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SPECIAL REPORT

Stimulation of cyclic AMP formation in the circular smooth muscle of human colon by activation of 5-HT_4-like receptors

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5-HT stimulated cyclic AMP generation in human colonic circular smooth muscle in a concentration-dependent fashion (EC_{50} = 229.1 nM). DAU 6236 also increased cyclic AMP formation and was a partial agonist relative to 5-HT. GR 113808 inhibited the cyclic AMP formation induced by 5-HT with a $-\log K_i$ value of 9.1 and an apparent pA_2 value of 9.2. Ondansetron and methysergide failed to inhibit cyclic AMP formation induced by 5-HT. These results indicate that the 5-HT_4 receptors of human colonic circular muscle mediate relaxation and inhibition of spontaneous contractions via formation of cyclic AMP.

Keywords: 5-HT (5-hydroxytryptamine, serotonin); 5-HT_4 receptors; human colon; cyclic AMP; GR 113808; DAU 6236

Introduction Recently a 5-HT_4-like receptor has been identified and characterized pharmacologically in the human colon (Tam *et al.*, 1994; McLean *et al.*, 1995). The second messenger mediating the action of this receptor is unknown but 5-HT_4 receptors have been shown to be positively linked to adenylyl cyclase in numerous preparations of human and animal tissues (see Hoyer *et al.*, 1994). Thus, the present study was undertaken to determine whether cyclic AMP is involved in the 5-HT_4 receptor-induced responses in the human colon.

Methods Our previously described methods (McLean *et al.*, 1995) were used to measure the isotonic responses of isolated strips of human colonic circular muscle, suspended in Krebs-Henseleit solution at 37°C and gassed with 5% CO_2 in O_2.

Incubation of muscle strips for cyclic AMP production Cyclic AMP formation was measured in tissues exposed to a single concentration of an agonist either in the absence or presence of antagonist(s). The tissues were then frozen in liquid nitrogen, pulverized, and homogenized in 1.5 ml cold 6% trichloroacetic acid (TCA).

Extraction and determination of cyclic AMP in tissue extracts The homogenate was centrifuged and the supernatant treated with 0.5 M tri-n-octylamine dissolved in 1,1,2 trichloro-trifluoroethane to remove the TCA. The cyclic AMP content of the neutralised aqueous phase was determined by radioimmunossay following the methods of Marley *et al.* (1991). Cyclic AMP levels were normalized to account for tissue protein content measured by the Bradford protein assay.

Data analysis Concentration-effect curves for 5-HT and concentration-inhibition curves for GR 113808 were expressed as percentage cyclic AMP stimulation above basal levels. 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. The number of observations is indicated by *n*.

The significance of differences between values was determined by Student's unpaired *t* test and for multiple comparisons, Dunnett's *t* test. The criterion for statistical significance was set at $P<0.05$.

Materials Adenosine 3':5'-cyclic monophosphate (cyclic AMP) 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. All compounds were dissolved in distilled water with the exception of methysergide hydrogen maleate (Sandoz, Basle, Switzerland) which was dissolved in 90% ethanol and diluted with (+)-tartaric acid 0.1% in distilled water.

Results 5-HT and DAU 6236 stimulated the production of cyclic AMP in human colonic circular smooth muscle (Figure 1). The 5-HT-induced stimulation was concentration-dependent (EC_{50} = 229.1 ± 4.3 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} ($n=4$). A supramaximal 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 5-HT.

GR 113808 (0.1–1000 nM) inhibited the cyclic AMP formation induced by 5-HT (1 μM) in a concentration-dependent fashion. The concentration-inhibition curve for GR 113808 was determined by a non-linear curve fitting programme and is shown in Figure 2a. The $-\log IC_{50}$ value for GR 113808 was 8.36 ± 0.32 ($n=4$), with a subsequent $-\log K_i$ value of 9.1. At a

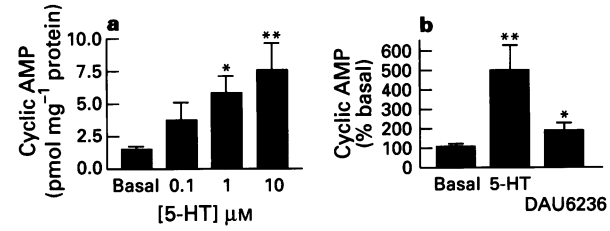

Figure 1 (a) Stimulation of cyclic AMP generation by 5-HT, and (b) comparison of the cyclic AMP generation induced by 5-HT (10 μM) and DAU 6236 (10 μM) in human colonic circular muscle. Each column represents the mean ± s.e.mean for $n \geq 4$ determinations. *$P<0.05$ (Student's *t* test); **$P<0.05$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

