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5-CT stimulation of adenylyl cyclase activity in guinea-pig hippocampus: evidence for involvement of 5-HT_7 and 5-HT_{1A} receptors

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1 A number of compounds, including the selective 5-HT_7 receptor antagonist SB-258719, were investigated for their effect on [^{3}H]-5-carboxamidotryptamine (5-CT) radioligand binding and 5-CT-stimulated adenylyl cyclase activity in guinea-pig hippocampal membranes, in order to confirm the presence of functionally coupled 5-HT_7 receptors in this tissue.

2 The [^{3}H]-5-CT radioligand binding profile was consistent with binding predominantly to 5-HT_7 receptors. The affinity of SB-258719 (pK_i 7.2 ± 0.1) was similar to its reported human 5-HT_7 receptor affinity.

3 In the adenylyl cyclase functional assay, 5-CT was a potent and full agonist compared to 5-HT, whereas 8-hydroxy-dipropylaminotetralin (8-OH-DPAT) was a partial agonist (intrinsic activity 0.4 ± 0.1). The rank order of potency for agonists (5-CT > 5-HT ~ 8-OH-DPAT) was consistent with activation of 5-HT_7 receptors. SB-258719 (5 μM) and methiothepin (1 μM) surmountably antagonized the response to 5-CT, consistent with competitive antagonism. The pK_B for SB-258719 (7.2 ± 0.1) was in good agreement with its reported antagonist potency at the human cloned 5-HT_7 receptor.

4 In the functional assay, WAY-100635 (100 nM) and cyanopindolol (1 μM) induced a biphasic 5-CT response curve, consistent with selective antagonism of a component of the response to 5-CT. The estimated pK_B values for WAY-100635 and cyanopindolol (9.6 and 8.4 respectively) were in good agreement with their reported 5-HT_{1A} receptor affinities.

5 The data are consistent with the presence of 5-HT_7 receptors in guinea-pig hippocampus which are positively coupled to adenylyl cyclase. In addition, 5-HT_7 receptor-mediated stimulation of adenylyl cyclase activity in this tissue appears to be augmented by a mechanism involving 5-HT_{1A} receptor activation.

Keywords: Guinea-pig hippocampus; adenylyl cyclase; [^{3}H]-5-CT binding; 5-HT_7 and 5-HT_{1A} receptors; receptor cross-talk

Abbreviations: 5-CT, 5-carboxamidotryptamine; 8-OH-DPAT, 8-hydroxy-dipropylaminotetralin

Introduction

5-HT receptors have been divided into seven major classes (5-HT_{1-7}) based on their structural, functional and pharmacological characteristics (Hoyer *et al*., 1994). The 5-HT_7 receptor has been cloned from a number of species, including guinea-pig and man (Tsou *et al*., 1994; Bard *et al*., 1993) and like 5-HT_4 and 5-HT_6 receptors, is positively coupled to adenylyl cyclase when expressed in cell lines (Hoyer *et al*., 1994). Receptor binding studies have identified a [^{3}H]-5-CT binding site in guinea-pig brain which displays a pharmacological profile consistent with that for recombinant (guinea-pig and human) 5-HT_7 receptors (To *et al*., 1995; Boyland *et al*., 1996) and with the highest density in limbic and thalamic regions (e.g. To *et al*., 1995).

The role of 5-HT_7 receptors in the brain has not been clearly established, although they may be involved in circadian rhythm control (Lovenberg *et al*., 1993). Furthermore, definitive evidence for functional coupling of 5-HT_7 receptors in brain has been absent, partly due to a lack of selective ligands. However, a 5-HT receptor which is positively coupled to adenylyl cyclase and which shows pharmacological similarities to a 5-HT_7 receptor has been reported to be present in guinea-pig hippocampus (Tsou *et al*., 1994). A 5-HT_{1A}-like receptor has also been reported to mediate stimulation of adenylyl cyclase activity in both rat and guinea-pig hippocampus (Markstein *et al*., 1986; Shenker *et al*., 1987). Due to similarities between the pharmacological profiles for 5-HT_7 and 5-HT_{1A} receptors, it has been suggested that the 5-HT_{1A}-like response reported in guinea-pig hippocampus may actually reflect 5-HT_7 receptor stimulation (Hoyer *et al*., 1994).

Recently, SB-258719 has been reported to be a selective antagonist at the human cloned 5-HT_7 receptor, displaying at least 100 fold selectivity in binding studies versus other cloned 5-HT receptor subtypes (Forbes *et al*., 1998; Thomas *et al*., 1998). In the present study we have investigated the effect of SB-258719 and a number of other compounds, including the selective 5-HT_{1A} antagonist WAY-100635 (Fletcher *et al*., 1996), on 5-CT-stimulated adenylyl cyclase activity in guinea-pig hippocampus in comparison with receptor binding data using the same tissue. We provide evidence to support the involvement of both 5-HT_7 receptors and 5-HT_{1A} receptors in the 5-CT-induced stimulation of adenylyl cyclase activity in this tissue. These data have been presented previously in abstract form (Thomas *et al*., 1999).

