Effects of 5-HT₃ receptor antagonists on 5-HT and nicotinic depolarizations in guinea-pig submucosal neurones

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- 1 Intracellular recordings were made from neurones of the guinea-pig submucosal plexus. The effects of several 5-hydroxytryptamine₃ (5-HT₃) receptor antagonists on depolarizations produced by ionophoretic application of 5-HT and acetylcholine, as well as on fast excitatory postsynaptic potentials (fast e.p.s.ps) produced by nerve stimulation were examined.
- 2 ICS 205-930, GR 38032F, MDL 72222, cocaine and curare all inhibited the fast e.p.s.p. as well as the depolarizations in response to 5-HT and acetylcholine (ACh) ionophoresis in a dose-dependent fashion.
- 3 IC₅₀ values for ICS 205-930, GR 38032F, MDL 72222, cocaine and curare in inhibiting the 5-HT mediated depolarizations were 12 nM, 100 nM, 3 μ M, 3 μ M and 20 μ M, respectively.
- 4 IC₅₀ values for ICS 205-930, GR 38032F, MDL 72222, cocaine and curare in inhibiting the nicotinic depolarizations were 4 μ M, 12 μ M, 11 μ M, 6 μ M and 17 μ M, respectively. Similar IC₅₀ values were obtained for inhibition of the fast e.p.s.ps by these antagonists.
- 5 The nicotinic receptor blocker, hexamethonium, inhibited the nicotinic depolarization and the fast e.p.s.p. with IC₅₀ values of 10 μ M. Hexamethonium (10 μ M–5 mM) did not alter the depolarization induced by 5-HT.
- 6 These results demonstrate that the pharmacological profile of 5-HT₃ receptors present on submucosal neurones is identical to that of 5-HT₃ receptors on myenteric neurones and, thus, provide evidence that the enteric neuronal 5-HT₃ receptor forms a receptor subtype distinct from that characterized in other parts of the autonomic nervous system.

Introduction

5-Hydroxytryptamine (5-HT) has multiple actions on both pre- and post-synaptic membranes in autonomic neurones; this multiplicity of action is due largely to the presence of multiple 5-HT receptor subtypes (Bradley *et al.*, 1986; Peroutka, 1988). The cellular mechanism responsible for the 5-HT₃ response in autonomic ganglia is the best characterized of all 5-HT receptor responses: activation of 5-HT₃ receptors produces a rapidly desensitizing, 'nicotinic-like' depolarization resulting from an increased conductance to monovalent cations (Higashi & Nishi, 1982). More recently, patch-clamp studies from excised membranes have shown directly that the ganglionic 5-HT₃ receptor is a ligand-gated ion channel distinct from the nicotinic ion channel (Derkach *et al.*, 1989).

The pharmacology of 5-HT₃ receptors also has developed rapidly since the introduction of MDL 72222 (Fozard, 1984) and now several substances in addition to this one, particularly ICS 205-930, GR 38032F and zacopride, are known to be highly selective 5-HT₃ receptor antagonists (see Richardson & Engel, 1986; Peroutka, 1988). This selectivity pertains to the capacity of these antagonists to recognize 5-HT₃ receptors over other 5-HT subtypes and amine receptors such as dopamine and noradrenaline (Peroutka, 1988). Indeed, the development of these 5-HT₃ receptor antagonists has enabled these responses to be studied in isolation from other 5-HT responses and has been the major stimulus for the growing number of studies on the action of 5-HT in the autonomic nervous system (Mawe *et al.*, 1986; Surprenant & Crist, 1988; Wallis & Dun, 1988; Elliott & Wallis, 1988; Galligan *et al.*, 1988). 5-HT₃ antagonists have been shown to be selective for 5-HT₃ receptors over muscarinic acetylcholine receptors (Fozard, 1984; Richardson *et al.*, 1985; Surprenant & Crist, 1988) but the extent to which this selectivity pertains to the nicotinic acetylcholine receptor has received relatively little attention. This is an important question to examine in view of the similarities in the cellular mechanisms of 5-HT₃ and nicotinic responses as well as the well known action of curare in blocking both types of receptor response (Neild, 1978; Skok & Selyanko, 1979; Higashi & Nishi, 1982). Therefore, we under-

