Enhancement of cyclic AMP accumulation mediated by 5-HT after chronic amitriptyline treatment in NG 108-15 cells

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1 The effects of chronic *in vitro* administration of amitriptyline, a tricyclic antidepressant, on 5-hydroxytryptamine (5-HT) receptor-mediated adenylyl cyclase activity was studied in the neuroblastoma × glioma hybrid cell line, NG 108-15.

2 Treatment of NG 108-15 cells with 8 µM amitriptyline for 3 days increased forskolin-stimulated (0.1 µM) adenosine 3':5'-cyclic monophosphate (cyclic AMP) accumulation. Addition of 5-HT (0.1–100 µM) increased forskolin-stimulated cyclic AMP accumulation in amitriptyline-treated cells in a concentration-dependent manner. However, 5-HT did not affect forskolin-stimulated cyclic AMP accumulation in untreated cells.

3 The 5-HT₄ receptor agonist, 5-methoxytryptamine, significantly enhanced forskolin-stimulated cyclic AMP accumulation in amitriptyline-treated cells. In contrast, amitriptyline treatment failed to modify 8-hydroxy-2-(di-n-propylamine) tetralin-induced inhibition of forskolin-stimulated cyclic AMP accumulation.

4 Pretreatment of cells with pertussis toxin did not affect the 5-HT-induced enhancement of cyclic AMP accumulation.

5 The 5-HT-induced enhancement of cyclic AMP accumulation in amitriptyline-treated cells was attenuated by the 5-HT₄ receptor antagonists, GR 113808 and ICS 205-930, with relatively low potency. However, spiperone, SCH 23390, and pindolol were completely ineffective against this 5-HT-induced enhancement.

6 Chronic treatment with amitriptyline did not modify the cyclic AMP production stimulated by prostaglandin E₁ or cholera toxin. This treatment also had no effect on GTPγS-, NaF-, and Mn²⁺-stimulated cyclic AMP accumulation in isolated cell membranes.

7 Chronic treatment with the 5-HT receptor antagonists, pindolol or ICS 205-930, did not inhibit the 5-HT-induced enhancement of cyclic AMP accumulation.

8 Chronic treatment with other antidepressant drugs, imipramine, mianserin or paroxetine, elicited the 5-HT-induced enhancement of cyclic AMP accumulation.

9 Taken together, these results suggest that chronic amitriptyline treatment of NG 108-15 cells causes 5-HT to enhance forskolin-stimulated cyclic AMP accumulation by enhancing 5-HT receptor-mediated stimulation of adenylyl cyclase and not by reducing 5-HT-mediated inhibition of adenylyl cyclase. The 5-HT-induced enhancement of cyclic AMP accumulation in amitriptyline-treated cells may result from changes at the level of the 5-HT receptor rather than at the level of G_i proteins or adenylyl cyclase. It is unlikely that this enhancement of cyclic AMP accumulation is caused by long-term antagonism of the 5-HT receptor by amitriptyline.

Keywords: Adenylyl cyclase; amitriptyline; antidepressant; cyclic AMP; 5-HT; 5-HT receptor; forskolin; NG 108-15 cell

Introduction

Clinical efficacy of antidepressant drugs requires chronic administration, suggesting that the therapeutic effects are produced by the long-term development of adaptive changes in neurotransmission, rather than by acute biochemical changes such as blockade of monoamine reuptake. Several lines of evidence suggest that antidepressant drugs may exert their clinical effects by producing adaptive changes in the number or function of central 5-hydroxytryptamine (5-HT) receptor subtypes (Peroutka & Snyder, 1980; Willner, 1985). Pharmacological studies have shown that chronic administration of antidepressants leads to a down-regulation of 5-HT_{2A} receptors in the cerebral cortex of rats (Peroutka & Snyder, 1980; Kellar *et al.*, 1981) and a decrease in 5-HT_{2A} receptor-mediated activation of phosphoinositide turnover (Kendal & Nahorski, 1985). However, other studies have shown that chronic administration of selective 5-HT reuptake inhibitors did not cause a down-regulation of cortical 5-HT_{2A} receptors (Hyttel *et al.*, 1984; Sanders-Bush *et al.*, 1989).

The effect of chronic antidepressant treatment on 5-HT receptors that are coupled to adenylyl cyclase signal transduction, such as 5-HT_{1A} and 5-HT_{1B} receptors, has also been studied. Recent reports have shown that 5-HT_{1A} receptor-mediated inhibition of adenylyl cyclase in rat hippocampus is either attenuated (Newman & Lerer, 1988; Newman *et al.*, 1992) or unaffected (Varrault *et al.*, 1991) by antidepressant treatments. Such treatments also have no effect on the density of 5-HT_{1A} receptor binding sites (Peroutka & Snyder, 1980; Welner *et al.*, 1989; Newman *et al.*, 1990). In addition, no changes in the density and affinity of 5-HT_{1B} receptor binding sites have been found after chronic treatment with any one of several antidepressants (Montero *et al.*, 1991). The range of conflicting reports of the effect of chronic administration of antidepressants on 5-HT₁ and 5-HT₂ receptor families suggests that antidepressant drugs may exert their effects by inducing changes in some other 5-HT receptor subtype.