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Methods

Radioligand binding

Guinea-pig hippocampal membranes were prepared by homogenizing (Polytron, 15 s, setting 5) hippocampal tissue in 20 volumes (based on wet weight of tissue) of 50 mM Tris (pH 7.4 at 37°C) containing 0.5 mM EDTA. Following centrifugation (50,000 × *g*, 12 min 4°C) and resuspension in the same medium, the membranes were incubated at 37°C for 20 min. After three further centrifugation and resuspension steps, membranes were stored at −80°C prior to use. Membranes (40–55 μg protein tube^{-1}) were incubated in Tris HCl buffer (50 mM, pH 7.4 at 37°C) containing $MgCl_2$ (5 mM), $CaCl_2$ (4 mM) and ascorbic acid (0.5 mM) in the presence of either [^{3}H]-5-CT (0.5 nM) or [^{3}H]-8-OH-DPAT (1 nM) and with or without test drugs. Pindolol (10 μM) and sumatriptan (1 μM) were included to inhibit [^{3}H]-5-CT binding to 5-HT$_{1A}$/5-HT$_{1B}$ and 5-HT$_{1D}$ receptors respectively. Non-specific binding was defined in the presence of 10 μM 5-HT. Incubations (45 min at 37°C) were started by addition of membranes and stopped by rapid filtration through Whatman GF/B grade filters (pre-soaked with 0.3% polyethyleneimine) followed by 5 × 1 ml ice-cold buffer washes. Bound radioactivity was determined by liquid scintillation counting.

Adenylyl cyclase assay

Guinea-pigs (male, Dunkin Hartley, 250–400 g) were killed by cervical dislocation and hippocampal tissue was removed and homogenized (Braun glass-Teflon homogeniser, 10 strokes at 200 r.p.m.) in 40 volumes of ice-cold buffer containing: sucrose (300 mM), Tris HCl (40 mM, pH 7.4 at 37°C), EDTA (1 mM), EGTA (1 mM), dithiothreitol (1 mM) and GDP (1 mM). The homogenate was filtered through a nylon mesh filter (Millipore Swinnex, type NY8H), diluted 3 fold with buffer and centrifuged (50,000 × *g*, 12 min, 4°C). The pellet was resuspended in 40 volumes of buffer, incubated for 20 min at 37°C and diluted 3 fold in buffer. Following centrifugation, the pellet was resuspended in buffer (minus GDP) and washed a further three times. Finally the pellet was resuspended in buffer (minus GDP) at a concentration of 75 mg tissue ml^{-1} (original wet weight) and used directly in the adenylyl cyclase assay.

Adenylyl cyclase activity in the guinea-pig hippocampal membranes was determined by measuring the conversion of [α-^{33}P]-ATP to [^{33}P]-cAMP. The reaction was performed in Tris HCl buffer (40 mM, pH 7.4 at 37°C) containing $MgCl_2$ (5 mM), GTP (50 μM), ATP (200 μM), phosphocreatine (20 mM), creatine phosphokinase (40 units ml^{-1}), sucrose (120 mM), EDTA (0.4 mM), EGTA (0.4 mM), dithiothreitol (0.4 mM); 1-methyl-3-isobutylxanthine (IBMX) (1 mM), cAMP (1 mM) and ascorbic acid (0.2 mM) in a total assay volume of 50 μl. Incubation (37°C, 12 min) was started by addition of 20 μl of membrane suspension (60–80 μg protein) to tubes containing incubation buffer, [α-^{33}P]-ATP (1–1.5 μCi tube^{-1}, specific activity 2000 Ci mmol^{-1}) and test drugs where appropriate. The response to 5-HT was measured in the presence of GR-113808 (1 μM) to prevent stimulation of 5-HT$_4$ receptors (Grossman *et al*., 1993). Incubation was stopped by addition of 100 μl of 0.5 M HCl containing ATP (40 mM), cAMP (10 mM) and [^{3}H]-cAMP (10,000 d.p.m. tube^{-1}, specific activity 27 Ci mmol^{-1}) for calculation of column recovery. Tubes were stored on ice prior to isolating [^{33}P]-cAMP according to the method of Salomon (1979). Samples were counted using a dual label protocol and the tritium signal was used to correct for per cent column recovery.

5-HT measurements

The level of endogenous 5-HT in the membrane preparation was measured by h.p.l.c. with electrochemical detection using a method similar to that described by Hutson *et al*. (1991).

Data analysis

For receptor binding assays, the concentration of drug inhibiting specific [^{3}H]-5-CT or [^{3}H]-8-OH-DPAT binding by 50% (IC_{50}) was determined and pK_i values (−log of the inhibition constant) were calculated from the IC_{50} values as described by Cheng & Prusoff (1973), using K_d values of 0.5 nM for [^{3}H]-5-CT and 1 nM for [^{3}H]-8-OH-DPAT.

Drug concentration-response curves from adenylyl cyclase assays were fitted to a 4-parameter logistic equation assuming either one or two interaction sites (Grafit, Erithacus Software). Agonist potency was expressed as the pEC_{50} (−log EC_{50}). Statistical analysis of apparently biphasic 5-CT curves generated in the presence of WAY-100635 or cyanopindolol was carried out using the method of Bates & Watts (1988). Apparent pK_B values ($-\log_{10}$ of the antagonist equilibrium dissociation constant) for antagonism were determined using the equation: pK_B = (−log ([antagonist]/(concentration ratio-1))) where concentration ratio = ratio of the agonist EC_{50} values in the presence and absence of antagonist. For both WAY-100635 and cyanopindolol, two pK_B values were calculated by comparing the EC_{50} from the control 5-CT curve with the EC_{50} derived from the two components of the (biphasic) 5-CT curve in the presence of antagonist. The pK_B for WAY-100635 determined from the IC_{50} of the drug to reverse the response to 100 nM 5-CT was calculated using the Cheng-Prusoff equation (Cheng & Prusoff, 1973) incorporating the agonist EC_{50} as described by Craig (1993). Data represent the mean ± s.e.mean of at least three separate experiments each performed using triplicate (adenylyl cyclase activity) or duplicate (receptor binding) determinations.

