskolin (Sigma, Australia) and rolipram (4-(3-cyclopentyloxy-4-methoxyphen-yl-2-pyrrolidone, Schering, Berlin, Germany) were dissolved in dimethylsulphoxide.

Results

Effects of 5-HT receptor agonists

Figure 1 shows that 5-HT and DAU 6236 caused a concomitant increase in cyclic AMP and relaxation in the circular smooth muscle of human colon. The 5-HT-induced cyclic AMP stimulation was concentration-dependent (EC_{50} = 175.5 (95% CL 22.9 – 1343) nM, n = 4), with a supramaximal relaxant concentration (10 μM) producing an approximately 5 fold increase above the basal level of 1.54 ± 0.24 pmol mg^{-1}, n = 4. The relaxant effect was also concentration-dependent (EC_{50} = 274.9 (95% CL 126.4 – 597.8) nM, n = 4).

A maximally active relaxing concentration of DAU 6236 (10 μM) produced a significant increase in cyclic AMP levels relative to basal levels ($P < 0.05$, n = 4) but was a partial agonist with an intrinsic activity of 0.37 relative to the 5-HT-induced maximal increase in cyclic AMP levels. The DAU 6236 (10 μM)-induced relaxant effect which accompanied this increase in cyclic AMP was 16.4% of the relaxant effect elicited by 5-HT (10 μM) (see Figure 1b).

Effects of 5-HT receptor antagonists

GR 113808 (0.1 – 1000 nM) inhibited the cyclic AMP formation and the relaxation induced by 5-HT (1 μM) in a concentration-dependent fashion (Figure 2a). A concentration-inhibition curve for GR 113808 was determined by a non-linear curve fitting programme. The pIC_{50} value for GR 113808 from the cyclic AMP data was 8.36 ± 0.32 (n = 4), with a subsequent pK_i value of 9.1. The values determined from the relaxation data were pIC_{50} = 8.25 ± 0.66 and pK_i = 8.9 (n = 4). At a concentration of 10 nM, GR 113808 produced a parallel rightward shift of the concentration-cyclic AMP elevation effect curve to 5-HT, yielding a concentration-ratio of 17.3 ± 2.8 and an apparent pA_2 value of 9.2 ± 0.1 (n = 4). The pA_2 determined from the relaxant effect data was 9.5 ± 0.1 (n = 4).

The combination of ondansetron (10 μM) and methysergide (10 μM) failed to inhibit the formation of cyclic AMP or the relaxation induced by 5-HT (1 μM, $P > 0.05$, Figure 2b).

Effects of phosphodiesterase inhibitors

Concentration-effect curves to the 5-HT-induced relaxation in the presence and absence of IBMX (non-selective phosphodiesterase inhibitor) are shown in Figure 3. In this series of experiments, the relaxant effect of 5-HT was concentration-dependent with an EC_{50} value of 250.1 nM (95% CL 144.0 – 435.5). Application of IBMX (0.1 – 300 μM) produced a concentration-dependent relaxation (EC_{50} = approx. 30 μM in 2 tissues). IBMX (1 μM), without affecting the basal tone, caused a significantly leftward shift ($P < 0.05$, two-way ANOVA) of the concentration-effect curve to 5-HT with a concentration-ratio of 4.1 (EC_{50} = 61.1 nM (95% CL 33.2 – 112.2) in the presence of IBMX, n = 5) without affecting the absolute maximum response to 5-HT ($P > 0.05$). At the higher concentration of 10 μM, IBMX also caused a leftward shift of the concentration-effect curve to 5-HT (concentration-ratio = 3.8) but this shift was no greater than the shift observed at 1 μM ($P > 0.05$). Rolipram (type IV selective phosphodiesterase inhibitor) also caused a concentration-dependent relaxation with a significantly higher potency but caused a smaller maximum relaxant effect value relative to IBMX (EC_{50} = 564.8 nM (186.7 – 1709.0); E_{max} = 28.9 cm.s (95% CL 17.5 – 40.3), n = 3). Rolipram (200 nM), without affecting the basal tone, also