Recent studies have described several new categories of 5-HT receptor subtypes (5-HT₄, 5-HT₆ and 5-HT₇), in both the peripheral and the central nervous system, that are

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positively coupled to adenylyl cyclase but are distinct from 5-HT_{1A} or 5-HT_{1B} receptors (Dumuis *et al.*, 1988; Bard *et al.*, 1993; Monsma *et al.*, 1993). In mouse colicilar neurones and guinea-pig hippocampal membranes, 5-HT stimulates adenylyl cyclase activity, but selective 5-HT₁ and 5-HT₂ receptor agonists do not. Similarly, this stimulatory effect of 5-HT is not blocked by 5-HT₁ or 5-HT₂ receptor antagonists (Dumuis *et al.*, 1988; Bockaert *et al.*, 1990). However, there have been no studies of the effects of chronically administered tricyclic antidepressants on this type of 5-HT-stimulated adenylyl cyclase activity. Therefore, we examined the chronic effect of tricyclic antidepressants on 5-HT-stimulated adenylyl cyclase signal transduction in the neuroblastoma × glioma hybrid cell line, NG 108-15.

Methods

Cell culture

NG 108-15 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum, 100 µM hypoxanthine and 16 µM thymidine in a humidified atmosphere of 90% air, 10% CO₂ at 37°C. Confluent cultures were subcultured at ratios of 1:10 to 1:20 in 35-mm culture dishes or 100-mm culture dishes (for the membrane preparation), and were exposed to vehicle or amitriptyline (0.25–10 µM) for 1–5 days. Media were replaced 2 days after the subculture.

Cyclic AMP determination

The DMEM was removed, and the cells (35-mm dish) were washed twice with 2.5 ml of HEPES-buffered Krebs-Ringer solution (composition, mM; NaCl 150, KCl 5, MgSO₄ 1, CaCl₂ 2, glucose 10, HEPES 10, pH 7.4) at 37°C and were incubated with 1.0 ml of buffer containing various agents for 10 min at 37°C. The reactions were terminated by adding 250 µl of ice-cold 20% perchloric acid and the mixtures were kept on ice for 20 min. The cells were removed by scraping and transferred into tubes. The extracts were centrifuged at 2000 g for 15 min at 4°C. The supernatants were neutralized with 1.53 M KOH containing 75 mM HEPES and were kept on ice for 60 min. Then the solutions were centrifuged at 2000 g for 15 min at 4°C, and the concentration of cyclic AMP in the supernatant was measured by radioreceptor binding assay (Amersham kit).

For cyclic AMP determination in membrane fractions, the cells (100-mm dish) were washed with phosphate buffered saline and harvested. A membrane fraction was prepared after centrifugation at 4°C at 900 g for 5 min and resuspension by homogenization with a polytron (Kinematica) in 10 mM Tris buffer containing 2 mM EGTA (pH 7.4). The resulting homogenate was centrifuged at 48 000 g for 20 min and the membrane pellet was resuspended in 10 mM Tris buffer containing 2 mM EGTA. The reaction buffer contained 80 mM Tris buffer (pH 7.4), 5 mM MgSO₄, 0.5 mM EGTA, 0.5 mM IBMX, 0.3% bovine serum albumin, 5 mM phosphocreatine, 50 units ml⁻¹ creatine phosphokinase, 0.5 mM ATP and either GTPγS, NaF, forskolin or MnCl₂, and was preincubated for 3 min at 37°C. After preincubation, reactions were initiated by the addition of the membrane fraction and incubated for an additional 10 min at 37°C. Reactions were stopped by boiling for 3 min. Samples were centrifuged at 13 000 g for 5 min and the concentration of cyclic AMP was measured by radioreceptor binding assay.

Protein assay

After removal of the reaction mixtures, the culture dishes were rinsed with 1.0 ml of water and the rinses were added to the pellets which remained in the tubes. The tubes were centrifuged again at 2000 g for 15 min at 4°C. Protein deter-

minations were performed in the resulting pellet with the Pierce BCA protein assay kit (Pierce, Rockford, IL, U.S.A.).