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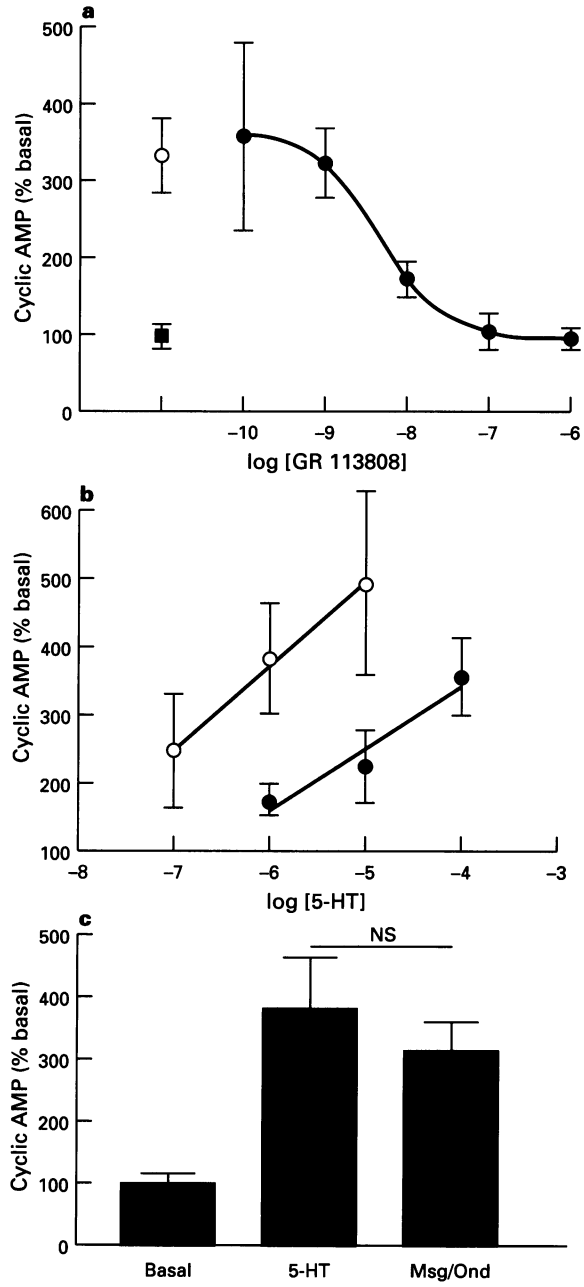

Figure 2 (a) Concentration-inhibition curve for GR 113808 (●) against 5-HT (1 μM) -induced cyclic AMP production; (■) basal; (○) 5-HT alone. (b) Concentration-effect curves to 5-HT-induced cyclic AMP production in the absence (○) and presence of GR 113808 (10 nM, ●). (c) Stimulation of cyclic AMP production by 5-HT (1 μM) in the absence and presence of methysergide (Msg, 10 μM) and ondansetron (Ond, 10 μM). Single line (NS) connecting bars indicates no significant difference ($P>0.05$). Each column/point represents the mean ± s.e.mean ($n \geq 4$). All data are expressed as the percentage cyclic AMP stimulation above basal levels in human colonic circular muscle.

concentration of 10 nM, GR 113808 produced a parallel rightward shift of the concentration-effect curve to 5-HT (Figure 2b) yielding a concentration-ratio of 17.3 ± 2.8 and an apparent pA_2 value of 9.2 ± 0.1 ($n=4$).

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

Discussion The present findings show that the activation of the 5-HT_4-like receptor which causes relaxation and inhibition of spontaneous contractions of human colonic circular muscle is associated with cyclic AMP formation.

To date all reported biochemical linkages to central and non-neuronal peripheral 5-HT_4 receptors have been through a positive coupling to adenylyl cyclase, ranging from receptors present on mouse embryonic colliculi neurones to cells in human atria and more recently in the human cortex (see Hoyer *et al.*, 1994).

The potency of 5-HT determined from its cyclic AMP generating action is similar to that from its relaxant action in the human colon (EC_{50} values of 229.1 and 250.1 nM respectively) which has been shown to be mediated by a 5-HT_4-like receptor (Tam *et al.*, 1994; McLean *et al.*, 1995). The 5-HT_4 receptor agonist, DAU 6236, produced a small increase in cyclic AMP levels with an intrinsic activity of 0.37 relative to 5-HT. This value is similar to that obtained from our previous whole tissue studies (intrinsic activity relative to 5-HT = 0.28, McLean *et al.*, 1995). These results indicate that the biochemical response to 5-HT_4 receptor activation is associated with the same receptor reserve as that associated with the relaxant response.

The 5-HT_4 receptor antagonist, GR 113808, which has a pA_2 of 9.0 in 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. This also compares favourably with affinity values in other tissues including the rat oesophagus and guinea-pig proximal colon ($pA_2 = 9.2$ and 9.5 respectively, see Hoyer *et al.*, 1994) and human atria ($pK_B = 8.8$, Kaumann, 1993).

Further evidence in favour of a 5-HT_4 receptor mediating the described 5-HT-induced cyclic AMP formation is that the stimulatory effect of 5-HT was not altered by methysergide and ondansetron which would be expected to block 5-HT_1, 5-HT_2, and 5-HT_3 receptors.

The results of this study are further confirmation that 5-HT_4 receptor coupling is mediated by cyclic AMP and is the first account of a 5-HT_4 receptor coupled to the nucleotide in the human alimentary tract.

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