Drugs

5-hydroxytryptamine HCl (5-HT), 5-carboxamidotryptamine (5-CT), methiothepin mesylate and pindolol were obtained from Sigma-Aldrich (Poole, U.K.). Cyanopindolol hemifurmarate was obtained from Tocris Cookson. SB-258719 ((R)-3,*N*-Dimethyl-*N*-[1-methyl-3-(4-methylpiperidin-1-yl)propyl]-benzene-sulphonamide), GR-113808 and WAY-100635 were synthesized at SmithKline Beecham (Harlow, U.K.). [α-^{33}P]-ATP, [^{3}H]-cAMP and [^{3}H]-8-OH-DPAT were obtained from NEN Du Pont. [^{3}H]-5-CT was obtained from Amersham (U.K.). Stock drug solutions were prepared fresh on the day of assay in de-ionized water or DMSO (the final assay concentration of DMSO not exceeding 0.4%). Drug dilutions were prepared in 40 mM Tris buffer (pH 7.4 at 37°C) containing 0.5 mM ascorbic acid.

Results

[^{3}H]-5-CT and [^{3}H]-8-OH-DPAT binding

[^{3}H]-5-CT binding to guinea-pig hippocampal membranes was inhibited by 5-CT, 5-HT and 8-OH-DPAT (Table 1, Figure 1A) with a rank order of potency corresponding to that reported for the human cloned 5-HT$_7$ receptor (Thomas *et al*., 1998). SB-258719 and methiothepin were moderately potent inhibitors. The pK_i for SB-258719 (7.2 ± 0.1) was in agreement

with its reported affinity for the human recombinant 5-HT$_7$ receptor. GR-113808, WAY-100635 and cyanopindolol (Hoyer *et al*., 1994) were all weak inhibitors of binding. In contrast to 5-CT, 5-HT and methiothepin, SB-258719 did not completely inhibit [^{3}H]-5-CT binding, producing a maximal inhibition of specific binding of approximately 75% (Figure 1A). The Hill slope for SB-258719 (0.9 ± 0.1) was consistent with interaction with a single binding site. This profile would be consistent with the presence of a minor component of [^{3}H]-5-CT binding reflecting binding to non-5-HT$_7$ sites under the assay conditions used. However, the Hill slope values for the other compounds tested were also close to unity, ranging from 0.83 ± 0.1 (5-HT) to 1.1 ± 0.1 (8-OH-DPAT). This suggests that, for the compounds tested, potencies to inhibit [^{3}H]-5-CT binding to 5-HT$_7$ and SB-258719-insensitive binding sites were not markedly different. The maximal binding density (B_{max}) for [^{3}H]-5-CT binding to 5-HT$_7$ and SB-258719-insensitive binding sites combined was 116 ± 19 fmoles mg protein^{-1}. [^{3}H]-8-OH-DPAT binding to guinea-pig hippocampal membranes displayed a higher density, with B_{max} of 670 ± 130 fmoles mg protein^{-1}. [^{3}H]-8-OH-DPAT binding was potently inhibited by WAY-100635 (pK_i 9.5 ± 0.1) and 8-OH-DPAT (pK_i 8.8 ± 0.1), consistent with binding to 5-HT$_{1A}$ receptors (Figure 1B). In contrast, SB-258719 did not inhibit [^{3}H]-8-OH-DPAT binding (pK_i < 5, n = 3).

Adenylyl cyclase assay

5-CT stimulated adenylyl cyclase activity in guinea-pig hippocampal membranes from a basal level of 137 ± 3.8 pmoles mg^{-1} protein to 166 ± 5.2 pmoles mg^{-1} protein (21 ± 1.5% stimulation) and with a pEC$_{50}$ of 8.4 ± 0.2 (Figure 2, Table 1). 5-HT (in the presence of 1 μM GR-113808) produced a similar maximal stimulation compared to 5-CT (pEC$_{50}$ 7.7 ± 0.1). 8-OH-DPAT was a partial agonist compared to 5-CT and 5-HT (pEC$_{50}$ 7.5 ± 0.4, intrinsic activity 0.4 ± 0.1). The rank order of agonist potency was 5-CT > 5-HT8-OH-DPAT.

Table 1 Comparison of drug effects on [^{3}H]-5-CT binding and adenylyl cyclase activity in guinea-pig hippocampus

Compound	pK_i*	pEC$_{50}$† (IA) or pK_B‡
5-CT	8.8 ± 0.1	†8.4 ± 0.2 (1.0)
5-HT	7.8 ± 0.2	†7.7 ± 0.1
8-OH-DPAT	6.6 ± 0.1	†7.5 ± 0.4 (0.4)
Methiothepin	7.4 ± 0.1	7.4 ± 0.2 (1 μM)
SB-258719	7.2 ± 0.1	7.2 ± 0.1 (5 μM)
WAY-100635	< 5.5	< 7.0, 9.6 (0.1 μM)
Cyanopindolol	< 5.0	< 6.0, 8.4 (1 μM)
GR-113808	< 5.0	< 7.0 (0.1 μM)

*pK_i (−log inhibition constant) from [^{3}H]-5-CT binding experiments. Binding to guinea-pig hippocampus was measured in the presence of pindolol (10 μM) and sumatriptan (1 μM) to inhibit binding to 5-HT$_{1A/1B}$ and 5-HT$_{1D}$ receptors respectively; †pEC$_{50}$ for stimulation of adenylyl cyclase activity (intrinsic activity compared to 5-HT shown in brackets). ‡pK_B (−log antagonist equilibrium dissociation constant) for antagonism of 5-CT-stimulated adenylyl cyclase activity. The concentration of antagonist used (μM) is shown in parentheses. WAY-100635 and cyanopindolol induced biphasic 5-CT concentration-response curves and pK_B values shown were those determined from the two components of the curve. Data are the mean ± s.e.mean from at least three separate experiments each performed using duplicate (binding) or triplicate (adenylyl cyclase) determinations.