Materials

5-HT, 5-methoxytryptamine (5-MeOT), carbachol and prostaglandin E₁ (PGE₁) were obtained from Sigma Chem. Co. (St. Louis, MO, U.S.A.). Amitriptyline, imipramine, mianserin, noradrenaline (NA) and forskolin were obtained from Wako Pure Chem. (Osaka, Japan). Pindolol, R(+)-7-chloro-8-hydroxy-3-methyl-1-phenyl-2,3,4,5-tetrahydro-1H-3-benzazepine (SCH 23390), spiperone, 8-hydroxy-2-(di-n-propylamino)tetralin (8-OH-DPAT) and clonidine were obtained from Research Biochemicals Inc. (Natick, MA, U.S.A.). 3-Isobutyl-1-methylxanthine (IBMX) was obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.). Guanosine 5'-(3-O-thio)triphosphate (GTPγS) was obtained from Boehringer-Mannheim (Mannheim, Germany). Pertussis toxin was obtained from Kaken Seiyaku Co. (Tokyo, Japan). Cholera toxin was obtained from List Biological Laboratories Inc. (Campbell, CA, U.S.A.). Cyclic AMP assay kits were obtained from Amersham Co. (Arlington Heights, IL, U.S.A.). (3α-Tropanyl)-1H-indole-3-carboxylic acid ester (ICS 205-930) was donated by Sandoz Ltd. (Basel, Switzerland). {1-[2-(Methylsulphonylamino)ethyl]-4-piperidinyl}methyl 1-methyl-1H-indole-3-carboxylate (GR 113808A) was donated by Glaxo (Middlesex, U.K.). Paroxetine was donated by SmithKline Beecham (Surrey, U.K.). All other chemicals used were of analytical grade.

Data analysis

Data are shown as mean ± s.e.mean of the number (*n*) of separate experiments. The differences in cyclic AMP content stimulated by agonists between control and amitriptyline-treated were determined by Student's *t* test. Comparisons of the data expressed as the percentage of control were made using the Mann-Whitney U-test.

Results

Forskolin (0.01 to 10 µM) stimulated cyclic AMP accumulation in a dose-dependent manner in NG 108-15 cells. When the cells were treated with 8 µM amitriptyline for 1 to 5 days, forskolin-stimulated cyclic AMP accumulation increased markedly, with the maximal increase occurring after 3 days of treatment (data not shown). After 3 days of 8 µM amitriptyline treatment, exposure to 0.1 µM forskolin for 10 min increased cyclic AMP levels by 720%, while untreated (control) cells responded to this same forskolin treatment with only a 470% increase (Figure 1). In treated cells, the addition of 10 µM 5-HT enhanced this forskolin-stimulated cyclic AMP accumulation by 64% over the effect of forskolin alone. In control cells, the addition of 5-HT to the forskolin-stimulated cells did not significantly change the levels of the forskolin response (Figure 1). Amitriptyline treatment caused a slight, but statistically insignificant, increase in basal cyclic AMP levels. Protein contents per dish were not altered by the treatment with amitriptyline up to 10 µM (data not shown).

When the cells were treated with various concentrations of amitriptyline (0.25–10 µM), significant increases in cyclic AMP accumulation stimulated by forskolin in the presence of 5-HT were obtained even with low concentrations of amitriptyline (0.5–2 µM) (Figure 2). These low amitriptyline concentrations are similar to those required for clinical efficacy. On the other hand, significant increases of the forskolin response in the absence of 5-HT were obtained on treatment with >4 µM amitriptyline. Therefore, 8 µM amitriptyline was used in the majority of the experiments to maximise the increase of cyclic AMP accumulation. In amitriptyline-treated cells,

the 5-HT induced enhancement of forskolin-stimulated cyclic AMP accumulation was concentration-dependent over a range of $1\text{ }\mu\text{M}$ to $100\text{ }\mu\text{M}$ 5-HT (Figure 3).

To investigate the ability of various receptors, including 5-HT receptor subtypes, to modulate cyclic AMP production in amitriptyline-treated cells, we examined the effects of the following substances on forskolin-stimulated cyclic AMP accumulation: 5-MeOT (a 5-HT₄ receptor agonist), 8-OH-DPAT (a 5-HT_{1A} receptor agonist), carbachol (a muscarinic receptor agonist), clonidine (an α_2 -adrenoceptor agonist) and NA. The addition of 5-MeOT ($10\text{ }\mu\text{M}$) to the forskolin exposure significantly increased cyclic AMP accumulation in amitriptyline-treated cells (22.8% over the control; Figure 4). On the other hand, the application of 8-OH-DPAT ($10\text{ }\mu\text{M}$) reduced forskolin-stimulated cyclic AMP accumulation in control and amitriptyline-treated cells by 39.3% and 29.4%, respectively, indicating that the effects of 8-OH-DPAT were not modified by amitriptyline treatment. The application of either carbachol (1 mM), clonidine ($10\text{ }\mu\text{M}$), or NA ($10\text{ }\mu\text{M}$)

Figure 1 Modulation by 5-hydroxytryptamine (5-HT) of forskolin-stimulated cyclic AMP accumulation in NG 108-15 cells treated with amitriptyline. Cells were treated with (hatched columns) or without (open columns) $8\text{ }\mu\text{M}$ amitriptyline for 3 days. The media were replaced with HEPES-buffered Krebs-Ringer solution and the cells were incubated with $10\text{ }\mu\text{M}$ 5-HT, $0.1\text{ }\mu\text{M}$ forskolin alone, or forskolin plus 5-HT for 10 min at 37°C . Data represent the mean $\pm$ s.e.mean values of nine separate experiments. * $P < 0.05$, ** $P < 0.01$ versus amitriptyline-untreated.