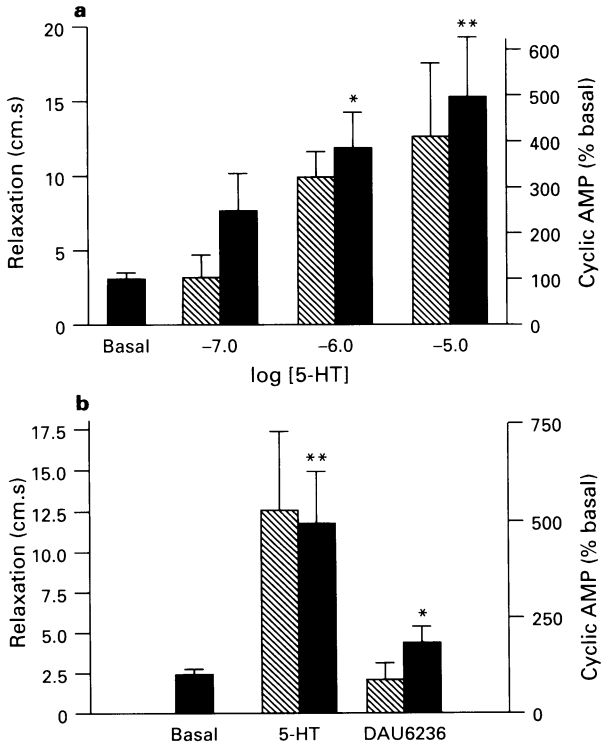

Figure 1 (a) Stimulation of cyclic AMP generation (solid columns) and relaxation (hatched columns) by 5-HT, and (b) comparison of the cyclic AMP generation (solid columns) and relaxation (hatched columns) induced by 5-HT (10 μM) and DAU 6236 (10 μM) in human colonic circular muscle. Each column represents the mean ± s.e. mean for $n \geq 4$ determinations. *$P < 0.05$ (Student's t test); ** $P < 0.05$ (ANOVA, Dunnett's t test) compared to basal.

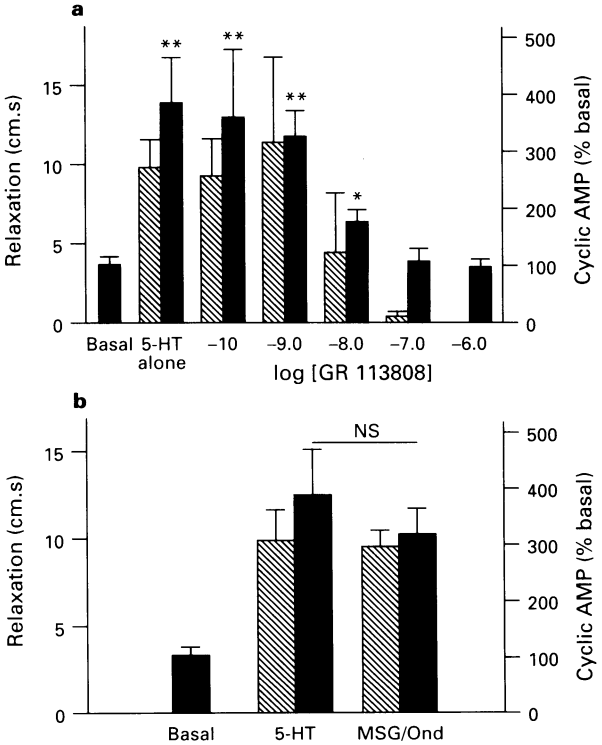

Figure 2 (a) The effect of GR 113808 against 5-HT (1 μM)-induced cyclic AMP production (solid columns) and relaxation (hatched columns) in human colonic circular muscle. Each column is the mean ± s.e.mean (n = 4). (b) Stimulation of cyclic AMP production (solid columns) and relaxation (hatched columns) by 5-HT (1 μM) in the absence and presence of methysergide (MSG, 10 μM) and ondansetron (Ond, 10 μM) in human colonic circular muscle. Each column represents the mean ± s.e.mean ($n \geq 4$). Data are expressed as the percentage cyclic AMP stimulation above basal levels and as the integral of the relaxation response. *$P < 0.05$ (Student's t test); ** $P < 0.05$ (ANOVA, Dunnett's t test) compared to basal. Single line (NS) connecting columns indicates no significant difference ($P > 0.05$).

caused a significantly leftward shift ($P<0.05$, two-way ANOVA) of the concentration-effect curve to 5-HT with a concentration-ratio of 5.5 ($EC_{50}=45.88$ nM (95% CL 17.8–118.2) in the presence of rolipram, $n=4$) without affecting the maximum response to 5-HT ($P>0.05$).

