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The interaction of antidepressant drugs with central and peripheral (enteric) 5-HT_3 and 5-HT_4 receptors

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1 A combined study of receptor binding in central neuronal cell membranes and functional responses in isolated segments of guinea-pig small intestine allowed characterization of the interaction of four antidepressant drugs with central and peripheral 5-HT_3 and 5-HT_4 receptors.

2 Clomipramine, paroxetine and fluoxetine inhibited [^{3}H]-DAU 6215 binding to 5-HT_3 recognition sites in NG 108-15 cells with IC_{50} values in the range 1.3–4 μM. Litoxetine had an IC_{50} of 0.3 μM. The specific binding of [^{3}H]-GR 113808 to 5-HT_4 recognition sites in pig striatal membranes was inhibited by all four antidepressants with negligible potency (IC_{50} values $\geqslant$ 20 μM).

3 In whole ileal segments, concentration-response curves to 5-HT were biphasic, with the high- and low-potency phases involving 5-HT_4 and 5-HT_3 receptors, respectively. Curves to 2-methyl-5-hydroxytryptamine (2-methyl-5-HT: a 5-HT_3 receptor agonist) and 5-methoxytryptamine (5-MeOT: a 5-HT_4 receptor agonist) were monophasic. All antidepressants were used at concentrations lacking anticholinoceptor properties, as demonstrated in both electrically stimulated longitudinal muscle-myenteric plexus preparations (LMMPs) and in unstimulated LMMPs following addition of acetylcholine (100 nM).

4 Fluoxetine (0.1–1 μM) and litoxetine (0.3–3 μM) antagonized both the high- and low-potency phases of the 5-HT curve. Schild analysis for the low-potency phase yielded pA_2 estimates of 6.6 ± 0.3 (Schild slope of 1.1) and of 6.6 ± 0.1 (Schild slope of 1.1), respectively. At higher concentrations (3 μM), fluoxetine markedly inhibited the 5-HT response maximum. Clomipramine (10–300 nM) inhibited, by a mechanism independent of concentration, both phases of the 5-HT curve with a reduction of the maximum response. Paroxetine (1 μM) was ineffective on the high-potency phase, but caused a rightward shift of the low-potency phase (pK_B: 6.1 ± 0.01).

5 Responses to 2-methyl-5-HT were inhibited by 1 μM fluoxetine (pK_B: 5.4 ± 0.02). Like clomipramine (30 and 100 nM), litoxetine (1 and 3 μM) produced rightward displacements of 2-methyl-5-HT-induced contractions, which were virtually independent of antidepressant concentration (pK_B values: 6.0 ± 0.02 and 5.5 ± 0.01, respectively). At higher concentrations, fluoxetine (3 μM) and clomipramine (300 nM) markedly reduced the 2-methyl-5-HT response maximum. Paroxetine (1 μM) was ineffective.

6 Responses to 5-MeOT were shifted to the right by fluoxetine (0.1–1 μM) and litoxetine (1 and 3 μM) in a concentration-dependent manner. At higher concentrations, fluoxetine (3 μM) markedly reduced the 5-MeOT response maximum, an effect also observed with 100 and 300 nM clomipramine. Paroxetine (1 μM) was ineffective.

7 In unstimulated LMMPs, the excitatory effects evoked by 5-HT, 2-methyl-5-HT and 5-MeOT and the antagonism produced by 300 nM clomipramine were comparable to those obtained in whole ileal segments. This suggests that 5-HT contained in the mucosa of whole preparations does not interfere with agonist-induced contractile responses and with the inhibitory effect of antidepressant drugs.

8 In conclusion, our results show that clomipramine, fluoxetine, paroxetine and litoxetine possess low to moderate potency/affinity at both central and peripheral (enteric) 5-HT_3 receptors. In contrast, all four antidepressants are virtually ineffective at central 5-HT_4 receptors. Inhibition of 5-HT_4 receptor-mediated ileal contractions by fluoxetine, litoxetine and clomipramine may result from allosteric antagonism or, more likely, from post-receptor blockade of second messenger generation. The interaction of antidepressants with central and peripheral 5-HT_3 and 5-HT_4 receptors may be relevant for both potential therapeutic action and adverse effects at gastrointestinal level.

Keywords: 5-HT_3 receptors; 5-HT_4 receptors; NG 108–15 cells; pig corpus striatum; guinea-pig ileum; antidepressant drugs (clomipramine, fluoxetine, paroxetine, litoxetine)

Introduction

It is generally accepted that central 5-hydroxytryptaminergic pathways are involved in the pathogenesis of depression. By blocking 5-hydroxytryptamine (5-HT) reuptake from nerve terminals, antidepressant drugs, such as clomipramine, fluoxetine and paroxetine, enhance central 5-hydroxytryptaminergic transmission, which can be regarded as an initial step in the therapeutic action of these compounds. In fact, other mechanisms have been reported, including changes in 5-HT_{1A} and 5-HT_2 receptor density and/or sensitivity as a consequence of chronic antidepressant treatment (see Cowen, 1990, for review).

More recently, two additional receptor types, the 5-HT_3 and 5-HT_4 receptors, have been identified in the central nervous system (CNS) (see Peters *et al.*, 1992; Bockaert *et al.*, 1994 for reviews). The 5-HT_3 receptor sites are ligand gated ion channels which mediate the release of a number of neurotransmitters, while 5-HT_4 receptors are positively coupled to adenylyl cyclase and appear to mediate slow excitatory responses to 5-HT in brain (see Zifa & Fillion,

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1992 for review). Although antidepressants possess low to moderate affinity for central 5-HT$_3$ binding sites (Hoyer *et al.*, 1989; Kilpatrick *et al.*, 1989; Schmidt & Peroutka, 1990), potent antagonists at these receptors (tropisetron and ondansetron) have been found, at least in one animal model of depression, to be as active as conventional antidepressants (Martin *et al.*, 1992). This suggests a potential involvement of 5-HT$_3$ receptors in depressive disorders (Greenshaw, 1993). Conversely, the role of 5-HT$_4$ receptors is still obscure.

Both 5-HT$_3$ and 5-HT$_4$ receptors are present in peripheral tissues (Eglen *et al.*, 1990; Tonini *et al.*, 1991; Ford & Clarke, 1993). In the guinea-pig ileum, 5-HT acts mainly by facilitating the neuronal release of acetylcholine (Ford & Clarke, 1993). The resulting concentration-contractile response curve is typically biphasic, with the high (submicromolar) and low (micromolar) potency phase mediated by 5-HT$_4$ and 5-HT$_3$ receptors, respectively (Buchheit *et al.*, 1985; Clarke *et al.*, 1989; Eglen *et al.*, 1990). These receptors, like those in the CNS, may represent a peripheral target for antidepressant drugs.