Figure 2 Effect of various amitriptyline concentrations on enhancement by 5-hydroxytryptamine (5-HT) of forskolin-stimulated cyclic AMP accumulation in NG 108-15 cells. Cells were treated with various concentrations (0.25 to $10\text{ }\mu\text{M}$) of amitriptyline for 3 days. The cells were incubated with $0.1\text{ }\mu\text{M}$ forskolin alone (○), or forskolin plus $10\text{ }\mu\text{M}$ 5-HT (●) for 10 min at 37°C . Data are presented as percentages of the values obtained with forskolin alone or forskolin plus 5-HT of control cells, and are mean $\pm$ s.e.mean values for three to six separate experiments. * $P < 0.05$, ** $P < 0.01$ versus control.

also inhibited forskolin-stimulated cyclic AMP accumulation to the same extent in both control and amitriptyline-treated cells.

To determine whether the 5-HT-induced enhancement of cyclic AMP accumulation is due to a decrease in the inhibition of adenylyl cyclase by an inhibitory guanine nucleotide binding regulatory protein (Gi protein), cells were preincubated for 20 h with pertussis toxin, 100 ng ml^{-1} . There was no significant difference in the degree of 5-HT-induced enhancement of forskolin-stimulated cyclic AMP accumulation between control and toxin-treated cells (Table 1). However, treatment with pertussis toxin abolished the ability of 8-OH-DPAT to inhibit forskolin-stimulated cyclic AMP accumulation.

The effects of various 5-HT receptor antagonists on 5-HT-induced enhancement of cyclic AMP accumulation in amitriptyline-treated cells were examined. Recent reports have

Figure 3 Concentration-dependent increases by 5-hydroxytryptamine (5-HT) of cyclic AMP accumulation in amitriptyline-treated NG 108-15 cells. Cells were treated with (●) or without (○) $8\text{ }\mu\text{M}$ amitriptyline for 3 days. The cells were incubated with $0.1\text{ }\mu\text{M}$ forskolin alone, or forskolin plus 5-HT (0.1 to $100\text{ }\mu\text{M}$) for 10 min at 37°C . Data are presented as percentages of the values obtained with forskolin alone of control or amitriptyline-treated cells, and are mean $\pm$ s.e.mean values for three to five separate experiments. ** $P < 0.01$ versus forskolin alone of amitriptyline-treated cell.

Figure 4 Effects of chronic amitriptyline treatment on regulation of forskolin-stimulated cyclic AMP accumulation by 5-hydroxytryptamine (5-HT) receptor, muscarinic receptor and adrenoceptor agonists in NG 108-15 cells. Cells were treated with (solid columns) or without (open columns) $8\text{ }\mu\text{M}$ amitriptyline for 3 days, and then incubated with 5-HT ($10\text{ }\mu\text{M}$), 5-MeOT ($10\text{ }\mu\text{M}$), 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) ($10\text{ }\mu\text{M}$), carbachol (1 mM), clonidine ($10\text{ }\mu\text{M}$), or noradrenaline (NA, $10\text{ }\mu\text{M}$), in the presence of $0.1\text{ }\mu\text{M}$ forskolin. Data are expressed as percentages of forskolin-stimulated cyclic AMP levels in control or amitriptyline-treated cells, and are mean $\pm$ s.e.mean values for three to six separate experiments. * $P < 0.05$ versus forskolin alone, ^b $P < 0.01$ versus control.

Table 1 Effect of pertussis toxin pretreatment on regulation by 5-hydroxytryptamine (5-HT) or 8-hydroxy-2-(di-n-propylamino) tetralin (8-OH-DPAT) of forskolin-stimulated cyclic AMP accumulation in amitriptyline-treated NG 108-15 cells

	Cyclic AMP (pmol mg ⁻¹ protein)			
	Treatment			
	Amitriptyline (-)	(+)	(-)	(+)
	Pertussis toxin (-)	(-)	(+)	(+)
Basal	74 ± 5	76 ± 6	75 ± 6	74 ± 6
Forskolin	473 ± 26 (100)	680 ± 48 (100)	480 ± 25 (100)	696 ± 32 (100)
Forskolin + 5-HT	520 ± 36 (110)	1149 ± 68 (169) ^a	499 ± 10 (104)	1127 ± 37 (162) ^a
Forskolin + 8-OH-DPAT	330 ± 23 (69.8) ^b	488 ± 27 (71.8) ^b	514 ± 23 (107) ^c	807 ± 68 (116) ^c

Cells were treated with or without 8 μ M amitriptyline for 3 days, and treated with pertussis toxin (100 ng ml⁻¹) in the presence or absence of amitriptyline for 20 h at the end of amitriptyline treatment. After the treatment, the cells were incubated with 0.1 μ M forskolin alone, forskolin plus 10 μ M 5-HT, or forskolin plus 10 μ M 8-OH-DPAT. Data represent the mean $\pm$ s.e. mean values of three separate experiments. Numbers in parentheses are cyclic AMP levels as a percentage of the respective forskolin values. ^a P < 0.01 versus forskolin, ^b P < 0.05 versus forskolin, ^c P < 0.01 versus (-) pertussis toxin.