Effects of protein kinase and adenylyl cyclase inhibitors

The effects of H7 (100 nM; non specific protein kinase inhibitor), H89 (200 nM, protein kinase A selective inhibitor) and SQ 22536 (100 μM; adenylyl cyclase inhibitor; Haslam *et al.*, 1978) on the 5-HT-induced relaxation are shown in Figure 4. Each compound inhibited the action of 5-HT, without affecting the basal tone, evoking a significant rightward shift ($P<0.05$, two-way ANOVA) of the concentration-effect curve to 5-HT with concentration-ratios of 2.7 (H7), 4.2 (H89) and 10.1 (SQ 22536). EC_{50} values were 274.9 nM (126.4–597.8, control 5-HT); 754.8 nM (497.9–1144.0, H7), 1.14 μM (0.57–2.31, H89) and 10.1 μM (7.4–13.8, SQ 22536) ($n \geqslant 4$). No alteration of the maximum response to 5-HT was observed with H7; however, a significant reduction was observed in the presence of H89 and SQ 22536 ($E_{max}=96.0\%$ (89.2–102.8, control 5-HT); 61.3% (55.0–67.6, H89); 80.1% (74.5–85.7 SQ 22536), $P<0.05$, $n \geqslant 4$). In one separate tissue, no response was observed to 5-HT ($\leqslant$ 50 μM) in the presence of H89.

Effects of forskolin

Forskolin stimulated cyclic AMP production and caused a marked relaxation. The maximum relaxant effect of forskolin (6 μM) was 13.8±1.9 cm.s which was not significantly different from the maximum relaxant effect of 5-HT (10 μM, 12.7±4.9 cm.s, $n=4$, $P>0.05$). However, the cyclic AMP levels stimulated by forskolin (6 μM, 49.3±6.6 pmol mg^{-1}) were markedly greater than those stimulated by 5-HT (10 μM, 7.6±2.0 pmol mg^{-1}, $n=4$, $P<0.05$, Figure 5).

Discussion

The present study confirms and extends our previous findings that 5-HT stimulates cyclic AMP formation in human colonic circular muscle via activation of 5-HT$_4$ receptors (McLean & Coupar, 1996). The possibility that cyclic AMP is the second messenger involved in mediating the 5-HT-induced response was confirmed by the use of phosphodiesterase inhibitors. In addition, data are provided to show that the receptor is positively coupled to adenylyl cyclase and also that the cyclic AMP produced activates cyclic AMP-dependent protein kinase (protein kinase A, PKA). These findings were based on the use of an adenylyl cyclase inhibitor and protein kinase inhibitors. The study also shows that a good correlation exists between the 5-HT-induced relaxation and cyclic AMP elevation providing further evidence that the 5-HT-induced relaxation is mediated via cyclic AMP.

The biochemical linkage to the 5-HT$_4$ receptor in numerous preparations is well established as being through a positive coupling to adenylyl cyclase. This includes the 5-HT$_4$ receptors present on mouse embryonic colliculi neurones and on membranes from guinea-pig hippocampus (Dumuis *et al.*, 1988), cells in porcine atria (Kaumann *et al.*, 1991) and more recently