took this study in order to examine the actions of a number of 5-HT₃ receptor antagonists on the 5-HT₃ and the nicotinic depolarizations in submucosal neurones and on the nicotinic excitatory synaptic potential produced by nerve stimulation of submucosal fibres. These studies have allowed us to determine the potency of action of these antagonists on both the 5-HT₃ and the nicotinic responses, as well as their relative selectivity of action at these two distinct receptors in submucosal neurones.

Methods

Experiments were carried out on submucosal plexus preparations obtained from the small intestine of guinea-pigs (175–250 g). Methods of dissection, intracellular recording and stimulation have been described previously (Surprenant & Crist, 1988; Surprenant & North, 1988). The preparation was continuously superfused with a physiological saline solution containing (mM): NaCl 126, NaH₂PO₄ 1.2, MgCl₂ 1.2, CaCl₂ 2.5, KCl 5, NaHCO₃ 25, glucose 11; gassed with 95% O₂ and 5% CO₂.

Acetylcholine and 5-HT were applied by ionophoresis from the same microelectrodes as were used for intracellular recording; the concentration of agonist in the pipette was 100 μ M. Stimulus parameters were set so as to produce responses whose durations were, as far as was possible, comparable to the fast e.p.s.p. (50–200 ms); duration of the ionophoretic pulses ranged from 0.5 ms to 15 ms. Small amounts of backing current (<5 nA) were applied if signs of leakage existed. In some experiments dopamine (1 M solution) was applied by ionophoresis. Ionophoretic pulses were delivered at a frequency of 0.05 Hz throughout the course of the experiment. Fast excitatory postsynaptic potentials (e.p.s.ps) were recorded in response to single nerve stimuli delivered at a frequency of 0.3 Hz.

Antagonist concentration-inhibition curves were constructed by applying antagonists in cumulative doses, at least 5 min superfusion was allowed between measurements; preliminary experiments in which single concentrations of

antagonists were used indicated that this length of time was sufficient for antagonists to achieve their maximum effect. Intracellular recordings were usually maintained for sufficient time (typically 3–4 h) to examine several antagonists on a single cell. All results are expressed as means $\pm$ s.e.means; tests of significance were carried out by Student's *t* test.

Drugs

The following drugs were used: (from Sigma) atropine, hexamethonium, 5-hydroxytryptamine creatinine sulphate (5-HT), cocaine hydrochloride, dopamine, acetylcholine, curare and physostigmine; (from Glaxo UK) 1 α H,3 α ,5 α H-tropan-3-yl-3,5-dichlorobenzoate methanesulphonate (MDL 72222) and 1,2,3,9-tetrahydro-9-methyl-3-[(2-methyl-1H-imidazol-1-yl)-methyl]-4H-carbazol-4-one hydrochloride (GR 32032F; odansetron); (from Reckitt & Colman) idazoxan; (from Sandoz) (3 α -tropanyl) H-indole-3-carboxylic acid ester (ICS 205-930).

Results

Depolarizations evoked by 5-HT, ACh and nerve stimulation

Our aim was to examine the actions of antagonists on responses which were as similar to those produced by neurally released transmitter as we could obtain (Figure 1). Resting membrane potentials of submucosal neurones studied ranged from -50 mV to -63 mV ($n = 43$); all responses were recorded at a membrane potential of -90 mV (by applying steady hyperpolarizing current through the intracellular microelectrode). In this study the fast e.p.s.p. produced by single nerve stimuli averaged 21 ± 1.1 mV amplitude with half-duration of 57 ± 4 ms ($n = 18$); average amplitude and half-duration of the 5-HT potential was 26 ± 1 mV and 132 ± 21 ms ($n = 14$); that for the ACh potential was 33 ± 2 mV and 180 ± 30 ms ($n = 17$). All neurones responded to 5-HT and ACh with these fast depolarizations ($n = 43$); the amplitudes of the 5-HT and ACh potentials were constant ($\pm 5\%$) for periods of hours when evoked by ionophoretic pulses delivered at frequencies of less than once per 15–20 s

Figure 1 Examples of depolarizations evoked by nerve stimulation (fast excitatory postsynaptic potential (e.p.s.p.)), and ionophoretic pulses of acetylcholine (ACh) and 5-hydroxytryptamine (5-HT) in submucosal neurones; the actions of 5-HT₃ antagonists on these short duration responses were examined in this study. All responses recorded at a membrane potential of -90 mV.