demonstrated that GR 113808 is a highly potent antagonist of the 5-HT₄ receptor in guinea-pig and rat brain membranes (Grossman *et al.*, 1993; Kaumann, 1993). In addition, high concentrations of ICS 205-930 have been shown to antagonize competitively the 5-HT₄ receptor in many neuronal and peripheral tissues (Dumuis *et al.*, 1988; Kaumann *et al.*, 1990; Craig & Clarke, 1990). We found that GR 113808 (>5 μ M) significantly attenuated 5-HT-induced enhancement of forskolin-stimulated cyclic AMP accumulation. However, the maximum inhibition by GR 113808 was only 65%, even at a concentration of 30 μ M. In addition, ICS 205-930 (30 μ M) decreased the 5-HT-induced enhancement by 47% (Figure 5). However, spiperone (an antagonist of 5-HT₁ and 5-HT₂ receptors), SCH23390 (an antagonist of the 5-HT₂ receptor) and pindolol (an antagonist of the 5-HT₁ receptor) all failed to inhibit the 5-HT-induced enhancement, even at concentrations of up to 30 μ M.

The effects of amitriptyline treatment on cyclic AMP accumulation induced by the activation of other receptors that are positively coupled to adenylyl cyclase activity were examined. Cyclic AMP accumulation in NG 108-15 cells was strongly stimulated by PGE₁ (0.1 μ M), but the amount of cyclic AMP accumulation was not altered by amitriptyline treatment. The addition of 10 μ M 5-HT to the PGE₁ exposure did not modify the PGE₁-stimulated cyclic AMP accumulation in either control or amitriptyline-treated cells (Table 2). In addition, the possible involvement of post-receptor components of signal transduction was examined by studying the effects of amitriptyline treatment on cholera toxin-induced cyclic AMP production. Cholera toxin stimulates adenylyl cyclase by the ADP-ribosylation of α subunit of a stimulatory guanine nucleotide binding regulatory protein (Gs protein). We found that amitriptyline treatment failed to modify the amount of cyclic AMP produced by cholera toxin (Table 2). We also examined the effect of chronic amitriptyline treatment on the direct stimulation of G proteins by GTP γ S or NaF in membranes of NG 108-15 cells. Neither the cyclic AMP produced by GTP γ S (10 μ M) nor NaF (10 mM) was affected by amitriptyline treatment. The addition of 10 μ M 5-HT to the GTP γ S exposure did not modify the GTP γ S-stimulated cyclic AMP accumulation in either control or treated membranes (Table 2). Direct activation of adenylyl cyclase by 20 mM Mn²⁺ was also unaffected by amitriptyline treatment. However, amitriptyline treatment enhanced forskolin-stimulated cyclic AMP accumulation in the presence and absence of 10 μ M 5-HT in both isolated membranes and intact cells.

To determine whether chronic amitriptyline treatment up-regulates the 5-HT receptors by receptor antagonism, cells were chronically treated with pindolol or ICS 205-930 in the presence or absence of amitriptyline for 3 days. Neither pindolol nor ICS 205-930 affected forskolin-stimulated cyclic AMP accumulation in either the presence or absence of 5-HT.

Figure 5 Effect of various 5-hydroxytryptamine (5-HT) receptor antagonists on 5-HT-induced enhancement of cyclic AMP accumulation in amitriptyline-treated cells. Cells were treated with or without 8 μ M amitriptyline for 3 days. After washing, the cells were preincubated with GR 113808A (30 μ M), ICS 205-930 (30 μ M), spiperone (30 μ M), SCH 23390 (30 μ M), or pindolol (30 μ M) for 10 min and then were stimulated with 0.1 μ M forskolin alone, or forskolin plus 10 μ M 5-HT for 10 min at 37°C in the presence of each antagonist. The addition of these antagonists did not affect the forskolin responses. Data are expressed as percentages of the values obtained with forskolin plus 5-HT in the absence of the antagonist, and are mean $\pm$ s.e. mean values for three separate experiments. ** P < 0.01 versus forskolin plus 5-HT of amitriptyline-treated cell.

Neither of these drugs also altered the effects of amitriptyline treatment (Table 3).

We examined the ability of chronic treatment with the antidepressant drugs, imipramine, mianserin, or paroxetine, to modify forskolin-stimulated cyclic AMP accumulation and its enhancement by 5-HT. Each of these antidepressants (8 μ M for 3 days) mimicked the effect of amitriptyline on forskolin-stimulated cyclic AMP accumulation and its enhancement by 5-HT (Figure 6).