The response to 5-CT was characterized further using a number of 5-HT receptor antagonists. GR-113808, tested at 100 nM to selectively antagonize 5-HT$_4$ receptors, did not significantly antagonize the 5-CT response, confirming a lack of involvement of 5-HT$_4$ receptors (Figure 3). In contrast, methiothepin (1 μM), was a surmountable antagonist (pK_B 7.4 ± 0.2) (Figure 3). The selective 5-HT$_7$ receptor antagonist SB-258719 (5 μM) also antagonized surmountably the response to 5-CT (Figure 4), consistent with competitive antagonism (pK_B 7.2 ± 0.1). The Hill slope of the 5-CT concentration-response curve determined in the presence of SB-258719 was similar to that in the absence of SB-258719 (0.79 ± 0.1 and 0.78 ± 0.1 respectively).

In addition to antagonizing the effect of 5-CT, SB-258719 and methiothepin both appeared to produce a small inhibition of basal adenylyl cyclase activity (Figures 3 and 4). Measurement, by h.p.l.c., of the level of endogenous 5-HT in a series of membrane preparations used in the adenylyl cyclase assays revealed a concentration of 11 ± 2 nM (mean of three determinations). Modifications to the membrane preparation, including increasing both the number of washing steps and the length of preincubation, failed to remove this apparent 'tonic' stimulation of adenylyl cyclase activity (data not shown).

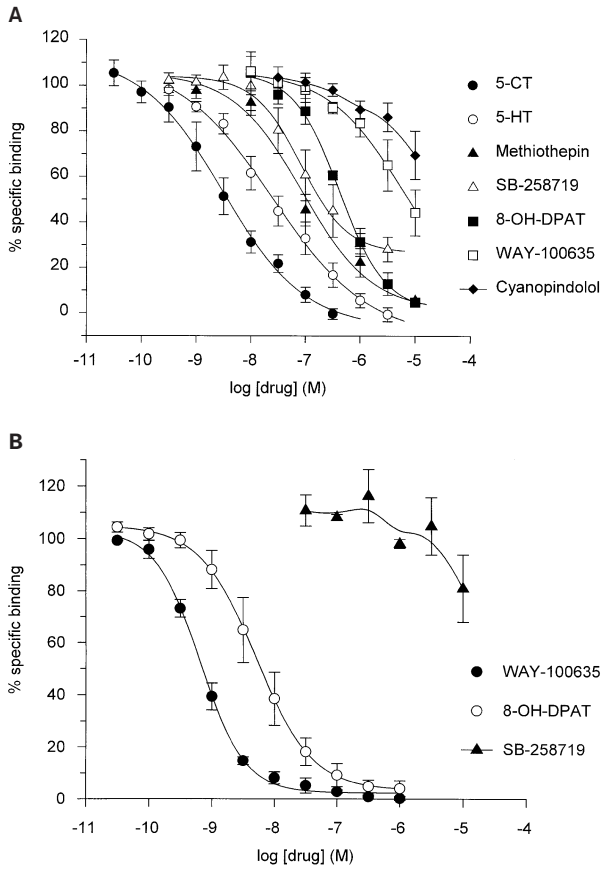

Figure 1 (A) Inhibition of [^{3}H]-5-CT binding to guinea-pig hippocampal membranes measured in the presence of (±) pindolol (10 μM) and sumatriptan (1 μM) to inhibit binding to 5-HT$_{1A/1B}$ and 5-HT$_{1D}$ receptors respectively. Data points represent the mean ± s.e.mean of at least three separate experiments each performed using duplicate determinations. Hill slopes ranged from 0.83 ± 0.1 (5-HT) to 1.1 ± 0.1 (8-OH-DPAT). Results are expressed as per cent of specific binding where non-specific binding was defined using 10 μM 5-HT. (B) Inhibition of [^{3}H]-8-OH-DPAT binding to guinea-pig hippocampal membranes. Data points represent the mean ± s.e.mean of at least three separate experiments each performed using duplicate determinations. Results are expressed as per cent specific binding where non-specific binding was defined using 10 μM 5-HT.

It has been reported that guinea-pig hippocampal membranes contain a population of 5-HT$_{1A}$-like receptors which mediate stimulation of adenylyl cyclase activity (Shenker *et al*., 1987). The effects of WAY-100635 and cyanopindolol, which are both 5-HT$_{1A}$ receptor antagonists displaying at least 1000 fold selectivity for 5-HT$_{1A}$ versus 5-HT$_7$ receptors, were therefore investigated. In the presence of WAY-100635 (tested at 100 nM to selectively antagonize 5-HT$_{1A}$ receptors) the 5-CT concentration-response curve displayed a biphasic profile, consistent with antagonism by WAY-100635 of a component of the 5-CT response and representing approximately 55% of the total response (Figure 5). In contrast to SB-258719, WAY-100635 did not significantly alter basal activity. The 5-CT curve in the presence of WAY-100635 could be curve-fitted using a two site model (RMSq = 14.5 for two site fit vs 15.4 for one site fit; Bates & Watts, 1988), giving pK_B values for WAY-100635 of <7.0 and 9.6 (95% confidence interval 9.0 – 10.2), for the first and second components of the curve respectively. The latter pK_B value is consistent with the previously reported 5-HT$_{1A}$ receptor affinity of WAY-100635 (Fletcher *et al*., 1996). Cyanopindolol (1 μM), like WAY-100635, did not alter basal activity, but induced an apparently biphasic 5-CT stimulation curve (Figure 5) which could also be fitted using a two site model (RMSq = 3.29 for two site fit vs 3.17 for one site fit). The pK_B values for cyanopindolol were <6 and 8.4 (95% confidence interval 7.7 – 9.1) respectively, for the first and second components of the curve, the latter value being similar to its reported antagonist potency at the 5-HT$_{1A}$ receptor (pK_B 8.1, Hoyer *et al*., 1994).