This study was designed to evaluate whether clomipramine (McTavish & Benfield, 1990), fluoxetine (Benfield *et al.*, 1986), paroxetine (Dechant & Clissold, 1991) and litoxetine (Angel *et al.*, 1993), which block 5-HT reuptake with a noradrenaline/5-HT uptake blocking ratio ranging from 20 to 320 (Benfield *et al.*, 1986; Thomas *et al.*, 1987; Scatton *et al.*, 1988), interact with central and peripheral (enteric) 5-HT$_3$ and 5-HT$_4$ receptors. In particular, binding of these drugs to central receptors was assessed by using two different models, the NG 108–15 neuroblastoma-glioma cells and the pig corpus striatum homogenate, which are suitable for studying 5-HT$_3$ and 5-HT$_4$ receptors, respectively (Giraldo *et al.*, 1992; Rizzi *et al.*, 1994). Functional studies were aimed at investigating the effects of antidepressant drugs on the contractile responses elicited by 5-HT, 2-methyl-5-hydroxytryptamine (agonist at 5-HT$_3$ receptors) and 5-methoxytryptamine (agonist at 5-HT$_4$ receptors) in the guinea-pig isolated ileum.

Methods

5-HT$_3$ receptor binding in NG 108-15 hybrid cells

NG 108-15 neuroblastoma-glioma hybrid cells were cultured as described by Hoyer & Neijt (1987). Crude membrane fractions were prepared according to the method of Bradbury *et al.* (1990), with slight modifications. Briefly, subconfluent cultures were washed twice with phosphate-buffered saline and lysed in 2 mM Tris HCl/1 mM EDTA (pH 7.1) solution for 30 min at 0°C. The suspension was homogenized and centrifuged (400 *g*, 5 min) to remove the nuclei. The supernatant was centrifuged at 30000 *g* for 20 min, and the pellet was resuspended and centrifuged as above. Membranes were suspended in 50 mM HEPES buffer (pH 7.4), divided into 0.5 ml aliquots and stored at −80°C until use.

Displacement experiments were performed by incubating the homogenate, diluted to about 150 μg protein ml^{-1} final concentration, at 30°C for 30 min in the presence of 0.3 nM [^{3}H]-DAU 6215 (Giraldo *et al.*, 1992) and different concentrations of the test compounds dissolved in the assay buffer. Incubation volume was 1.0 ml. The specific binding of [^{3}H]-DAU 6215 (defined as the binding displaceable by 3 μM MDL 72222) was about 95% of total binding. The reaction was terminated by rapid filtration using an IH-110 INOTECH cell harvester (type G7 glass filters, INOTECH). The filters were transferred into plastic vials, 4.0 ml scintillation cocktail (Filter Count, Packard) was added, and radioactivity was counted by liquid scintillation spectrometry (Contron Betamatic V). Protein content was determined by the method of Bradford (1976).

5-HT$_4$ receptor binding in pig corpus striatum

Pig corpora striata were removed and kept on ice for about 2 h before a cold solution of 50 mM HEPES buffer (pH 7.4) was added (w/v 1:10). The tissue was homogenized in an Ultra-Turrax (30 s at full speed) followed by homogenization in a Potter-Elvehjem glass-on-Teflon homogenizer. The homogenate was divided into 5 ml aliquots, and stored at −80°C until use. Displacement experiments were performed by incubating 980 μl of the homogenate (final tissue dilution 1:70) at 30°C for 30 min in the presence of 0.1 nM [^{3}H]-GR 113808 (Grossman *et al.*, 1993) and different concentrations of the test compounds dissolved in the assay buffer. Incubation volume was 1.0 ml. Specific [^{3}H]-GR 113808 binding (defined as the binding displaceable by 10 μM BIMU 1) was about 80% of total binding. The incubation was stopped by rapid filtration as described above.

The inhibition of specific binding by competing ligands was analyzed graphically to estimate IC_{50} values (concentration of antidepressant displacing 50% of specifically bound radioligand) by a nonlinear least squares regression analysis.

Experimental animals

Fasted male Dunkin-Hartley guinea-pigs weighing 480–600 g, were killed by CO_2 asphyxiation. A segment of ileum, 8 cm in length was excised about 1–2 cm from the ileo-caecal junction and the luminal contents were flushed out with warm Krebs-Henseleit solution (composition in mM: NaCl 118, KCl 5.6, $CaCl_2.2H_2O$ 2.5, $MgSO_4.7H_2O$ 1.19, NaH_2PO_4 1.3, $NaHCO_3$ 25, glucose 10; pH 7.4).

Electrically stimulated longitudinal muscle-myenteric plexus preparations (LMMPs)

Longitudinal muscle-myenteric plexus preparations (LMMPs), prepared as described by Paton & Zar (1968), were mounted isometrically (tension 5 mN) in 5 ml organ baths containing oxygenated (95% O_2 + 5% CO_2) Krebs-Henseleit solution. Each preparation was allowed to equilibrate at 37°C for at least 60 min before experiments were started.

Electrical field stimulation was delivered by means of two platinum electrodes placed at the top and the bottom of the chamber. Maximal nerve-mediated acetylcholine contractions were evoked by rectangular pulses with the following parameters: 0.1 Hz, 40–60 V, pulse duration 0.5 ms. After at least 10 min of reproducible 'twitch' contractions, cumulative concentration-response curves to each antidepressant drug were obtained. Drug-induced changes in 'twitch' height were expressed as percentage of the control contractions taken as 100% response.

In a separate set of unstimulated LMMPs, antidepressant drugs were tested against muscarinic contractile responses induced by 100 nM acetylcholine (ACh), the magnitude of which was equivalent to that of the 'twitch' contractions.

The above experiments were carried out to determine the range of antidepressant concentrations devoid of anti-acetylcholine activity, to be used in functional (contractility) studies with 5-HT and 5-HT-related agonists.

Functional studies in whole resting ileal segments and unstimulated LMMPs

Segments of whole ileum (1.5–2 cm long) were set up isometrically (tension 10 mN) in 10 ml organ baths containing oxygenated Krebs-Henseleit solution at 37°C. Tissues were allowed to equilibrate for 60 min with a 15 min wash cycle.