Discussion

In the present study, chronic amitriptyline treatment of NG 108-15 cells was found to cause 5-HT to enhance forskolin-stimulated cyclic AMP accumulation. This 5-HT-induced enhancement was not affected by pertussis toxin treatment. The application of 5-MeOT, a 5-HT receptor agonist, also significantly enhanced forskolin-stimulated cyclic AMP accumulation in amitriptyline-treated cells. These findings suggest that the 5-HT-induced enhancement of cyclic AMP accumulation in amitriptyline-treated cells may occur through the activation of a 5-HT receptor subtype that is positively coupled to adenylyl cyclase. Such a 5-HT receptor subtype in NG 108-15 cells has not been described pre-

Table 2 Effect of amitriptyline treatment on cholera toxin-, prostaglandin E_1 -, GTP γ S-, NaF-, and Mn^{2+} -stimulated cyclic AMP accumulation in NG 108-15 cells and isolated membranes

	Cyclic AMP (pmol mg^{-1} protein)	
	Control	Amitriptyline-treated
Intact cells		
Basal	86.9 $\pm$ 7.5	95.5 $\pm$ 8.8
Cholera toxin 1 μg ml^{-1}	349.0 $\pm$ 22.7	324.4 $\pm$ 30.3
Prostaglandin E_1 0.1 μM	318.6 $\pm$ 11.9	292.7 $\pm$ 18.8
+ 5-HT 10 μM	291.8 $\pm$ 10.6	296.1 $\pm$ 7.5
Membrane		
Basal	162.7 $\pm$ 18.2	209.4 $\pm$ 15.9
GTP γ S 10 μM	1326.8 $\pm$ 71.6	1540.4 $\pm$ 90.0
+ 5-HT 10 μM	1374.8 $\pm$ 78.1	1575.4 $\pm$ 73.0
Forskolin 0.1 μM	786.0 $\pm$ 29.7	977.8 $\pm$ 78.8*
+ 5-HT 10 μM	842.6 $\pm$ 45.1	1218.1 $\pm$ 52.2**
NaF 10 mM	755.2 $\pm$ 99.2	794.4 $\pm$ 24.2
$MnCl_2$ 20 mM	4173.3 $\pm$ 554.8	4949.5 $\pm$ 383.9

Cells were treated with or without 8 μM amitriptyline for 3 days. The media were replaced with HEPES-buffered Krebs-Ringer solution, and the cells were incubated with 1 μg ml^{-1} cholera toxin for 30 min, or 0.1 μM prostaglandin E_1 , or prostaglandin E_1 plus 10 μM 5-HT for 10 min at 37°C. Membranes were prepared as described in Methods, and were incubated with 10 μM GTP γ S, 20 mM NaF, 0.1 μM forskolin, or 20 mM $MnCl_2$ for 10 min at 37°C. Data represent the mean $\pm$ s.e.mean values of three separate experiments. * P < 0.05. ** P < 0.01 versus control.

Table 3 Effect of chronic treatment with pindolol or ICS 205-930 in the presence or absence of amitriptyline on regulation by 5-hydroxytryptamine (5-HT) of forskolin-stimulated cyclic AMP accumulation

	Cyclic AMP level (% of control)	
	Forskolin	Forskolin + 5-HT
Control	100	100
Pindolol 10 μM	116 $\pm$ 8	108 $\pm$ 10
ICS 205-930 10 μM	117 $\pm$ 10	84 $\pm$ 4
Amitriptyline 8 μM	167 $\pm$ 11**	231 $\pm$ 14**
+ Pindolol	162 $\pm$ 11**	220 $\pm$ 12**
+ ICS 205-930	161 $\pm$ 8**	208 $\pm$ 10**

Cells were treated with or without 10 μM pindolol or 10 μM ICS 205-930 in the presence or absence of 8 μM amitriptyline for 3 days, and then incubated with 0.1 μM forskolin alone, or forskolin plus 10 μM 5-HT. Data represent percentages of the control values obtained in forskolin alone or forskolin plus 5-HT and are mean $\pm$ s.e.mean values of three separate experiments. ** P < 0.01 versus control.

viously. However, we observed that 5-MeOT increased basal cyclic AMP levels in the absence of forskolin (data not shown), suggesting the existence of a 5-HT receptor subtype positively coupled to adenylyl cyclase in NG 108-15 cells. The fact that 5-HT alone had no effect on cyclic AMP levels may be due to coincident stimulation and inhibition of adenylyl cyclase by 5-HT.

The presence of 5-HT-induced stimulation of cyclic AMP accumulation suggests the involvement of a 5-HT $_4$ receptor (Bockaert *et al.*, 1992). Several studies have indicated that the 5-HT $_4$ receptor positively coupled to adenylyl cyclase is neither a member of the 5-HT $_1$ nor the 5-HT $_2$ receptor families, and that it exists in various tissues, including nervous tissue (Ford & Clarke, 1993). In mouse collicular neurones and guinea-pig hippocampal membranes, the 5-HT $_4$ receptor-mediated activation of adenylyl cyclase was not blocked by antagonists for the 5-HT $_{1A}$, 5-HT $_{1C}$, 5-HT $_{1D}$, 5-HT $_2$, or 5-HT $_3$ receptors, with the exception of ICS 205-930, which blocked the 5-HT $_4$ receptor-mediated effect with low

