Pharmacological Characterization of an Adenylyl Cyclase-Coupled 5-HT Receptor in Aplysia: Comparison With Mammalian 5-HT Receptors

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Cohen, Jonathan E., Chiadi U. Onyike, Virginia L. McElroy, Allison H. Lin, and Thomas W. Abrams. Pharmacological characterization of an adenylyl cyclase-coupled 5-HT receptor in Aplysia: comparison with mammalian 5-HT receptors. J Neurophysiol 89: 1440–1455, 2003. First published November 20, 2002; 10.1152/jn.01004.2002. We attempted to identify compounds that are effective in blocking the serotonin (5-hydroxytryptamine, 5-HT) receptor(s) that activate adenylyl cyclase (AC) in Aplysia CNS. We call this class of receptor 5-HTapAC. Eight of the 14 antagonists tested were effective against 5-HTapAC in CNS membranes with the following rank order of potency: methiothepin > metergoline > fluphenazine > clozapine > cyproheptadine > risperidone > ritanserin > NAN-190, GR-113808, olanzapine, Ro-04-6790, RS-102221, SB-204070, and spiperone were inactive. Methiothepin completely blocked 5-HT stimulation of AC with a Ki of 18 nM. Comparison of the pharmacological profile of the 5-HTapAC receptor with those of mammalian 5-HT receptor subtypes suggested it most closely resembles the 5-HT6 receptor. AC stimulation in Aplysia sensory neuron (SN) membranes was also blocked by methiothepin. Methiothepin substantially inhibited two effects of 5-HT on SN firing properties that are mediated by a AMP-dependent reduction in S-K+ current: spike broadening in tetraethylammonium/nifedipine and increased excitability. Consistent with cyproheptadine blocking 5-HT stimulation of AC, cyproheptadine also blocked the 5-HT-induced increase in SN excitability. Methiothepin was less effective in blocking AC-mediated modulatory effects of 5-HT in electrophysiological experiments on SNs than in blocking AC stimulation in CNS or SN membranes. This reduction in potency appears to be due to effects of the high ionic strength of physiological saline on the binding of this antagonist to the receptor. Methiothepin also antagonized AC-coupled dopamine receptors but not AC-coupled small cardioactive peptide receptors. In conjunction with other pharmacological probes, this antagonist should be useful in analyzing the role of 5-HT in various forms of neuromodulation in Aplysia.

INTRODUCTION

Serotonin (5-hydroxytryptamine, 5-HT) is an important modulatory neurotransmitter in the gastropod mollusk Aplysia californica, playing an important role in such behaviors as feeding (Rosen et al. 1989), locomotion (Mackey and Carew 1983), and the defensive withdrawal reflexes (Glanzman et al. 1989). 5-HT is released by behavioral stimuli that initiate nonassociative and associative learning (Mackey et al. 1989; Marinesco and Carew 2001) and triggers the alteration of properties of mechanosensory neurons (SNs) that provide different input to the circuits for the defensive gill, siphon, and tail withdrawal reflexes (Byrne and Kandel 1996). 5-HT stimulation of adenylyl cyclase (AC) acts via cAMP-dependent protein kinase to increase SN excitability and spike duration (Baxter and Byrne 1990; Goldsmith and Abrams 1992; Hochner and Kandel 1992; Klein et al. 1986) and to produce short-, intermediate-, and long-term facilitation of the synaptic connections between these SNs and postsynaptic neurons (Ghirardi et al. 1992, 1995; Schacher et al. 1988; Scholz and Byrne 1988). 5-HT activation of protein kinase C (PKC) and mitogen-activated protein kinase also contributes to facilitation of SN synapses (Braha et al. 1993; Byrne and Kandel 1996; Manseau et al. 2001; Martin et al. 1997; Sacktor et al. 1988; Sugita et al. 1994).

In studying the neuromodulatory roles of multiple 5-HT-activated second-messenger cascades, it would be advantageous to have selective pharmacological antagonists for the 5-HT receptors that activate AC in Aplysia CNS. In contrast, the 5-HT receptor antagonist cyproheptadine is widely used in Aplysia but affects multiple 5-HT receptor subtypes (Goldsmith and Abrams 1992; Sossin et al. 1994). To date, five G-protein-coupled 5-HT receptors have been cloned from Aplysia. Two of these, Ap5-HTB1 and Ap5-HTB2, are coupled to phospholipase C (PLC) (Li et al. 1995). Two other 5-HT receptors, 5-HTap1 and 5-HTap2, inhibit AC (Angers et al. 1998; Barbas et al. 2002). A fifth Aplysia 5-HT receptor, for which a partial cDNA clone has been obtained, is strongly expressed in the gill and weakly expressed in the CNS (Williams et al. 1997). 5-HT receptors that activate AC have not yet been cloned in Aplysia or any other gastropod mollusc (see Tierney 2001). We therefore pharmacologically characterized, in biochemical assays, the 5-HT receptor(s) that activate AC in