Non-cumulative concentration-response curves to 5-HT, 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) and 5-methoxytryptamine (5-MeOT) were constructed in separate tissues using 0.5 log unit increments at 15 min intervals. Each

agonist concentration was removed as soon as the maximum effect was reached. After completion of concentration-response curves, tissues were washed for 30 min with Krebs-Henseleit solution containing a given antidepressant concentration, which was left in the bath during the construction of subsequent agonist curves. Only one antidepressant concentration was tested in each ileal preparation. Reversibility of the inhibitory effect caused by antidepressants on agonist-induced contractions was tested by repeating concentration-response curves in tissues rinsed with normal Krebs-Henseleit solution for at least 60 min.

In order to allow direct between-agonist comparisons, a series of concentration-response curves to 5-HT, 2-methyl-5-HT and/or 5-MeOT were constructed in single preparations. For between-agonist comparisons, responses were expressed as a percentage of the maximal response to 5-HT.

In separate experiments using unstimulated LMMPs, clomipramine (300 nM) was tested against contractions induced by 5-HT, 2-methyl-5-HT and 5-MeOT. This procedure was designed to evaluate whether the presence (whole ileal segments) or the absence (LMMPs) of mucosal 5-HT may influence the effects of drugs with 5-HT reuptake blocking properties.

Data analysis

Curves were analyzed by fitting them to a logistic equation of the form: $\text{Effect} = E_{maximum}/1 + e^{\{-2.303 \times \text{slope} \times (\log [A] - \log [A_{50}])\}}$ where: $E_{maximum}$ = maximum response; [A] = molar agonist concentration; [A_{50}] = molar agonist concentration inducing 50% of the maximum response. All data were fitted either to a single logistic expression or to the sum of two logistics. Goodness of fit to a single or double logistic expression was evaluated by the F-test of the residual variances using a significance criterion of $P<0.05$ (SAS Institute Inc., 1988).

Agonist potency values were expressed as $-\log$ EC for monophasic curves and as $-\log EC_1$ and $-\log EC_2$ for the first and second phase of biphasic curves, where EC indicates molar agonist concentration inducing 50% of the maximum effect. Antidepressant-induced change of agonist curve was calculated as a percentage of the maximum effect of agonist obtained before antidepressant addition. Antagonist pA_2 estimates were calculated following Schild regression analysis, using agonist concentration-ratios (CR) determined at EC_{50} levels in control and test curves. Confidence limits (CL) at 95% probability for the slope of regression were evaluated by using a computer programme (PHARM/PCS, Version 4.1) based on a manual of pharmacological calculations (Tallarida & Murray, 1986). Apparent affinity estimates (pK_B) from single antagonist concentrations were calculated by the Gaddum (1957) equation. All data in the text are expressed as means ± s.e.mean. Differences between means were analyzed by Student's two-tail t test. Values of $P<0.05$ were taken as statistically significant.

Drugs

5-Hydroxytryptamine hydrochloride and acetylcholine chloride were obtained from Sigma; 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) maleate, 5-methoxytryptamine (5-MeOT) hydrochloride, and clomipramine hydrochloride were obtained from RBI; fluoxetine hydrochloride, paroxetine hydrochloride hemihydrate and litoxetine hydrochloride were kindly donated by Eli-Lilly Italia S.p.A., SmithKline Beecham (Great Britain) and Synthelabo Recherche (LERS) (France), respectively. BIMU 1 (3-ethyl-2,3-dihydro-N-(8-methyl-8-azabicyclo [3.2.1] oct-3-yl)-2-oxo-1H-benzimidazole-1-carboxamide hydrochloride) and MDL 72222 (1αH,3α, 5αH-tropan-3-yl-3,5-dichlorobenzoate) were synthesized by Boehringer Ingelheim, Italia. The radiolabelled ligands [^{3}H]-DAU 6215 (N-[endo-8-methyl-8-azabicyclo (3.2.1) oct-3-yl]-2,3-dihydro-2-oxo-1H-benzimidazol-1-carboxamide, hydrochloride) (83 Ci mmol^{-1}) and [^{3}H]-GR 113808 ([1-[2-[(methylsulphonyl)amino]ethyl]-4-piperidinyl]methyl-1-methyl-1H-indole-3-carboxylate) (82–85 Ci mmol^{-1}) were from Amersham International (UK). All drugs were dissolved in distilled water.

Results

Binding studies

IC_{50} values of antidepressants determined in membranes from NG 108-15 cells and pig striatum are shown in Table 1.

Clomipramine, paroxetine and fluoxetine inhibited [^{3}H]-DAU 6215 binding to 5-HT$_3$ recognition sites in NG 108-15 cells with IC_{50} values in the range 1.3–4 μM. Litoxetine was the most active compound showing an IC_{50} of 0.3 μM. The specific binding of [^{3}H]-GR 113808 to 5-HT$_4$ recognition sites in pig striatal membranes was inhibited by the antidepressant drugs with low potency (IC_{50} values ⩾ 20 μM).

Functional studies

5-HT (1 nM–30 μM), 2-methyl-5-HT (0.1–30 μM) and 5-MeOT (3 nM–10 μM) induced concentration-dependent contractions in whole segments of guinea-pig ileum. Curves to 5-HT were better fitted to a biphasic than to a monophasic model: ($F = 7.004$ $P<0.005$). The first phase occurred at 5-HT concentrations ranging from 1 nM to 0.3 μM, while the second phase in the range of 1 and 30 μM. $-\log EC_1$ and $-\log EC_2$ values for 5-HT were 7.99 ± 0.03 and 5.83 ± 0.02 for the first and second phase, respectively (Figure 1). The concentration-response curve to the selective 5-HT$_3$ receptor agonist, 2-methyl-5-HT was monophasic ($-\log EC = 5.45 \pm 0.01$) and the maximum response was 64.0 ± 2.7% of that

Table 1 Potency values (IC_{50}) of antidepressant drugs at 5-HT$_3$ receptors in NG 108-15 neuroblastoma-glioma cells and at 5-HT$_4$ receptors in pig corpus striatum homogenate

Test substance	*NG 108-15 cells* IC_{50} (nM)	*Corpus striatum* IC_{50} (nM)
Clomipramine	1308 ± 85	31500 ± 1200
Fluoxetine	4000 ± 150	42800 ± 1500
Paroxetine	2154 ± 110	66600 ± 1850
Litoxetine	315 ± 65	19600 ± 850

[^{3}H]-DAU 6215 and [^{3}H]-GR 113808 were used as ligands of 5-HT$_3$ and 5-HT$_4$ receptors, respectively. Data are expressed as means ± s.e.mean of 3 experiments.