(see Methods). Some cells also exhibited a slow depolarization whose onset followed the decay of the fast depolarization (e.g. Figure 2c). This slow 5-HT-mediated depolarization has been shown previously to be insensitive to blockade by ICS 205-930 and curare (Surprenant & Crist, 1988). Neurones which exhibited this slow, non 5-HT₃ response, were not used for analysis of the actions of 5-HT₃ antagonists.

Inhibition of responses by ICS 205-930 and GR 38032F

ICS 205-930 and GR 38032F inhibited both the 5-HT and ACh potentials as well as the fast e.p.s.p. in a dose-dependent and reversible fashion in all neurones examined (Figures 2 and 3). Figure 3 summarizes the results obtained from all experiments in which the actions of ICS 205-930 and GR 38032F on the 5-HT and ACh potential were examined. The concentrations of ICS 205-930 and GR 38032F which resulted in 50% inhibition (IC_{50}) of the 5-HT potential were 12 nM and 100 nM respectively. Tenfold higher concentrations were required to inhibit the ACh potentials (Figure 3; Table 1) with IC_{50} values for ICS 205-930 and GR 38032F inhibition of the nicotinic response being 4 μ M and 12 μ M respectively. The threshold concentration for inhibition of the fast e.p.s.p. by ICS 205-930 was 600 nM ($n = 4$).

Inhibition of responses by curare and hexamethonium

Curare produced equivalent inhibitions of the ACh potential, the 5-HT potential and the fast e.p.s.p., with IC_{50} values for

Figure 2 Inhibition of the fast 5-hydroxytryptamine (5-HT) depolarization by ICS 205-930 (a) and GR 38032F (b). 5-HT potentials evoked by repetitive ionophoretic pulses (frequency 0.05 Hz) in control solution and in the presence of increasing concentrations of ICS 205-930 and GR 38032F; each potential was obtained 5 min after the introduction of a new concentration of antagonist, by which time the maximum effect of the antagonist had been reached. All recordings in (a) and (b) are from the same cell. (c) Example of both fast and slow depolarizations recorded from a proportion of neurones in response to 5-HT ionophoresis. High concentrations of cocaine (60 μ M) blocked the fast but not the slow depolarization.

Figure 3 Inhibition of 5-hydroxytryptamine (5-HT, two leftmost curves) and acetylcholine (ACh, two rightmost curves) potentials by ICS 205-930 (○) and GR 38032F (●) in guinea-pig submucosal neurones. Each point is the mean from 4–8 experiments; vertical lines indicate s.e.mean.

all three responses being approximately 20 μM (Figure 4a; Table 1). Hexamethonium inhibited the ACh potential and the fast e.p.s.p. to the same degree (IC_{50} of 5 μM, Figure 4b), but had no effect on the 5-HT potential at concentrations of 500 μM ($n = 3$) or 5 mM ($n = 2$).

Inhibition of responses by MDL 72222 and cocaine

Cocaine and its close structural analogue, MDL 72222 (Fozard, 1984), inhibited both 5-HT and ACh potentials; the IC_{50} values for cocaine and MDL 72222 in inhibiting the 5-HT potential were the same (Figure 4c,d; Table 1). MDL 72222 was slightly, but significantly, more potent in blocking the 5-HT potential than the ACh potential (Figure 4d; Table 1). We also noted that neither cocaine (Figure 2c) nor MDL 72222 had any effect on the slow depolarization evoked in some cells by 5-HT ($n = 4$).