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At pH 7.3, 100 M H9262 mediated, 5-HT-dependent neuromodulation in receptors, methiothepin should be useful in studying cAMP-multiple 5-HT receptors. However, in conjunction with spiperone, which is more effective in inhibiting the 5-HT receptor subtype. Of the 14 compounds tested, methiothepin, a dibenzapine, was the most effective in inhibiting selective for specific mammalian 5-HT receptor subtypes. The pharmacology of the 5-HT receptor in Aplysia CNS that activates AC resembled most closely the pharmacology of the 5-HT receptor subtype. Of the 14 compounds tested, methiothepin, a dibenzapine, was the most effective in inhibiting 5-HT stimulation of AC. Unfortunately, methiothepin inhibits AC. The 5-HT receptor subtypes activate PLC. A seventh receptor subtype, 5-HT3, forms a nonselective cationic channel. Using 5-HT stimulation of AC in Aplysia CNS membranes as an assay, we tested nonselective high-affinity antagonists as well as several antagonists that are highly selective for specific mammalian 5-HT receptor subtypes. The pharmacology of the 5-HT receptor in Aplysia CNS that activates AC resembled most closely the pharmacology of the 5-HT receptor subtype. Of the 14 compounds tested, methiothepin, a dibenzapine, was the most effective in inhibiting 5-HT stimulation of AC. Unfortunately, methiothepin inhibits multiple 5-HT receptors. However, in conjunction with spiperone, an antagonist selective for the PLC-coupled 5-HT receptors, methiothepin should be useful in studying cAMP-mediated, 5-HT-dependent neuromodulation in Aplysia.

**Methods**

Aplysia CNS. To assess the efficacy of these antagonists under physiological conditions, we also examined the 5-HT-induced cAMP-dependent modulation of the electrophysiological properties of SNs.

Recently, selective, high-affinity ligands have been developed for most known subtypes of mammalian 5-HT receptors (Bonhaus et al. 1997; Roth et al. 1994; Sleigh et al. 1998; Wardle et al. 1994). Six subtypes of G-protein-coupled 5-HT receptors have been characterized in mammals (Hoyer and Martin 1997; Hoyer et al. 1994). Five of these receptor subtypes are coupled to AC: the 5-HT1A, 5-HT1B, and 5-HT3 receptors, which activate AC, and the 5-HT1C and 5-HT3 receptors, which inhibit AC. The 5-HT2 receptor subtypes activate PLC. A seventh receptor subtype, 5-HT3, forms a nonselective cationic channel. Using 5-HT stimulation of AC in Aplysia CNS membranes as an assay, we tested nonselective high-affinity antagonists as well as several antagonists that are highly selective for specific mammalian 5-HT receptor subtypes. The pharmacology of the 5-HT receptor in Aplysia CNS that activates AC resembled most closely the pharmacology of the 5-HT receptor subtype. Of the 14 compounds tested, methiothepin, a dibenzapine, was the most effective in inhibiting 5-HT stimulation of AC. Unfortunately, methiothepin inhibits multiple 5-HT receptors. However, in conjunction with spiperone, an antagonist selective for the PLC-coupled 5-HT receptors, methiothepin should be useful in studying cAMP-mediated, 5-HT-dependent neuromodulation in Aplysia.

**Preparation of tissue for AC assays**

For whole CNS membranes, trimmed ganglia were homogenized in a glass-raise homogenizer in homogenization buffer: 50 mM K-HEPES (pH 7.6), 75 mM KCl, 3 mM EGTA, 1 mM diethiothreitol (DTT), and protease inhibitors (10 μg/ml aprotinin, 1 mM benzamidine, 10 μg/ml leupeptin, and 25 μg/ml p-nitrophenyl-p'-guanidinobenzoate hydrochloride; 0.8 ml buffer/CNS). Any residual sheath was removed, and the material was homogenized in a glass-Teflon homogenizer. For SN membranes, the VC cluster was dissected from 18 deheaded pleural ganglia and homogenized in a glass-Teflon homogenizer in 1.5 ml homogenization buffer. All processing of membranes was at 0°C; centrifugations were at 4°C. The CNS or SN homogenate was centrifuged at 1,000 g for 2 min to remove any crude particulate material. The supernatant was then centrifuged at 16,000 g for 20 min. The pellet was resuspended in homogenization buffer and recentrifuged. The final pellet was homogenized in resuspension buffer [50 mM K-HEPES, pH 7.6, 75 mM KCl, 1 mM DTT and protease inhibitors (described in the preceding text)] (500 μl/CNS and 380 μl/18 SN clusters) and then assayed immediately.