Figure 6 Effect of imipramine, mianserin or paroxetine treatment on regulation by 5-hydroxytryptamine (5-HT) of forskolin-stimulated cyclic AMP accumulation in NG 108-15 cells. Cells were treated with either 8 μM imipramine, 8 μM mianserin, or 8 μM paroxetine for 3 days. Then the cells were incubated with vehicle (open columns), 0.1 μM forskolin (hatched columns), or forskolin plus 10 μM 5-HT (solid columns) for 10 min at 37°C. Data are expressed as percentages of the values obtained with vehicle, forskolin alone or forskolin plus 5-HT of control cells, and are mean $\pm$ s.e.mean values for three separate experiments. * P < 0.05, ** P < 0.01 versus control.

potency (Shenker *et al.*, 1987; Dumuis *et al.*, 1988; Bockaert *et al.*, 1990). The finding that 5-HT-induced enhancement of cyclic AMP accumulation in amitriptyline-treated cells was not inhibited by pindolol, spiperone, or SCH 23390, is consistent with the pharmacological profile of the 5-HT $_4$ receptor. However, the relatively low potency of ICS 205-930 and GR 113808 on the 5-HT-induced enhancement precludes the involvement of the 5-HT $_4$ receptor. We attempted to measure 5-HT $_4$ receptor binding in membranes from NG 108-15 cells using [3H]-GR 113808 (Grossman *et al.*, 1993). However, little specific binding of [3H]-GR 113808 was observed in either control or amitriptyline-treated membranes when either 5-HT or GR 113808 were used to define nonspecific binding (data not shown). This inability to measure specific binding to 5-HT $_4$ receptors may be due to a low density of these receptors on NG 108-15 cells.

On the other hand, there are a number of reports describing other 5-HT receptors positively coupled to adenylyl cyclase, which are pharmacologically different from the 5-HT $_4$ receptor, such as: the 5-HT $_6$ receptor (Monsma *et al.*, 1993), 5-HT $_7$ receptor (Bard *et al.*, 1993), neuroblastoma NCB20 5-HT receptor (Conner & Mansour, 1990), and mouse 5-HT $_4$ receptor (Plassat *et al.*, 1993). Interestingly, some of these receptors exhibit relatively high affinity for psychotropic drugs such as clozapine, amoxipine, or amitriptyline (Monsma *et al.*, 1993; Plassat *et al.*, 1993). More recently, 5-HT-mediated adenylyl cyclase activation has been observed in mouse neuroblastoma N18TG2 cells, and it exhibited a pharmacological profile resembling some aspects of the 5-HT $_6$ receptor (Unsworth & Molinoff, 1994). Since NG 108-15 cells are derived by fusing cells from N18TG2 cells and C6Bu-1 cells, it is possible that the same kind of 5-HT receptors found in N18TG2 cells may also exist in NG 108-15 cells.

We investigated whether the 5-HT-induced enhancement of cyclic AMP accumulation caused by amitriptyline treatment was due to decreased activation of a 5-HT receptor-G $_i$ protein pathway. We found that the inhibition of forskolin-stimulated cyclic AMP accumulation by 8-OH-DPAT (an agonist for G $_i$ protein-coupled 5-HT receptor) was not affected by amitriptyline treatment. This is consistent with previous reports that 5-HT $_{1A}$ receptor-induced inhibition of adenylyl cyclase is not altered by chronic desipramine treatment in rat forebrain and hippocampus (De Montigny & Aghajanian, 1978; Varraut *et al.*, 1991). We also found that amitriptyline treatment did not modify carbachol-, clonidine-, or NA-induced inhibition of forskolin-stimulated cyclic AMP

accumulation. Moreover, pretreatment with 100 ng ml⁻¹ pertussis toxin had no effect on the 5-HT enhancement of forskolin-stimulated cyclic AMP accumulation in either control or amitriptyline-treated cells. These results indicate that a 5-HT receptor coupled to G_i proteins does not participate in the 5-HT-induced enhancement of cyclic AMP accumulation in amitriptyline-treated cells.

It is possible that chronic amitriptyline treatment produces the 5-HT-induced enhancement through an increase in G_s protein activity. However, the present study shows that the cyclic AMP accumulation produced by cholera toxin and NaF was not affected by amitriptyline treatment. The GTPγS-stimulated cyclic AMP accumulation was slightly, but not significantly, increased in amitriptyline-treated membranes. These findings indicate that chronic amitriptyline treatment may not induce quantitative or functional changes in G_s proteins. Furthermore, PGE₁-stimulated cyclic AMP accumulation was also unaffected by amitriptyline treatment. Taken together, it is most likely that the 5-HT-induced enhancement of forskolin-stimulated cyclic AMP accumulation in amitriptyline-treated cells is due to modifications at the level of the 5-HT receptor rather than at the level of the G_s protein.