WAY-100635 was also investigated for its effect on the response to a near maximally-stimulating concentration of 5-CT (100 nM). WAY-100635 produced only partial inhibition of the 5-CT response (maximal inhibition 59 ± 9% at 1 μM, $P<0.05$ (Student's *t*-test) compared to the near complete inhibition produced by 5 μM SB-258719 (96 ± 9%)) (Figure 6). The pK_B for WAY-100635 of 9.4, calculated using the Cheng-Prusoff equation (see Methods), was also consistent with antagonism of a 5-HT$_{1A}$ receptor-mediated response.

Additional experiments were carried out in order to identify the receptor(s) mediating the response to 8-OH-DPAT, using the selective antagonists WAY-100635 and SB-258719. However, due to the small degree of stimulation by 8-OH-DPAT, it was not possible to determine whether the response was mediated *via* 5-HT$_{1A}$, 5-HT$_7$ or a combination of these receptors (data not shown).

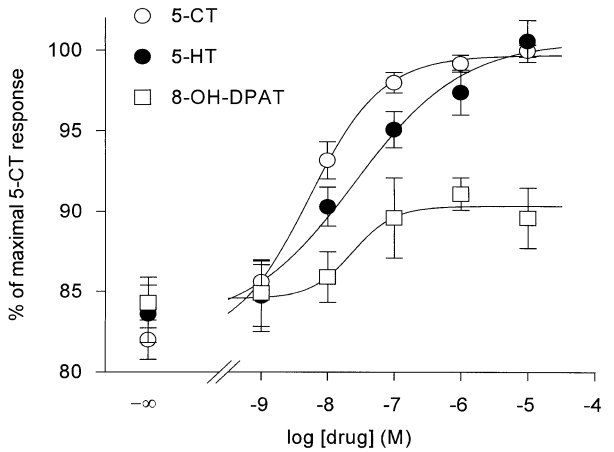

Figure 2 Stimulation of adenylyl cyclase activity in guinea-pig hippocampal membranes by 5-CT, 5-HT and 8-OH-DPAT. Stimulation by 5-HT was determined in the presence of GR-113808 (1 μM). Data points represent the mean ± s.e.mean of at least three separate experiments each performed using triplicate determinations. Results are expressed as per cent of the maximal 5-CT response.

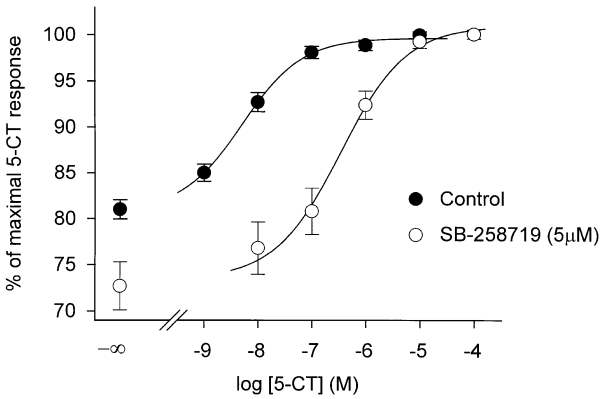

Figure 4 Stimulation of adenylyl cyclase activity in guinea-pig hippocampal membranes by 5-CT alone and in the presence of SB-258719 (5 μM). Data points represent the mean ± s.e.mean of three separate experiments each performed using triplicate determinations. Results are expressed as per cent of the maximal 5-CT response.

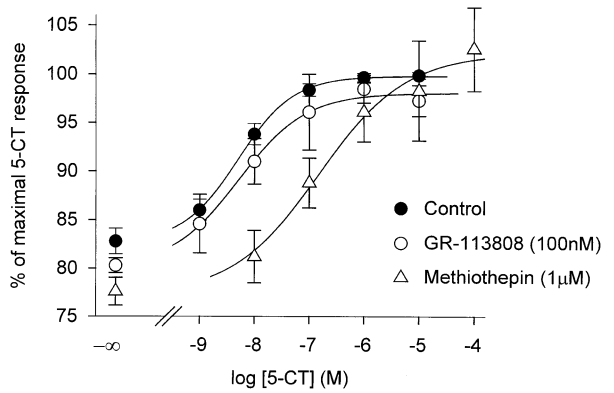

Figure 3 Stimulation of adenylyl cyclase activity in guinea-pig hippocampal membranes by 5-CT alone and in the presence of GR-113808 (100 nM) and methiothepin (1 μM). Data points represent the mean ± s.e.mean of three separate experiments each performed using triplicate determinations. Results are expressed as per cent of the maximal 5-CT response.

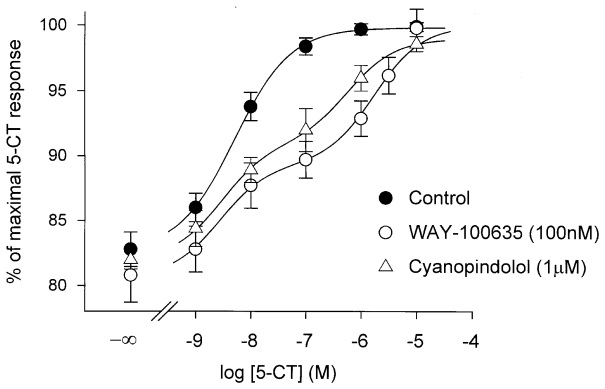

Figure 5 Stimulation of adenylyl cyclase in guinea-pig hippocampal membranes by 5-CT alone and in the presence of WAY-100635 (100 nM) or cyanopindolol (1 μM). Data points represent the mean ± s.e.mean of three separate experiments each performed using triplicate determinations. Results are expressed as per cent of the maximal 5-CT response.