**AC assays**

Assays of AC activity in membrane preparations were carried out in 80 μl for most experiments on CNS and in 60 μl for dose-response experiments and SN experiments. Assay times were 5 min for CNS or 8 min for SNs. Assay temperature was 30°C; this higher-than-physiological temperature increases product synthesis, making assays more reliable. AC assay solution included 10 μM [32P]-ATP (25 μCi/ml in whole CNS assays and 400 μCi/ml in SN assays), 50 μM [3H]-cAMP (∼3 × 106 cpm/ml), 10 μM GTP, 2.5 U/ml creatine phosphokinase, 5 mM creatine phosphate, 0.5 mM IBMX, 3 mM MgCl2, 75 mM KCl, 250 μM EGTA, 50 mM K-HEPES (pH 7.6), 1 mM DTT, and protease inhibitors (described in the preceding text). This buffer was designed to produce a total ionic strength of ~100 mM for standard biochemical assays, which is approximately sixfold lower than in Aplysia tissues. Assays were terminated by addition of unlabeled ATP and cAMP, plus sodium lauryl sulfate (Salomon 1979). Cyclic AMP was separated from precursor ATP as described by Salomon (1979). The [3H]-cAMP enabled normalization for recovery after chromatography. Perfused membrane AC assays were conducted as previously described (Jarrard et al. 1993); assay buffer was the same as in steady-state assays, except with 15 μCi/ml [32P]-ATP. Radioimmunoassays (RIAs) for cAMP were performed using a cAMP RIA kit (Biomedical Technologies, Stoughton, MA) according to the manufacturer’s instructions.

**Preparation of tissue for radioligand binding assays**

The membranes were prepared as in the AC assays except that homogenization of CNSs was in 2 mM Tris-Cl (pH 7.3) with 0.7 M sucrose and protease inhibitors (described in the preceding text), and membranes were thoroughly washed in a series of four 30 min centrifugations at 47,000 g. Pellets were resuspended in 2 mM Tris-Cl, 5 mM K-HEPES, 1 mM EGTA, 1 mM diethiothreitol (DTT), 1 μM aprotinin, 1 mM benzamidine, 10 μg/ml leupeptin, and 25 μg/ml p-nitrophenyl-p'-guanidinobenzoate hydrochloride; 0.8 ml buffer/CNS). Any residual sheath was removed, and the material was homogenized in a glass-Teflon homogenizer. For SN membranes, the VC cluster was dissected from 18 deheaded pleural ganglia and homogenized in a glass-Teflon homogenizer in 1.5 ml homogenization buffer. All processing of membranes was at 0°C; centrifugations were at 4°C. The CNS or SN homogenate was centrifuged at 1,000 g for 2 min to remove any crude particulate material. The supernatant was then centrifuged at 16,000 g for 20 min. The pellet was resuspended in homogenization buffer and recentrifuged. The final pellet was homogenized in resuspension buffer [50 mM K-HEPES, pH 7.6, 75 mM KCl, 1 mM DTT and protease inhibitors (described in the preceding text)] (500 μl/CNS and 380 μl/18 SN clusters) and then assayed immediately.

**Preparation of AC assays**

For whole CNS membranes, trimmed ganglia were homogenized in a glass-raise homogenizer in homogenization buffer: 50 mM K-HEPES (pH 7.6), 75 mM KCl, 3 mM EGTA, 1 mM diethiothreitol (DTT), and protease inhibitors (10 μg/ml aprotinin, 1 mM benzamidine, 10 μg/ml leupeptin, and 25 μg/ml p-nitrophenyl-p'-guanidinobenzoate hydrochloride; 0.8 ml buffer/CNS). Any residual sheath was removed, and the material was homogenized in a glass-Teflon homogenizer. For SN membranes, the VC cluster was dissected from 18 deheaded pleural ganglia and homogenized in a glass-Teflon homogenizer in 1.5 ml homogenization buffer. All processing of membranes was at 0°C; centrifugations were at 4°C. The CNS or SN homogenate was centrifuged at 1,000 g for 2 min to remove any crude particulate material. The supernatant was then centrifuged at 16,000 g for 20 min. The pellet was resuspended in homogenization buffer and recentrifuged. The final pellet was homogenized in resuspension buffer [50 mM K-HEPES, pH 7.6, 75 mM KCl, 1 mM DTT and protease inhibitors (described in the preceding text)] (500 μl/CNS and 380 μl/18 SN clusters) and then assayed immediately.
pH 7.3, without sucrose. The pellet after the first centrifugation was sonicated for 60 s (on ice) to remove any residual 5-HT. The final pellet was resuspended in 120 μg/CNS and aliquots stored in liquid nitrogen.