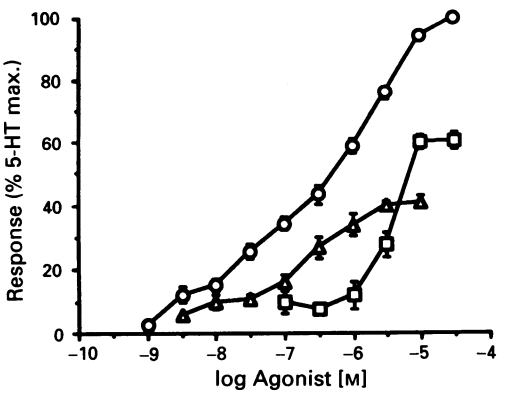

Figure 1 Concentration-response curves to 5-hydroxytryptamine (○), 2-methyl-5-hydroxytryptamine (□), and 5-methoxytryptamine (△) in isolated segments of whole guinea-pig ileum. Values are expressed as means ± s.e.mean, $n = 20$.

obtained with 30 μM 5-HT (Figure 1). The concentration-response curve to the selective 5-HT$_4$ receptor agonist 5-MeOT was also monophasic ($-\log EC = 6.92 \pm 0.02$). The maximum response ($41.0 \pm 1.5\%$) was not significantly different from that obtained with 0.3 μM 5-HT (Figure 1). In time control experiments, no evidence of desensitization was obtained, provided that there were frequent solution changes (every 5–10 min) and 30–60 min recovery periods between subsequent agonist concentration-response curves.

To assess the interaction of antidepressant drugs with 5-HT$_3$ and 5-HT$_4$ receptor-mediated contractions, these drugs were used at concentrations which were ineffective (or slightly effective) on neurogenic cholinergic 'twitch' contractions and on ACh-induced contractions in electrically stimulated and unstimulated LMMPs, respectively. In fact, the highest concentrations of clomipramine (300 nM), fluoxetine (3 μM), paroxetine (1 μM) and litoxetine (3 μM) tested did not reduce the amplitude of both indirect (Figure 2a) and direct ACh (100 nM)-mediated responses (Figure 2b) by more than 15%. None of these drugs incubated with the ileum before agonist administration changed the basal tone of the preparations. Clomipramine (10–300 nM) inhibited, by a mechanism not clearly dependent on concentration, both phases of the 5-HT curve with progressive reduction of maximum response up to 30% of control (Figure 3a). This prevented the evaluation of affinity estimates for the drug. Complete reversibility of the inhibitory effect caused by 300 nM clomipramine was obtained following a 60 min washing period. At low concentrations (30 and 100 nM), clomipramine slightly shifted the 2-methyl-5-HT concentration-response curve to the right in a concentration-independent manner (CR: 2.1 ± 0.02 and 2.1 ± 0.02 at 30 and 100 nM, respectively). At higher concentrations (300 nM), the drug caused a further rightward shift with marked (50%) depression of agonist response maximum (Figure 3b). At 100 and 300 nM, clomipramine concentration-dependently shifted the 5-MeOT concentration-response curve to the right, an effect associated with approximately 50% depression of maximum response (Figure 3c). The antagonist properties of clomipramine (including the reduction of agonist response maximum) were also observed in resting LMMPs. In these preparations, 300 nM clomipramine produced a rightward shift of 5-HT, 2-methyl-5-HT and 5-MeOT curves which was superimposable on that obtained in whole ileal segments (Figure 4).

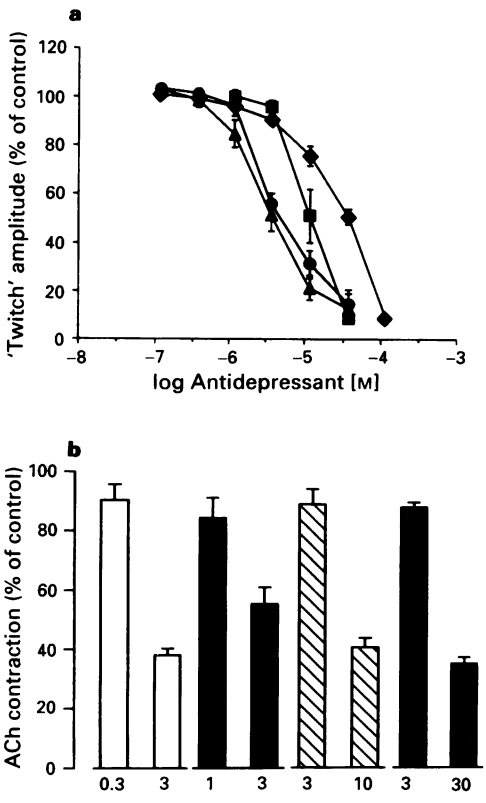

Figure 2 Concentration-response curves for clomipramine (▲), paroxetine (●), fluoxetine (■) and litoxetine (◆) in inhibiting nerve-mediated acetylcholine-mediated contractions to electrical field stimulation ('twitch') in longitudinal muscle-myenteric plexus preparations (LMMPs) from the guinea-pig ileum (a). In (b) the effects of 0.3 μM clomipramine (open column), 1 μM paroxetine (solid column), 3 μM fluoxetine (hatched column) and 3 μM litoxetine (stippled column) on contractile responses caused by 100 nM acetylcholine are shown. Higher clomipramine (3 μM), paroxetine (3 μM), fluoxetine (10 μM) and litoxetine (30 μM) concentrations significantly inhibited ($P < 0.05$) ACh-induced contractions. The latter antidepressant concentrations caused an approximately 50% reduction of twitch contraction amplitude (a). Values are expressed as means ± s.e.mean, $n = 4–6$.