Voltage-dependence of inhibition by antagonists

In order to determine whether any of the antagonists might exhibit a voltage-dependence to their inhibition, we measured the inhibition produced by the IC_{50} concentration of each antagonist on the 5-HT and ACh potentials evoked at -60 mV, -90 mV and -110 mV. It was usually not possible to hold the membrane potential more depolarized than

Figure 4 Inhibition of 5-hydroxytryptamine (5-HT) potentials (●), acetylcholine (ACh) potentials (○) and fast e.p.s.ps (■) by curare (a), hexamethonium (b), cocaine (c) and MDL 72222 (d) in guinea-pig submucosal neurones. Each point is the mean of 4–9 experiments (except in the case of hexamethonium on the 5-HT potential in which case $n = 2$); vertical lines show s.e.mean.

Table 1 Summary of IC_{50} values obtained for 5-hydroxytryptamine₃ (5-HT₃) antagonists examined in this study

Antagonist	IC_{50} values		Relative selectivity
	5-HT	ACh	
ICS 205-930	12 nM	4 μM	333
GR 38032F	100 nM	12 μM	120
MDL 72222	3 μM	11 μM	4
Cocaine	3 μM	6 μM	2
Curare	20 μM	17 μM	1

5-HT: acetylcholine (ACh) selectivity ratios are indicated for each antagonist.

approximately -55 mV because of the presence of a non-inactivating calcium current and calcium-activated potassium conductance at these potentials (Akasu & Tokimasa, 1989). ICS 205-930, GR 38032F and curare inhibited the 5-HT response to a similar degree at each membrane potential examined (Figure 5a). Both curare and hexamethonium produced a significantly greater inhibition of the ACh potential at -110 mV than at -60 mV (Figure 5b). However, this voltage-dependence of blockade of the nicotinic response was very weak (see Figure 5b) and thus appears similar to the nicotinic responses recorded in frog autonomic ganglia, where curare and hexamethonium inhibit nicotinic synaptic potentials in a voltage-independent manner (Lipscombe & Rang, 1988).

Dopamine activation of 5-HT₃ receptors

Depolarizations evoked by dopamine in mouse neuroblastoma cells have been shown to be mediated by 5-HT₃ receptors (Neijt *et al.*, 1986). In submucosal neurones, dopamine produces a membrane hyperpolarization by activating α_2 -adrenoceptors (Hirst & Silinsky, 1974; Surprenant & North, 1988). We examined whether dopamine could also activate 5-HT₃ receptors on submucosal neurones by applying ionophoretic pulses of dopamine onto these neurones after

Figure 5 Effects of membrane potential on inhibition of 5-hydroxytryptamine (5-HT) (a) and acetylcholine (ACh) (b) potentials. (a) The membrane potential did not alter the inhibition of the 5-HT response produced by ICS 205-930 (10 nM, ○), GR 38032F (100 nM, □) or curare (20 μM, ●); $n = 4-8$ for each point. (b) The inhibition of the ACh potential by hexamethonium (■) and curare (●) was significantly greater when the membrane potential was held at -110 mV than when it was held at -60 mV; $n = 4-8$ for each point.

blockade of α_2 -adrenoceptors by idazoxan (2 μ M). In the presence of idazoxan, long duration (100–350 ms) ionophoretic pulses depolarized the membrane by 5–15 mV; the dopamine-mediated depolarization was abolished by 200 nM ICS 205-930 ($n = 3$) but not by the dopamine receptor antagonist haloperidol (2 μ M).

Discussion

We have examined several agents which are known to inhibit 5-HT₃-mediated excitatory responses in autonomic ganglia and have determined their potency in inhibiting the 5-HT₃ depolarization in submucosal neurones (Table 1). We have also examined the ability of these antagonists to inhibit the nicotinic response in the same neurones and have demonstrated that they all block nicotinic responses with a distinct potency profile (Table 1). In submucosal neurones ICS 205-930 is over 300 fold selective in blocking 5-HT₃ over nicotinic responses, GR 38032F is greater than 100 fold selective for 5-HT₃ over nicotinic receptors while MDL 72222, cocaine and curare are approximately equipotent in blocking both 5-HT₃ and nicotinic receptors.