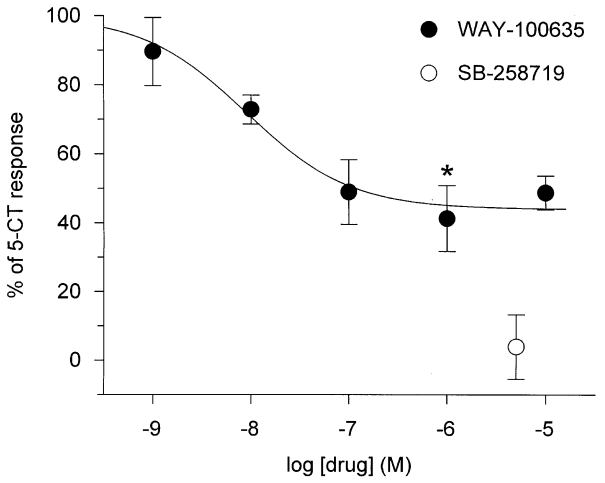

Figure 6 Effect of WAY-100635 (1 nM – 10 μM) and SB-258719 (5 μM) on adenylyl cyclase activity in guinea-pig hippocampal membranes in the presence of 5-CT (100 nM). *$P<0.05$ (Student's *t*-test), compared to inhibition by 5 μM SB-258719. Data points represent the mean ± s.e.mean of at least three separate experiments each performed using triplicate determinations. Results are expressed as per cent of the 5-CT response in the absence of added antagonist.

Discussion

A number of 5-HT receptor subtypes have been reported to mediate stimulation of adenylyl cyclase activity in guinea-pig hippocampus, including 5-HT$_4$ (Dumuis *et al*., 1988), 5-HT$_{1A}$-like (Shenker *et al*., 1987) and 5-HT$_7$-like receptors (Tsou *et al*., 1994). However, definitive evidence for functional coupling of 5-HT$_7$ receptors in brain has been absent, principally due to a lack of selective ligands acting at this receptor. Furthermore, similarities between the pharmacological profiles for 5-HT$_{1A}$ and 5-HT$_7$ receptors, and the fact that 5-HT$_{1A}$ receptors have been shown to be negatively coupled to adenylyl cyclase in guinea-pig hippocampus (e.g. De Vivo & Maayani 1986), has led to speculation that the 5-HT$_{1A}$-like cyclase stimulatory response reported in guinea-pig hippocampus may actually reflect 5-HT$_7$ receptor stimulation (Hoyer *et al*., 1994). In the present study, in order to better define the 5-HT$_7$-like functional response reported in guinea-pig hippocampus, we have investigated 5-CT stimulation of adenylyl cyclase activity in this tissue using a number of compounds including the selective 5-HT$_7$ receptor antagonist SB-258719 (Forbes *et al*., 1998; Thomas *et al*., 1998). In addition, we have compared these functional data with data obtained from receptor binding studies carried out in the same tissue.

Radioligand binding studies using [^{3}H]-5-CT have previously shown 5-HT$_7$ receptors to be present in a number of guinea-pig brain regions including cerebral cortex and hippocampus (To *et al*., 1995). In the present study, [^{3}H]-5-CT binding to guinea-pig hippocampal membrane revealed that the rank order of affinity for agonists (5-CT > 5-HT > 8-OH-DPAT), and affinity of methiothepin, were similar to that reported by To *et al*. (1995) for inhibition of [^{3}H]-5-CT binding to 5-HT$_7$ receptors in guinea-pig cortex. Furthermore, the affinity of SB-258719 (pK_i 7.2 ± 0.1) was in agreement with that reported for the human cloned 5-HT$_7$ receptor (Thomas *et al*., 1998). A small component of [^{3}H]-5-CT binding measured in the present study (representing approximately 25% of total binding) appeared to be inhibited by 5-CT, 5-HT and methiothepin, but not by SB-258719. This profile would be consistent with a minor component of [^{3}H]-5-CT binding to non-5-HT$_7$ binding sites (possibly 5-ht$_5$), at which 5-CT displays high affinity, methiothepin and 8-OH-DPAT display moderate affinity, while SB-258719 and WAY-100635 display low affinity. Due to the presence of this additional binding component, the actual binding density of 5-HT$_7$ receptors in guinea-pig hippocampus may therefore be slightly lower than the B_{max} determined in the present study using [^{3}H]-5-CT. The overall profile of inhibition of [^{3}H]-5-CT binding to guinea-pig hippocampal membranes was therefore consistent with binding predominantly to 5-HT$_7$ receptors under the assay conditions used. SB-258719 did not inhibit [^{3}H]-8-OH-DPAT binding to 5-HT$_{1A}$ receptors in guinea-pig hippocampus, consistent with its previously reported lack of affinity for the human cloned 5-HT$_{1A}$ receptor (pK_i < 5.1, Forbes *et al*., 1998) and suggesting that the 100 fold selectivity of SB-258719, for 5-HT$_7$ receptors determined in recombinant systems, is maintained in guinea-pig brain.

Agonist stimulation of adenylyl cyclase activity in guinea-pig hippocampus was investigated using 5-CT, 5-HT and 8-OH-DPAT. 5-CT was a potent full agonist compared to 5-HT (the latter tested in the presence of GR-113808 to prevent stimulation of 5-HT$_4$ receptors), whereas 8-OH-DPAT was a partial agonist, with a potency comparable to 5-HT. The rank order of agonist potency was therefore 5-CT > 5-HT ~ 8-OH-DPAT. This agonist profile is consistent with activation of 5-HT$_7$ receptors, being similar to the profile reported for the human cloned 5-HT$_7$ receptor (Thomas *et al*., 1998), and the previous studies of Tsou *et al*. (1994) in guinea-pig hippocampus. In contrast, this agonist profile is inconsistent with activation of either 5-HT$_4$ or 5-HT$_6$ receptors, since 5-CT displays low potency at these subtypes compared to 5-HT (Hoyer *et al*., 1994). However, an adenylyl cyclase-stimulatory 5-HT$_{1A}$-like receptor, which is activated by both 5-CT and 8-OH-DPAT, has been reported to be present in guinea-pig hippocampus (Shenker *et al*., 1987). Therefore, it is possible that the agonist profile described in the present study could reflect stimulation of either 5-HT$_7$ or 5-HT$_{1A}$ receptors, or a mixed 5-HT$_7$/5-HT$_{1A}$ receptor population.