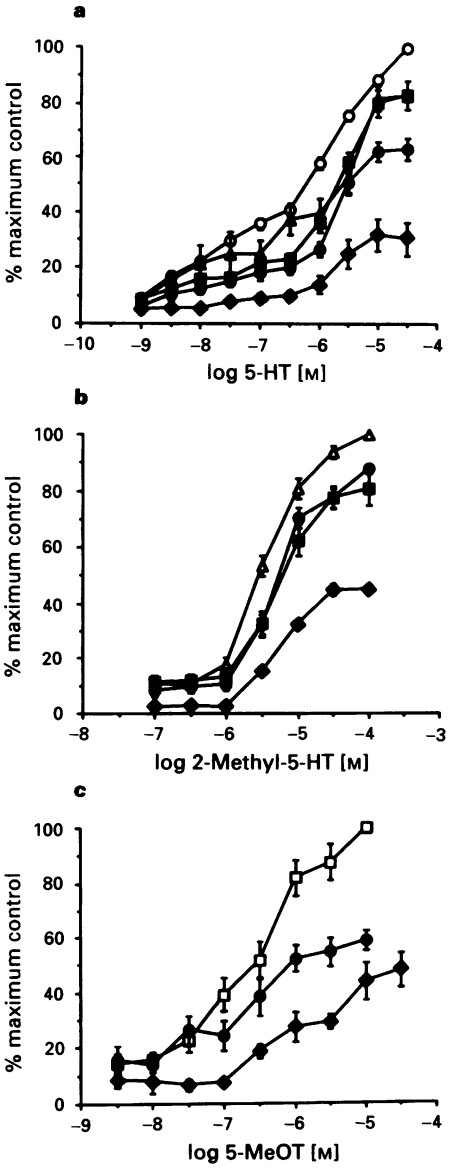

Figure 3 Effect of clomipramine on responses to 5-hydroxytryptamine (5-HT) (a), 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) (b) and 5-methoxytryptamine (5-MeOT) (c) in isolated segments of whole guinea-pig ileum. Control responses (open symbols); responses in the presence of 10 (▲), 30 (■), 100 (●) and 300 nM (◆) clomipramine. Values are expressed as means ± s.e.mean, $n = 6–14$.

In whole ileal segments, fluoxetine (0.1–1 μM) reduced both phases of the 5-HT curve in a concentration-related manner with some depression of maximum response (Figure 5a). By comparison of the responses at the original EC_{50} level for the second phase (EC_2), Schild analysis yielded pA_2 estimates of 6.6 ± 0.3 (Schild slope of 1.1 (0.8–1.2)). At 3 μM, fluoxetine further shifted the 5-HT curve to the right, with marked depression (70%) of maximum effect. The antagonism caused by 3 μM fluoxetine was fully reversed by 60 min washing. In contrast, responses to 2-methyl-5-HT were slightly affected by 1 μM fluoxetine. Use of the Gaddum equation yielded a pK_B value of 5.4 ± 0.02. A higher fluoxetine concentration (3 μM) caused a parallel rightward displacement of the 2-methyl-5-HT curve up to 10 μM. However, this effect was followed by a decreased responsiveness of the preparation (≥ 50%) to higher 2-methyl-5-HT concentrations (Figure 5b). Curves to 5-MeOT were concentration-dependently shifted to the right by fluoxetine (range 0.1–1 μM), with slight (20%) depression of maximum response. However, since these fluoxetine concentrations are 40–400 fold lower than those interacting with central 5-HT$_4$ recognition sites, it is unlikely that the target of fluoxetine action in the ileum is the '5-HT$_4$ receptor'. In fact, affinity estimates of GR 113808 (a 5-HT$_4$ receptor ligand) at both the central and peripheral receptors are superimposable (Grossman *et al.*, 1993; Ford & Clarke, 1993; Bockaert *et al.*, 1994), thus presumably excluding 5-HT$_4$ receptor heterogeneity. Based on this assumption, affinity values for fluoxetine (as well as for litoxetine, see below) were not calculated. When fluoxetine was used at 3 μM, a marked (≥ 80%) depression of maximum agonist response was observed (Figure 5c).

Paroxetine was used at 1 μM only, since higher concentration produced marked inhibitory effects on cholinoceptor-mediated contractions in LMMPs. At this concentration, paroxetine did not alter the first high-potency phase of the

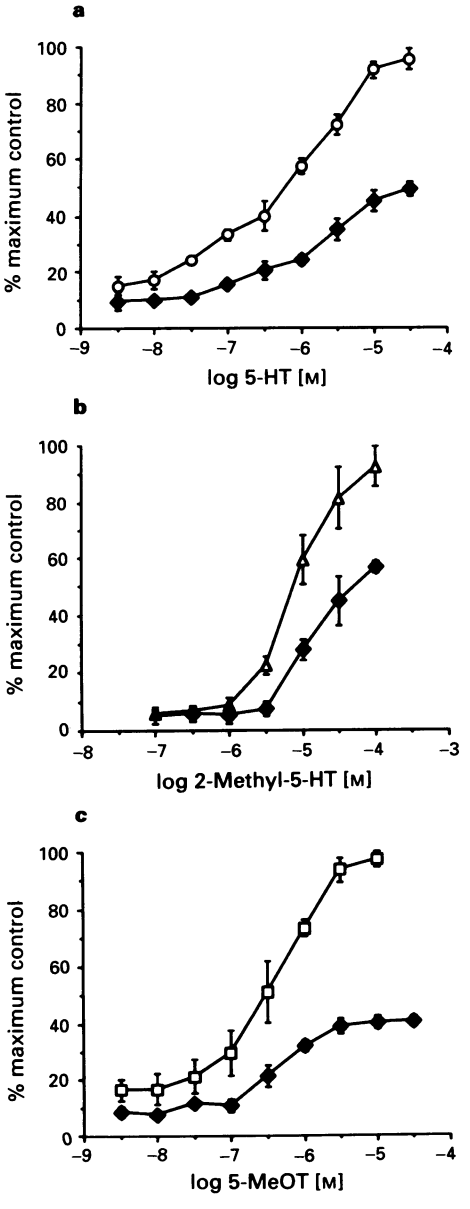

Figure 4 Effect of clomipramine on responses to 5-hydroxytryptamine (5-HT) (a), 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) (b) and 5-methoxytryptamine (5-MeOT) (c) in longitudinal muscle-myenteric plexus preparations (LMMPs) of guinea-pig ileum. Control responses (open symbols); responses in the presence of 300 nM (closed symbols) clomipramine. Note that the inhibitory effect caused by clomipramine is not significantly different from that obtained in whole segments at the same concentration of the antidepressant (Figure 3). Values are expressed as means ± s.e.mean, $n = 4–6$.