The present study shows that chronic amitriptyline treatment of NG 108-15 cells caused an increase in forskolin-stimulated cyclic AMP accumulation. This is consistent with previous reports that demonstrate that chronic antidepressant treatment enhances forskolin-stimulated cyclic AMP production in rat cerebral cortex and limbic system (Ozawa & Rasenick, 1989; De Montis *et al.*, 1990; Jolas *et al.*, 1994). Our results also showed that Mn²⁺-induced adenylyl cyclase activation was not modified by amitriptyline treatment. In isolated membranes of NG 108-15 cells, [³H]-forskolin binding seems to be biphasic, with one binding site exhibiting a high affinity ($K_d = 18$ nM) for the complex of adenylyl cyclase and G_sα proteins (Kim *et al.*, 1994). At a concentration of 0.1 μM (as used in this study), forskolin predominantly recognizes the high affinity binding site, indicating that the increase of forskolin-stimulated cyclic AMP accumulation by amitriptyline treatment may reflect an increase of the complex of adenylyl cyclase and G_s proteins rather than an increase in the population of the cyclase. In fact, Ozawa & Rasenick (1989) suggested that chronic *in vivo* treatment with antidepressants induces an increased coupling between G_s proteins and the catalytic subunits of adenylyl cyclase in rat cerebral cortex. This increased coupling between G_s proteins and adenylyl cyclase could contribute to the G protein-mediated stimulation of adenylyl cyclase. However, we did not find significant increases in GTPγS- and NaF-stimulated cyclic AMP accumulation in amitriptyline-treated membranes. With regard to this discrepancy, it should be considered that forskolin acts on adenylyl cyclase by G protein-mediated mechanisms unlike Mn²⁺ (Seamon *et al.*, 1981) and that the interaction between G_s proteins and adenylyl cyclase is complicated, e.g., multiple subtypes of α subunit with differential coupling efficiencies to different isoforms of adenylyl cyclase (Tang *et al.*, 1991; Tang & Gilman, 1992; Ishikawa *et al.*, 1993). Nonetheless, it is still questionable that the 5-HT-induced enhancement in amitriptyline-treated

cells occurs only in the presence of forskolin. However, it is notable that treatment with lower concentrations of amitriptyline (<2 μM) causes 5-HT to enhance significantly forskolin-stimulated cyclic AMP accumulation despite an unchanged forskolin response. This result indicates that amitriptyline treatment may exert its effects on 5-HT receptor function and the forskolin response through distinctly different mechanisms.

If chronic amitriptyline treatment increases 5-HT receptor function, it is possible that the 5-HT receptors are up-regulated due to receptor antagonism by amitriptyline. However, chronic treatment with pindolol or ICS 205-930 did not cause the enhancement of cyclic AMP accumulation by 5-HT. Neither pindolol nor ICS 205-930 affected 5-HT-induced enhancement of cyclic AMP accumulation produced by amitriptyline treatment. Thus, it is not likely that the effect of amitriptyline treatment is due to 5-HT receptor antagonism.

Chronic treatment with either the tricyclic antidepressant, imipramine, or other types of antidepressants such as mianserin and paroxetine, mimicked the effects of amitriptyline. Mianserin, a weak monoamine uptake inhibitor, also caused 5-HT to enhance forskolin-stimulated cyclic AMP accumulation, suggesting that the blockade of monoamine reuptake may not be involved in the 5-HT-induced enhancement of cyclic AMP accumulation. The therapeutic plasma concentrations of tricyclic antidepressants are estimated at about 0.5–1 μM under normal conditions, rising to >3 μM during an overdose (Braithwaite *et al.*, 1979; Friedel, 1982). The present study demonstrates that 5-HT can significantly potentiate cyclic AMP accumulation in the presence of similarly low antidepressant concentrations. This suggests that the effect of antidepressants on 5-HT-modulated cyclic AMP production may contribute to some of the therapeutic or adverse effects of antidepressants.

In the present study, we demonstrated that chronic amitriptyline treatment of NG 108-15 cells caused 5-HT to enhance forskolin-stimulated cyclic AMP accumulation, and that this enhancement was unaffected by pretreatment with pertussis toxin and insensitive to 5-HT₁ and 5-HT₂ receptor antagonists. In contrast, chronic amitriptyline treatment did not affect 8-OH-DPAT-induced inhibition of cyclic AMP accumulation. Cyclic AMP production by direct stimulation of G proteins and adenylyl cyclase was also unaffected by amitriptyline treatment. These results suggest that this 5-HT-induced enhancement of cyclic AMP accumulation may be due to increased activity of the 5-HT receptor that is positively coupled to adenylyl cyclase, and that this increased activity may result from the changes at the level of the 5-HT receptor rather than at the level of the G_s proteins or adenylyl cyclase. It is unlikely that the enhancement of cyclic AMP accumulation is caused by a reduction in the inhibition of adenylyl cyclase or by long-term antagonism of the 5-HT receptor by amitriptyline.

We are grateful to Dr H. Higashida of Kanazawa University and Dr A. Kagaya of Hiroshima University for providing NG 108-15 cells.

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(Received August 22, 1994)

Revised November 4, 1994

Accepted November 25, 1994)