In order to better define the receptor(s) involved in mediating 5-CT stimulation of adenylyl cyclase in guinea-pig hippocampus, the response to 5-CT was investigated further using a number of 5-HT receptor antagonists. The selective 5-HT$_4$ antagonist GR-113808 (100 nM) did not antagonize the response to 5-CT, confirming that 5-HT$_4$ receptors were not mediating the response. In contrast, methiothepin (1 μM), which displays high affinity for both 5-HT$_1$ and 5-HT$_7$ receptors (Hoyer *et al*., 1994), was a surmountable antagonist of the 5-CT response. The selective 5-HT$_7$ antagonist SB-258719 (5 μM) also produced a surmountable antagonism of the 5-CT response, consistent with competitive antagonism (Figure 4). The pK_B for SB-258719 of 7.2 ± 0.1 was in good agreement with its previously reported antagonist potency at the human cloned 5-HT$_7$ receptor (pA_2 7.2 ± 0.2, Thomas *et al*., 1998) and suggested that 5-HT$_7$ receptors were indeed mediating the 5-CT response.

In addition to antagonizing the 5-CT response, SB-258719 produced a small inhibition of basal adenylyl cyclase activity (Figure 4), an effect also seen for methiothepin. This inhibition of basal activity could be explained by antagonism by SB-258719 of 'tonic' 5-HT$_7$ receptor stimulation by endogenous 5-HT not completely removed during the membrane preparation procedure. Analysis of hippocampal membranes by h.p.l.c. revealed a membrane 5-HT concentration of 11 ± 2 nM. Since the observed EC_{50} for 5-HT in this system was 21 nM, this level of endogenous 5-HT could cause a measurable level of 5-HT$_7$ receptor activation and could therefore explain the inhibitory effect of SB-258719 on basal activity. Modifications to the

membrane preparation method (including increasing the number of washing steps and the pre-incubation time) failed to completely remove this inhibitory effect. Tsou *et al*. (1994), who also investigated 5-CT stimulation of adenylyl cyclase in guinea-pig hippocampus, included spiperone in the membrane pre-incubation step, perhaps to prevent association of endogenous 5-HT with the membranes. In our studies, inclusion of spiperone (10 μM) during the membrane pre-incubation resulted in a reduction in 5-CT potency in the subsequent adenylyl cyclase assay (data not shown), implying incomplete removal of spiperone by the membrane washing procedure. For this reason, spiperone was not routinely included in the membrane pre-incubation step. However, the inclusion of GDP in the initial stages of membrane preparation (see Method) was found to minimize basal activity measured in the subsequent adenylyl cyclase assay without altering 5-CT potency or degree of stimulation. It is possible that the addition of GDP may serve to oppose receptor-G-protein coupling and therefore high affinity agonist binding by maintaining G_s in the uncoupled (GDP-bound) state.

The data described above are consistent with the presence of 5-HT$_7$ receptors in guinea-pig hippocampus which are positively coupled to adenylyl cyclase and which, under the experimental conditions used in the present study, may be partially tonically activated. However, as already mentioned above, there is evidence that guinea-pig hippocampus may also contain a 5-HT$_{1A}$-like receptor positively coupled to adenylyl cyclase. Although the potency with which SB-258719 antagonized the response to 5-CT in the present study is inconsistent with a response mediated directly by 5-HT$_{1A}$ receptors, the possibility that a component of the response to 5-CT might involve 5-HT$_{1A}$ receptors was investigated.

WAY-100635 and cyanopindolol, which both display at least 1000 fold selectivity for 5-HT$_{1A}$ versus 5-HT$_7$ receptors (Fletcher *et al*., 1996; Hoyer *et al*., 1994) were investigated for their effect on the 5-CT stimulation of adenylyl cyclase. Neither WAY-100635 (100 nM) nor cyanopindolol (1 μM) (concentrations which antagonize 5-HT$_{1A}$ but not 5-HT$_7$ receptors), significantly altered basal adenylyl cyclase activity, but both compounds induced an apparently biphasic 5-CT concentration-response curve (Figure 5). This profile appeared consistent with potent antagonism of a component of the 5-CT response representing approximately 55% of the total response. For both compounds the data could be fitted according to a two-site model, allowing two pK_B values to be determined for each compound from the two components of the curve (see Methods). The estimated pK_B values for WAY-100635 and cyanopindolol from one component (<7 and <6 respectively) were consistent with antagonism of a 5-HT$_7$ receptor-mediated response. In contrast, the pK_B values for WAY-100635 and cyanopindolol calculated from the second component of the 5-CT response (9.6 and 8.4 respectively) agreed well with their reported 5-HT$_{1A}$ receptor affinities (WAY-100635 pK_i 9.9, Fletcher *et al*., 1996, cyanopindolol pK_B 8.1, Hoyer *et al*., 1994) and so were consistent with antagonism of a 5-HT$_{1A}$ receptor-mediated response. WAY-100635 was also investigated for its effect on the response to a near maximally stimulating concentration of 5-CT (100 nM). WAY-100635 (1 nM – 10 μM) partially inhibited the 5-CT response, in contrast to SB-258719 (5 μM) which completely reversed the 5-CT response. The estimated pK_B for WAY-100635 of 9.4 (calculated using the Cheng-Prusoff equation), was similar to its pK_B value reported above.