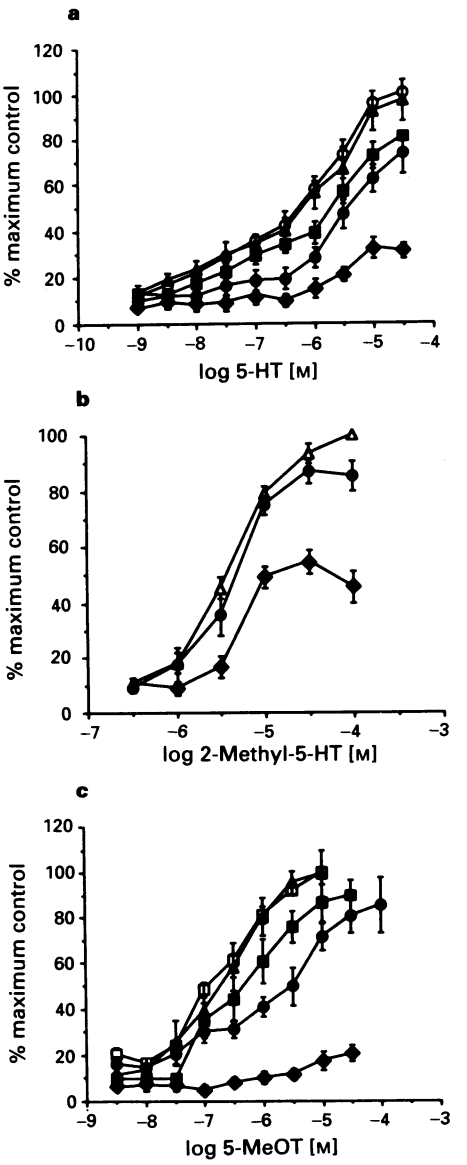

Figure 5 Effect of fluoxetine on responses to 5-hydroxytryptamine (5-HT) (a), 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) (b) and 5-methoxytryptamine (5-MeOT) (c) in isolated segments of whole guinea-pig ileum. Control responses (open symbols); responses in the presence of 0.1 (▲), 0.3 (■), 1 (●) and 3 μM (◆) fluoxetine. Values are expressed as means ± s.e.mean, $n = 6–14$.

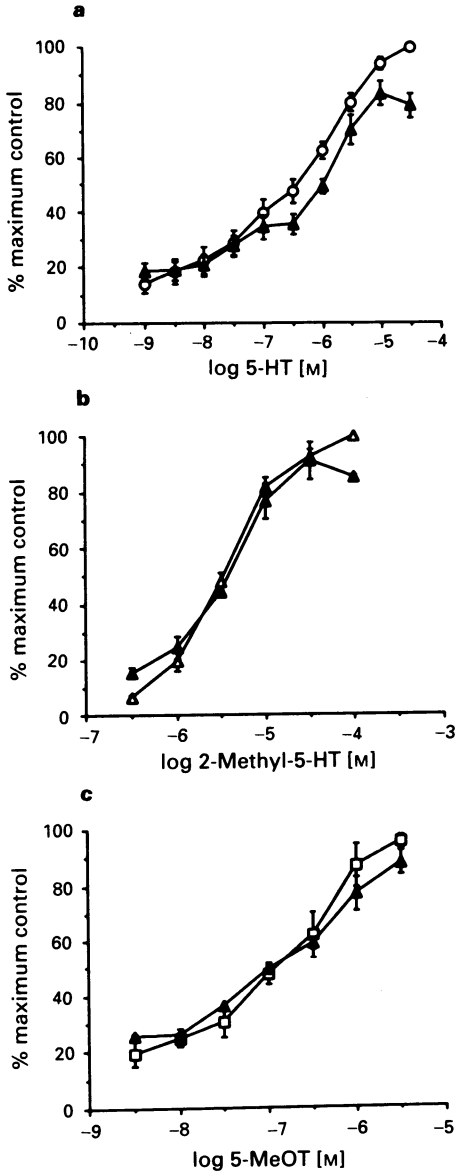

Figure 6 Effect of paroxetine on responses to 5-hydroxytryptamine (5-HT) (a), 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) (b) and 5-methoxytryptamine (5-MeOT) (c) in isolated segments of whole guinea-pig ileum. Control responses (open symbols); responses in the presence of 1 μM paroxetine (▲). Values are expressed as means ± s.e.mean, $n = 4-8$.

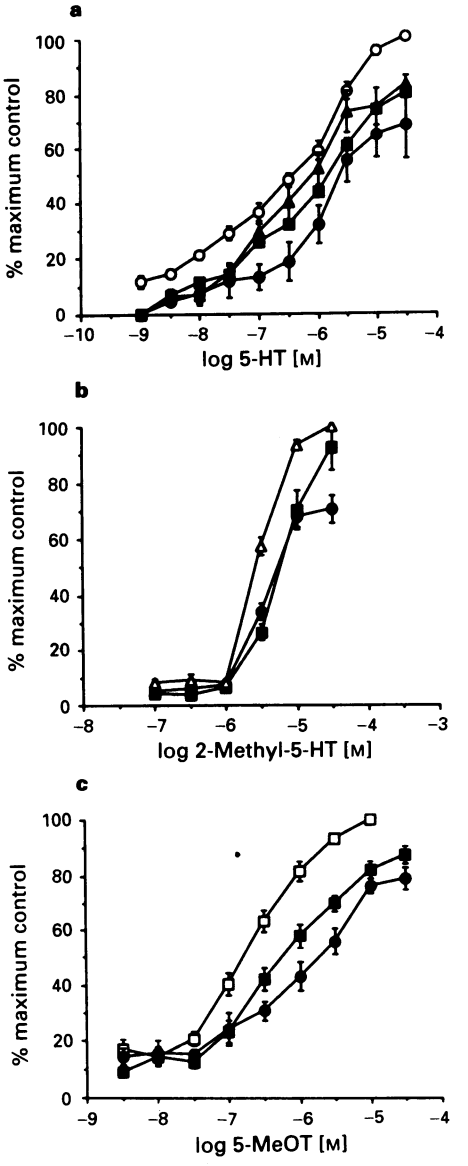

Figure 7 Effect of litoxetine on responses to 5-hydroxytryptamine (5-HT) (a), 2-methyl-5-hydroxytryptamine (2-methyl-5-HT) (b) and 5-methoxytryptamine (5-MeOT) (c) in isolated segments of whole guinea-pig ileum. Control responses (open symbols); responses in the presence of 0.3 (▲), 1 (■) and 3 μM (●) litoxetine. Values are expressed as means ± s.e.mean, $n = 6-18$.

5-HT curve, but caused a slight dextral shift of the second low-potency phase (pK_B: 6.1 ± 0.01). In contrast, the responsiveness of the preparations to 2-methyl-5-HT was unaffected by paroxetine, as was the concentration-response curve to 5-MeOT (Figure 6).