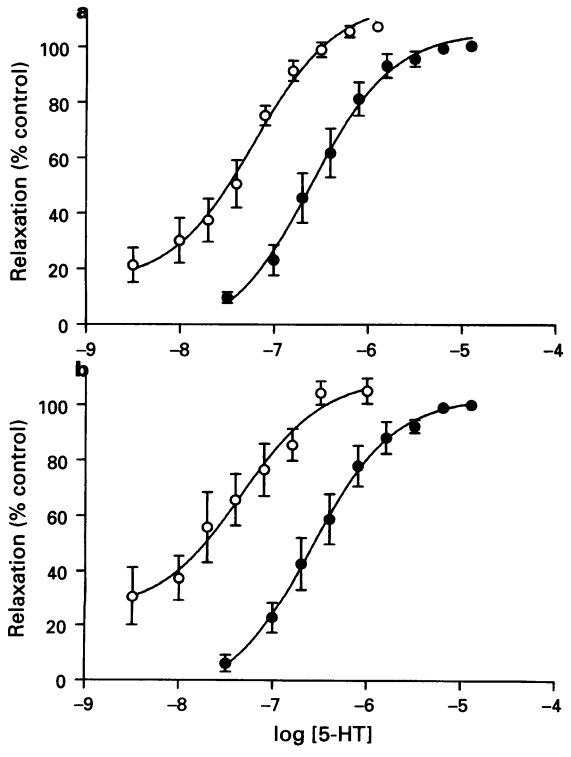

Figure 3 Cumulative concentration-effect curves to 5-HT in human colonic circular muscle in the absence (●) and presence of (a) IBMX (1 μM, ○); (b) rolipram (200 nM, ○). Data are mean±s.e. mean for $n \geqslant 4$ determinations.

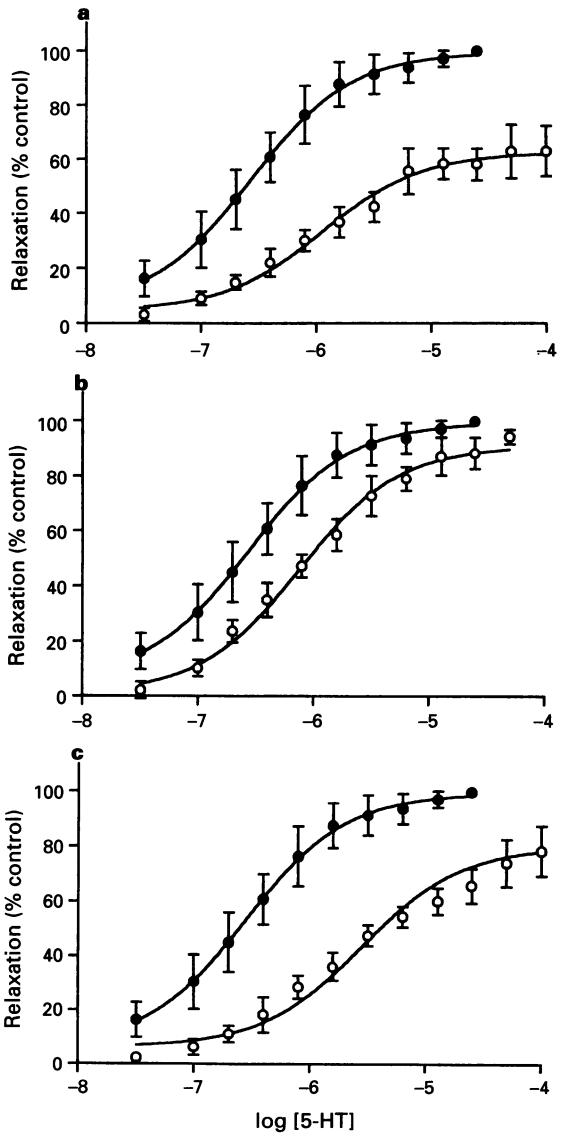

Figure 4 Cumulative concentration-effect curves to 5-HT in human colonic circular muscle in the absence (●) and presence of (a) H89 (200 nM, ○); (b) H7 (100 nM, ○); and (c) SQ 22536 (100 μM, ○). Data are mean±s.e. mean for $n \geqslant 4$ determinations.