The IC₅₀ values we obtained for ICS 205-930 (10 nM), GR 38032F (100 nM) and MDL 72222 (3 μ M) in blocking the post-synaptic 5-HT potential on submucosal neurones are the same as dissociation equilibrium constants (K_D values) determined from more indirect assays in which the contractile responses of the whole ileum to 5-HT have been measured (Fozard, 1984; Richardson *et al.*, 1985; Richardson & Engel, 1986). The type of assay used in 'whole ileum' experiments primarily reflects 5-HT-induced release of acetylcholine from myenteric neurones (Fozard & Mobarok 1978; Furness & Costa, 1987); in other words, it is an indirect assay of 5-HT₃ receptors present on myenteric neurones. Our results demonstrate that 5-HT₃ receptors on submucosal neurones and on myenteric neurones are identical.

Results from the present study provide further evidence supporting the existence of subtypes of 5-HT₃ receptors. The pharmacological profile of 5-HT₃ receptors present on autonomic sympathetic neurones, such as the rabbit superior cervical ganglion, and sensory neurones (e.g. rabbit nodose ganglion) is clearly distinct from that of enteric neurones. K_D values for ICS 205-930, GR 38032F and MDL 72222 at sympathetic and sensory neurone 5-HT₃ sites are in the range of 40 pM, 5 nM and 10 nM, respectively; these values are ten to one hundred fold lower than obtained in this and other studies for 5-HT₃ receptors on enteric neurones (Fozard, 1984; Richard-

son & Engel, 1986; Butler *et al.*, 1988). Thus, while there is currently debate concerning whether the 5-HT₃ receptor present on sympathetic ganglia is pharmacologically distinct from that present on parasympathetic vagal fibres and sensory neurones (Richardson & Engel, 1986; Peroutka, 1988), our results on submucosal neurones combined with those on whole ileum (i.e. myenteric neurones) leave little doubt that the 5-HT₃ receptor on enteric neurones is a pharmacologically distinct receptor subtype.

Additional support for the existence of at least two 5-HT₃ receptor subtypes has come from recent electrophysiological studies of 5-HT₃-induced whole cell and single channel currents in neuroblastoma cells and submucosal neurones (Neijt *et al.*, 1988; Lambert *et al.*, 1989; Derkach *et al.*, 1989). The ionic mechanism and properties of desensitization underlying the 5-HT₃ responses in both tissues are identical. However, unitary conductances, measured either directly by single-channel recordings (submucosal neurones) or indirectly by noise analysis of 5-HT inward currents (neuroblastoma cells), demonstrate that the 5-HT₃ receptor in submucosal neurones is a 15 pS cationic channel while that in neuroblastoma appears to be a very small, subpicoseiman, cationic channel. The pharmacological profile of the 5-HT₃ receptor present on neuroblastoma cells partially resembles that of sensory neurones (Neijt *et al.*, 1986; 1988); thus it will be important to determine unitary conductance values for 5-HT₃ responses in other mammalian autonomic neurones and, particularly, to examine the actions of antagonists at the single channel level.

Few comparative data are available concerning the relative potencies of 5-HT₃ antagonists in a single tissue. One recent study, in which the properties of ICS 205-930, GR 38032F and zacopride at 5-HT₃ receptors in the guinea-pig ileum were compared (Cohen *et al.*, 1989), yielded K_D values for ICS 205-930 and GR 38032F which were identical to those obtained in the present study on submucosal neurones. Relative potencies of 5-HT₃ antagonists in blocking nicotinic responses have not been obtained previously, although micromolar concentrations of MDL 72222 are known to block nicotinic responses in the guinea-pig ileum (Fozard, 1984). Our results demonstrating the relative selectivity between 5-HT and nicotinic responses should be of considerable practical use in investigations of these 5-HT₃ antagonists on *in vivo* and *in vitro* gastrointestinal functions.

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