Like fluoxetine, litoxetine (0.3–3 μM) reduced both phases of the 5-HT curve in a concentration-related manner with some depression of maximum response (Figure 7a), an effect reversed by prolonged (60 min) washing. Use of the Schild analysis yielded a pA_2 estimate of: 6.6 ± 0.1 (Schild slope of 1.1 (0.9–1.2)). At 1 and 3 μM, litoxetine produced slight rightward displacements of 2-methyl-5-HT-induced contractions, which were virtually independent of the antidepressant concentration used (pK_B values: 6.0 ± 0.02 and 5.5 ± 0.01, respectively) (Figure 7b). Conversely, these concentrations shifted to the right, in an apparently concentration-dependent manner, the response caused by 5-MeOT (Figure 7c).

Discussion

A combined study of receptor binding in central neuronal cell membranes and functional responses in guinea-pig isolated small intestine preparations allowed this study to characterize the interaction of four antidepressant drugs with central and peripheral 5-HT$_3$ and 5-HT$_4$ receptors.

Receptor binding studies

In NG 108-15 neuroblastoma-glioma cell membranes, clomipramine, fluoxetine, paroxetine and litoxetine displaced tritiated DAU 6215 (a ligand of 5-HT$_3$ receptors) from its binding sites. In agreement with previous evidence, the IC_{50} values for clomipramine, fluoxetine and paroxetine were in the range 1.3–4 μM, suggesting a low potency of these drugs at 5-HT$_3$ receptors (Hoyer *et al.*, 1989; Schmidt & Peroutka,

1990; Angel *et al.*, 1993). Conversely, litoxetine was 4–12 fold more potent than the aforementioned antidepressants ($IC_{50} = 0.3\ \mu M$), suggesting a moderate potency at 5-HT$_3$ receptors, as previously reported by Angel *et al.* (1993) in rat cortical membranes. As far as the influence of antidepressants at central 5-HT$_4$ receptors is concerned, the results of binding experiments in pig striatal membranes indicate that all four bind to central 5-HT$_4$ recognition sites with negligible potency (IC_{50} values $\geqslant 20\ \mu M$). Following this evidence, our study was expanded to assess the influence of antidepressant drugs on neurogenic contractions evoked by 5-HT$_3$ and 5-HT$_4$ receptors in enteric preparations.

Functional studies

As previously reported (Buchheit *et al.*, 1985; Fox & Morton, 1990; Eglen *et al.*, 1990; Butler *et al.*, 1990; Craig *et al.*, 1990), in isolated segments of guinea-pig ileum, the concentration-contractile response curve to 5-HT was biphasic in nature. It is well established that the high- and low-potency phases of the 5-HT curve are mediated by the 5-HT$_4$ and 5-HT$_3$ receptors, respectively. Both the 5-HT$_4$ receptor agonist 5-MeOT and the 5-HT$_3$ receptor agonist 2-methyl-5-HT exhibited monophasic curves with potency values in agreement with previous evidence (Eglen *et al.*, 1990; Fox & Morton, 1990; Butler *et al.*, 1990; Turconi *et al.*, 1991; Buchheit *et al.*, 1992). 5-MeOT behaved as a full agonist (see also Eglen *et al.*, 1990; Turconi *et al.*, 1991), while 2-methyl-5-HT showed partial agonist properties. The latter findings are in agreement with those of Butler *et al.* (1990), but at variance with those of Eglen *et al.* (1990) and Fox & Morton (1990), who reported that in the guinea-pig ileum, 2-methyl-5-HT induces a maximal response comparable to that of 5-HT.

All four antidepressants were devoid of intrinsic activity in ileal segments. At concentrations not exceeding $3\ \mu M$, clomipramine, fluoxetine and litoxetine inhibited both the high- and low-potency phases of 5-HT curve. Conversely, paroxetine slightly affected the low-potency phase only. The effect of antidepressants on each phase of the 5-HT curve will be discussed separately.

The parallel rightward displacement of the low-potency phase caused by antidepressants (with the exception of clomipramine and within low ranges of concentration) allowed the evaluation of their apparent affinity estimates at enteric 5-HT$_3$ receptors. The resulting affinity estimates of fluoxetine (range $0.1-1\ \mu M$, pA_2: 6.6), paroxetine ($-\log K_B$: 6.1) and litoxetine (pA_2: 6.6) are consistent with low to moderate potency of these drugs, as observed at central and peripheral (vagus nerve) 5-HT$_3$ recognition sites (Hoyer *et al.*, 1989; Kilpatrick *et al.*, 1989; Schmidt & Peroutka, 1990; Angel *et al.*, 1993). In contrast, the antagonism exerted by clomipramine on the second phase of 5-HT curve was hardly dependent on concentration, thus preventing the evaluation of affinity estimates of this drug at enteric 5-HT$_3$ receptors. However some discrepancies emerged from data obtained using central and peripheral models. For example, litoxetine was 12 fold more potent than fluoxetine at central 5-HT$_3$ receptors, while it possesses the same affinity as fluoxetine at peripheral 5-HT$_3$ sites. Furthermore, at variance with pure 5-HT$_3$ receptor antagonists, whose affinity is significantly lower in guinea-pig tissues suggesting a species variant of the 5-HT$_3$ receptor (Butler *et al.*, 1990; Kilpatrick & Tyers, 1992; Wong *et al.*, 1992; 1993), antidepressants do not show clear-cut differences between their potency/affinity values in central and peripheral (guinea-pig ileum) tissues.

The antagonism of litoxetine towards 5-HT$_3$ receptor-mediated contraction is in agreement with previous evidence obtained by Galzin *et al.* (quoted by Angel *et al.*, 1993) in the same experimental model. However, it is noteworthy that fluoxetine (like clomipramine and, to a minor extent, litoxetine) at the highest concentration tested, markedly reduced 5-HT response maximum, a feature which suggests unsurmountable antagonism rather than competitive antagonism. As mentioned above, in the guinea-pig ileum both 5-HT$_3$ and 5-HT$_4$ receptors participate in the contractile response of 5-HT, which is mainly dependent on acetylcholine release from cholinergic neurones (Eglen *et al.*, 1990; Tonini *et al.*, 1991; Ford & Clarke, 1993). However, the reduction of 5-HT response maximum caused by antidepressants cannot be ascribed to their potential anti-acetylcholine (antimuscarinic) activity (Thomas *et al.*, 1987), since the highest antidepressant concentrations used in functional studies were previously found to affect only slightly cholinoceptor-mediated contractions to electrical stimulation or to applied ACh in LMMPs. Another possible explanation is that the apparent unsurmountable antagonism of the 5-HT$_3$ and 5-HT$_4$ receptor-mediated responses observed with antidepressant compounds may be due to receptor desensitization. In fact, in the presence of the reuptake blockers, 5-HT, released from the enterochromaffin cells of the mucosa, may remain in contact with the receptors for a sufficiently long time to desensitize them, thus shifting the 5-HT curve in a non-competitive manner. This possibility however, has been ruled out by the observation that in LMMPs, clomipramine reduced responses to 5-HT (as well as to 2-methyl-5-HT and 5-MeOT) to an extent similar to that observed in whole ileal segments. Furthermore, in the latter preparations, paroxetine and litoxetine, which possess the highest potency/affinity in inhibiting 5-HT uptake in rat synaptosomes (Thomas *et al.*, 1987; Scatton *et al.*, 1988; Benfield *et al.*, 1986), were significantly less potent than clomipramine and fluoxetine in inhibiting contractile responses to 5-HT. This would further exclude any 5-HT reuptake blocking mechanism in the inhibition of 5-HT responses caused by antidepressant compounds.