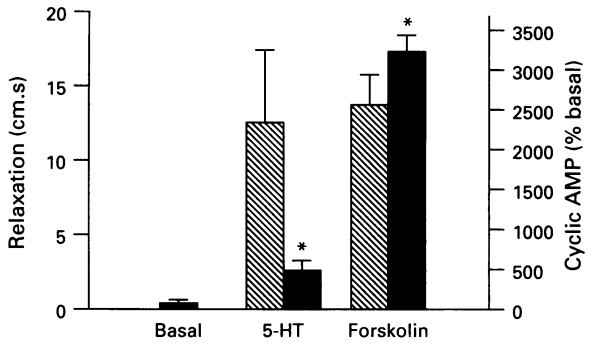

Figure 5 Comparison of cyclic AMP generation (solid columns) and relaxation (hatched columns) induced by 5-HT (10 μM) and forskolin (6 μM) in human colonic circular muscle. Each column represents the mean ± s.e. mean for $n=4$ determinations. *$P<0.05$ compared to basal.

in the human frontal cortex (Monferini *et al.*, 1993). In other human tissues 5-HT$_4$ receptors which are positively linked to adenylyl cyclase have been shown to be present on cells from atrial appendages (Kaumann *et al.*, 1990) and the adrenal gland (Lefebvre *et al.*, 1992). In the digestive tract, however, the rat oesophagus is the only preparation containing a 5-HT$_4$ receptor which has been shown to be positively coupled to adenylyl cyclase (Ford *et al.*, 1992). In fact, it has not been possible to show an involvement of cyclic AMP in the 5-HT$_4$ receptor-mediated response in the guinea-pig ileum (Linnik *et al.*, 1991; Kilbinger *et al.*, 1995).

Our current results show that human colonic circular muscle also possesses a 5-HT$_4$ receptor which mediates its response by increasing cyclic AMP formation. The first of the criteria as suggested by Sutherland (1972) is that an agonist that putatively acts by increasing cyclic AMP formation in a tissue must be shown to activate adenylyl cyclase. This has been satisfied by the use of the adenylyl cyclase inhibitor, SQ 22536 (Haslam *et al.*, 1978; Lippe & Ardizzone, 1991), which was observed to inhibit the 5-HT-induced relaxation.

The second criterion, that the agonist should increase cyclic AMP levels in a manner that correlates appropriately with the physiological response has also been satisfied, indeed, the concentration-effect curves for the relaxant and cyclic AMP elevating effects were coincident. The potency of 5-HT determined from its cyclic AMP-generating action was not significantly different from that of its relaxant action (EC_{50} values of 175.5 and 274.9 nM respectively). Previously we have shown the 5-HT$_4$ receptor agonist, DAU 6236, to be a partial agonist in functional whole tissue studies (intrinsic activity relative to 5-HT = 0.28, McLean *et al.*, 1995). In the present study DAU 6236 produced an increase in cyclic AMP levels but was a partial agonist relative to 5-HT, with an intrinsic activity of 0.37. This value is also similar to that obtained from the functional data in the present study (intrinsic activity relative to 5-HT = 0.16). These results suggest that the biochemical response to 5-HT$_4$ receptor activation may be in parallel with the relaxant response. However, more partial agonist studies are required to establish fully the correlation.

The affinity values determined for the 5-HT$_4$ receptor antagonist GR 113808 (Gale *et al.*, 1994; Grossman *et al.*, 1993; McLean *et al.*, 1995) from the cyclic AMP and functional data were also in agreement. GR 113808, which has a pA_2 of 9.0 in strips of human colon (McLean *et al.*, 1995) has a 1000 fold selectivity for the 5-HT$_4$ receptor. In the present study, GR 113808 reduced the 5-HT-induced increase in cyclic AMP with an apparent pA_2 value of 9.2 and a pK_i value of 9.1 which compares favourably with the affinity values calculated from the functional data (9.5 and 8.9 respectively). These values for GR 113808 are in accordance with other reported affinity values including inhibition of 5-HT-induced relaxation of the rat oesophagus and rat ileum ($pA_2 = 8.6$ and 9.3 respectively, McLean *et al.*, 1995), contraction of the guinea-pig distal colon ($pA_2 = 9.2$, Gale *et al.*, 1994), increase in contractile force of human atria ($pK_B = 8.8$, Kaumann, 1993), potentiation of neuromuscular cholinergic transmission in human detrussor muscle ($pA_2 = 8.9$, Tonini *et al.*, 1994) and has a reasonably similar affinity at 5-HT$_4$ receptors in brain tissues from guinea-pig and rat ($pK_i = 9.5$ and 9.6 respectively, Grossman *et al.*, 1993), in monkey urinary bladder ($pA_2 = 9.5$, Waikar *et al.*, 1994) and in guinea-pig ileal mucosa ($pA_2 = 9.6$, Leung *et al.*, 1995).