The above data are consistent with the 5-CT-induced stimulation of adenylyl cyclase activity in guinea-pig hippocampus being mediated *via* both 5-HT$_7$ and 5-HT$_{1A}$ receptors. However, the antagonist profile for SB-258719 appears to be inconsistent with such a dual mechanism. Firstly, SB-258719 produced a parallel rightward shift of the 5-CT concentration-response curve, consistent with the whole 5-CT-induced response being mediated by a single class of receptor. Secondly, the antagonist potency of SB-258719 is in good agreement with its potency at the human cloned 5-HT$_7$ receptor (Thomas *et al*., 1998). Furthermore, binding data generated in the present study suggest that the 100 fold selectivity of SB-258719 for 5-HT$_7$ versus 5-HT$_{1A}$ receptors reported previously in human recombinant systems (Forbes *et al*., 1998) is maintained in guinea-pig hippocampus. Long and short splice variants of the 5-HT$_7$ receptor (designated h5-HT$_{7(a)}$ and h5-HT$_{7(b)}$ respectively) have been reported to be present in hippocampus at similar expression levels and both splice variants display a similar affinity for 5-CT (Jasper *et al*., 1997). This raises the possibility that the profile of antagonism displayed by WAY-100635 and cyanopindolol could be explained by selective antagonism of the 5-CT response mediated *via* a specific 5-HT$_7$ splice variant. However, this possibility seems unlikely since, as shown in the present study, WAY-100635 and cyanopindolol are both weak inhibitors of [^{3}H]-5-CT binding to 5-HT$_7$ receptors in guinea-pig hippocampus and their antagonist potencies correlate well with their corresponding 5-HT$_{1A}$ receptor affinities.

An alternative explanation for the profile of antagonism by WAY-100635 and cyanopindolol is that 5-HT$_{1A}$ receptor activation in guinea-pig hippocampus does not directly stimulate adenylyl cyclase activity, but serves to augment 5-HT$_7$-mediated stimulation. It has been shown that activation of adenylyl cyclase isoform 2 by G_s coupled receptors can be amplified by $\beta\gamma$ subunits released following activation of G_i-proteins (Tang & Gilman, 1991). Importantly, this $\beta\gamma$-mediated amplification was shown to be conditional on G_s activation. Consistent with these findings, 5-HT$_{1A}$ receptors couple negatively to adenylyl cyclase *via* $G_{\alpha i}$, but do not appear to couple directly to $G_{\alpha s}$, as reported in studies using an *in vitro* reconstitution system in which the human 5-HT$_{1A}$ receptor was combined with various G-protein subtypes (Bertin *et al*., 1992). Although these initial studies were carried out using a purified system, receptor cross-talk involving $\beta\gamma$ subunits has subsequently been reported for a number of native tissue systems (for review see Selbie & Hill, 1998). Adenylyl cyclase isoform 2 is present in hippocampus and this mechanism could, as previously suggested by Hoyer *et al*. (1994), explain why hippocampal 5-HT$_{1A}$ receptors have been reported to mediate inhibition (*via* G_i) or activation (*via* $\beta\gamma$ subunits) of adenylyl cyclase activity (De Vivo & Maayani, 1986; Shenker *et al*., 1987).

If 5-HT$_{1A}$ receptor activation augments, and is conditional on, 5-HT$_7$-mediated activation of adenylyl cyclase in guinea-pig hippocampus, then selective 5-HT$_7$ antagonism would be predicted to inhibit both the 5-HT$_7$ and 5-HT$_{1A}$ receptor-mediated components of the response to 5-CT. In contrast, selective 5-HT$_{1A}$ receptor antagonism would inhibit the 5-HT$_{1A}$-mediated augmentation but not the direct 5-HT$_7$-mediated response to 5-CT. The antagonist profiles seen in the present study are consistent with this proposed mechanism, since the selective 5-HT$_7$ antagonist SB-258179 completely reversed the 5-CT stimulation of adenylyl cyclase activity, whereas the 5-HT$_{1A}$ antagonists WAY-100635 and cyanopindolol only partially antagonized the 5-CT response. This latter profile is therefore consistent with selective attenuation of a 5-HT$_{1A}$-mediated augmentation of the 5-HT$_7$-mediated response. The antagonist profile seen for WAY-100635 and cyanopindolol also suggests that the concentration of 5-CT

required to produce 5-HT$_{1A}$-mediated augmentation of the 5-HT$_7$-mediated response may be higher than that reported to inhibit forskolin-stimulated adenylyl cyclase activity (Schoeffter and Hoyer 1988). Assuming that $\beta\gamma$ subunits are indeed mediating the 5-HT$_{1A}$ augmentation effect, this profile would not be inconsistent with previous studies which have shown that higher concentrations of $\beta\gamma$, compared to G_α, are required to modulate adenylyl cyclase activity (Tang & Gilman 1991; Hoyer & Boddeke 1993).

If 5-HT$_7$ and 5-HT$_{1A}$ receptors act synergistically in guinea-pig hippocampus then they should be co-localized on the same neuronal membrane. Consistent with this, there is evidence that 5-HT$_{1A}$ and 5-HT$_7$ receptors may both be located pre- and post-synaptically in hippocampus (Hoyer *et al*., 1994; To *et al*., 1995). It is therefore possible that 5-HT$_7$ and 5-HT$_{1A}$ receptors act synergistically in guinea-pig hippocampus to finely control cyclic AMP production in pre- and/or post-synaptic neurones.

In conclusion, the data from the present study are consistent with the presence of 5-HT$_7$ receptors in guinea-pig hippocampus which are positively coupled to adenylyl cyclase. In addition, the data suggest that the 5-HT$_7$ receptor-mediated stimulation of adenylyl cyclase activity in this tissue can be augmented by a mechanism involving 5-HT$_{1A}$ receptor activation.

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