Unsurmountable antagonism, leading to reduction of maximum agonist response, could be expected as a result of pseudoirreversible competitive antagonism (Kenakin, 1987). This type of antagonism, for example, is exerted by the 5-HT$_1$/5-HT$_{2A}$ receptor antagonist, metergoline, on 5-HT-induced vasoconstrictor response in the rat isolated kidney (Bond *et al.*, 1989). Pseudoirreversible antagonism may partly explain our findings, since inhibition of 5-HT contractile responses produced by clomipramine, fluoxetine and litoxetine was reversed very slowly by washing. This may reflect slow dissociation kinetics of antidepressants from the 5-HT$_3$ receptor (and/or slow diffusion from tissues), and not true irreversibility.

To characterize further the antagonism of antidepressants at the 5-HT$_3$ receptor, additional studies were conducted with the selective 5-HT$_3$ receptor agonist 2-methyl-5-HT (Richardson *et al.*, 1985). However, the interaction of antidepressants with 2-methyl-5-HT-induced responses was less clearcut than that obtained with 5-HT as an agonist. In fact, fluoxetine at a concentration ($1\ \mu M$) which effectively antagonized the 5-HT$_3$ receptor-mediated component of 5-HT curve (Figure 4a) was poorly effective on 2-methyl-5-HT responses (pK_B: 5.4), while paroxetine was ineffective. Furthermore, concentrations of litoxetine (1 and $3\ \mu M$) which produced concentration-dependent dextral shift of the 5-HT curve, slightly antagonized 2-methyl-5-HT-induced contractions in an apparent concentration-independent manner (Figure 6b). Concentration-independent antagonism was also produced by clomipramine (30 and 100 nM), as observed in 5-HT experiments. High clomipramine, fluoxetine and litoxetine concentrations reduced 2-methyl-5-HT response maximum by an extent similar to that observed with 5-HT. Based on partial discrepancy of results with 5-HT and 2-methyl-5-HT emerging from our functional studies, other experimental models, such as the 5-HT$_3$ receptor-mediated vagal reflex bradycardia (von Bezold Jarish reflex), are required to assess the potential interaction of antidepressants with peripheral 5-HT$_3$ receptors. Very recently, imipramine and fluoxetine were found to inhibit the inward current mediated by 5-HT$_3$ receptor activation in rat nodose ganglion neurones, thus providing additional evidence for an interaction of

antidepressants with 5-HT$_3$ sites (Fan, 1994).

As mentioned above, all antidepressants, with the exception of paroxetine, inhibited the 5-HT$_4$ receptor-mediated high-potency phase of the 5-HT curve. This was a rather unexpected finding, since antidepressants had negligible potency at central 5-HT$_4$ receptors as measured by radioligand binding ($IC_{50} \geqslant 20\ \mu M$). Antagonism of antidepressant drugs at ileal 5-HT$_4$ receptors was further investigated with the selective agonist, 5-MeOT (Eglen *et al.*, 1990; Hill *et al.*, 1990; Fozard, 1990). Clomipramine, fluoxetine and litoxetine inhibited 5-MeOT-induced ileal contractions at concentrations 6–100 fold lower than those required to bind striatal 5-HT$_4$ recognition sites. In the case of fluoxetine (range 0.1–1 μM) and litoxetine, a concentration-related dextral shift of the 5-MeOT curve with slight reduction (20%) of maximum agonist effect was observed. However, this type of antagonism cannot be ascribed to competitive antagonism for two reasons. First, previous receptor binding and functional studies have demonstrated that central and peripheral 5-HT$_4$ receptors are a homogeneous receptor population, which is recognized with comparable affinity by 5-HT$_4$ receptor ligands (Grossman *et al.*, 1993; Ford & Clarke, 1993; Bockaert *et al.*, 1994). Second, high concentrations of clomipramine (300 nM) and fluoxetine (3 μM) (albeit much lower than those affecting 5-HT$_4$ receptor binding) caused a marked reduction (50–80%) of agonist response maximum. Therefore, the peripheral target of antidepressants might not be the 5-HT$_4$ receptor, but rather an allosteric site in the receptor macromolecule (never demonstrated, however, in central binding studies) or, more likely, post-receptor site(s), involving inhibition of transduction signalling pathways. In this respect, antidepressants have been found to modulate, at least after chronic treatment, post-receptor transduction mechanisms (i.e. G proteins) leading either to increased (Menkes *et al.*, 1983) or decreased cyclic AMP generation (Lesch *et al.*, 1991; 1992).

In conclusion, results from binding studies in NG 108-15 neuroblastoma-glioma cells suggest that the antidepressants clomipramine, fluoxetine, paroxetine possess low and litoxetine moderate potency at central 5-HT$_3$ receptors. Although less homogeneous data have been obtained in the guinea-pig ileum, antidepressants seem to possess comparable affinity also for peripheral enteric 5-HT$_3$ receptors. Binding experiments in pig striatum homogenates demonstrated that antidepressant drugs are virtually ineffective at central 5-HT$_4$ receptors. Nevertheless, in functional studies, submicromolar or micromolar concentrations of clomipramine, fluoxetine and litoxetine effectively inhibited 5-MeOT-induced ileal contractions, through a mechanism which may reflect either allosteric antagonism or post-receptor blockade of second messenger (i.e. cyclic AMP) generation.

Based on our findings, the interaction of antidepressants with central and peripheral 5-HT$_3$ and 5-HT$_4$ receptors may be relevant to their therapeutic action (Greenshaw, 1993; Angel *et al.*, 1993; Bockaert *et al.*, 1994) and may explain their constipating effect (Sanger *et al.*, 1991), which can be further exacerbated by anticholinoceptor properties (Benfield *et al.*, 1986; McTavish & Benfield, 1990; Dechant & Clissold, 1991; Leonard, 1992).

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