The basal cyclic AMP levels determined from control tissues in the present study were relatively low (1.54 ± 0.24 pmol mg^{-1}) when compared to other studies (eg. Sanders & Kaumann, 1992, human atria, basal levels = 18.2 ± 2.2 pmol mg^{-1}). This apparent discrepancy is possibly due to regional tissue variation, which is supported by the observation that other tissues from the digestive tract were observed to have similar basal levels to the human colon (rat oesophagus, 3.7; rat jejunum, 2.1; unpublished observations). It is also possible that differences in assay methodology could affect inter-study basal cyclic AMP levels.

The data obtained with IBMX and rolipram are consistent with Sutherland's (1972) fourth criterion that phosphodiesterase inhibitors should reproduce the physiological response or should act synergistically with the neurotransmitter to produce the response. At least five different cyclic nucleotide phosphodiesterase enzymes have now been identified on the basis of their functional characteristics such as substrate-specificity and susceptibility to selective inhibitors (Beavo & Reifsnyder, 1990; Nicholson *et al.*, 1991) and molecular cloning studies have revealed that several additional families exist (Michaeli *et al.*, 1993). IBMX (a non-selective phosphodiesterase inhibitor, Beavo, 1988) and rolipram (a type IV, cyclic AMP-specific phosphodiesterase inhibitor, Schneider *et al.*, 1986; Lowe & Cheng, 1992) not only mimicked the action of 5-HT dose-dependently, but, at a concentration which had no effect on basal tone, enhanced the ability of 5-HT to produce its relaxant effect. There is evidence that phosphodiesterase inhibitors may not discriminate between phosphodiesterase isozymes (Wells & Kramer, 1981), however, the biochemical determinations performed in this study confirmed the involvement of cyclic AMP in the 5-HT-induced response.

All known actions of cyclic AMP or its derivatives are assumed to operate via activation of cyclic AMP-dependent protein kinase A (PKA) (see Beebe & Corbin, 1986, for review). However, there are some exceptions whereby cyclic AMP analogues were reported to produce their effect independent of PKA activation (Francis *et al.*, 1988; Hei *et al.*, 1991). Certain criteria should be satisfied before a cyclic AMP-mediated response can be established to be carried out by the cyclic AMP-dependent protein kinase (Cohen, 1978; Krebs & Beavo, 1979; Beavo & Mumby, 1982). One important criterion is that the response is sensitive to protein kinase inhibition with a selective protein kinase inhibitor such as H89 (Chijiwa *et al.*, 1989). The findings that the non-selective protein kinase inhibitor, H7 (Fagni *et al.*, 1992) and the cyclic AMP-dependent protein kinase inhibitor, H89, inhibited the 5-HT-induced relaxation of the muscle supports further the proposal that the cyclic AMP formed by 5-HT$_4$ receptor activation produces its effect by activating protein kinase A.

The incidental finding that forskolin produced a relaxation which mirrored the 5-HT-induced relaxation but produced a markedly greater cyclic AMP elevation is possibly explained by the fact that 5-HT$_4$ receptors activate only the adenylyl cyclase to which it is coupled via its specific G-protein, whereas forskolin activates all adenylyl cyclases in the tissue hence producing a far greater amount of cyclic AMP.

In conclusion, the 5-HT$_4$ receptor which mediates relaxation and inhibition of the spontaneous contractions of human colonic circular smooth muscle, produces its effect by activating adenylyl cyclase, stimulating intracellular cyclic AMP accumulation which activates protein kinase A. This confirms that 5-HT$_4$ receptors are positively linked to adenylyl cyclase and is the first report of 5-HT$_4$ receptor coupling in human alimentary tract which is mediated by cyclic AMP.

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