**Radioligand binding assays**

Binding experiments with 0.2 nM d-[^125]I]-lysergic acid diethylamide (LSD) (2200 Ci/mmol, Dupont NEN) were performed with 25–50 μg of protein/sample either in 55 mM Tris-Cl, pH 7.3, with 5 mM MgSO₄ (low salt), or in (in mM) 460 NaCl, 10 KCl, 10 CaCl₂, 55 MgCl₂, and 10 Na-HEPES, pH 7.3, (physiological salt), in a final volume of 60 μl, at 37°C for 60 min. The binding reaction contained 10 μM paragpine (RBI, MA) and 1.8 mM ascorbic acid (JT Baker, Phillipsburg, NJ). LSD acts as a partial agonist at both 5-HT and DA receptors in Aplysia CNS (Drummond et al. 1980; Southall et al. 1997). To eliminate LSD binding to DA receptors, experiments were carried out in the presence of 300 μM cold DA. Nonspecific binding was defined as counts remaining in the absence of 10 μM cold LSD. Binding reactions were terminated by addition of 3 ml of wash buffer (50 mM Tris-HCl, pH 7.3) and rapid filtration under vacuum over Whatman GF/B glass fiber filters, pretreated for 30 min with 0.5% polyethylenimine (Sigma, St. Louis, MO) and 0.5% nonfat dry milk. Filters were rinsed with 3 × 10 ml of wash buffer and counted.

**Electrophysiology**

Desheathed pleural ganglia were secured with minute pins on wax in a recording chamber. Preparations were studied at room temperature. In experiments on excitability, ganglia were superfused (at room temperature) with normal culture medium (in mM): 460 NaCl, 10 KCl, 11 CaCl₂, 55 MgCl₂, and 10 Na-HEPES, pH 7.6, supplemented with nutrients [7 mM glucose, MEM essential and nonessential amino acids (0.2 × normal concentration, GIBCO Invitrogen, Carlsbad, CA), and MEM vitamin solution (0.7 × normal concentration, GIBCO Invitrogen)]. In experiments on spike broadening, ganglia were superfused with high Mg²⁺/high Ca²⁺ culture medium (6 × normal Ca²⁺, 1.6 × normal Mg²⁺) (Goldsmith and Abrams 1991) to reduce spontaneous activity from modulatory interneurons (in mM) 328 NaCl, 10 KCl, 66 CaCl₂, 88 MgCl₂, and 10 Na-HEPES, pH 7.6, supplemented with the same nutrients. The high-divalent culture medium does not alter transmitter release from the siphon SNs as compared with normal culture medium (Jiang and Abrams 1998) nor of the spike duration in TEA (B. A. Goldsmith and T. W. Abrams, unpublished results), suggesting that Ca²⁺ influx to SNs during action potentials is not altered. Spike broadening measurements were conducted in the presence of 100 mM TEA and 20 mM nifedipine (diluted 1,000-fold from a fresh nifedipine stock in DMSO).

SNs in the VC cluster were penetrated with 10–20 MΩ glass microelectrodes filled with 2 M K-acetate/400 mM KCl. Data for spike duration and excitability were obtained in parallel from two SNs in the VC cluster. The two stimulus intensities were alternated every three to four stimuli. SNs were first exposed to 1 μM 5-HT followed by 5-HT plus either 100 μM methiothepin or 200 μM cyproheptadine. Electrophysiological data were acquired digitally with a Modular Instruments interface and analyzed using Spike software (Hilal Associates, Englewood Cliffs, NJ).

**Calculation of effects of antagonists on AC activity**

Normalized stimulation of AC activity, expressed as percentage above basal activity was calculated, in the absence of antagonist, as

\[
\text{AC activity with transmitter} - \frac{\text{basal AC activity}}{\text{AC activity with antagonist & transmitter}} \times 100 \quad (1)
\]

or in the presence of antagonist, as

\[
\frac{\text{AC activity with antagonist & transmitter}}{\text{AC activity with antagonist without transmitter}} \times 100 \quad (2)
\]

In these equations, basal activity is defined as activity in the absence of exogenous transmitter. In Eq. 2, stimulation with antagonist is normalized to the same control basal activity (without antagonist) as in Eq. 1; thus any decrease in basal activity produced by an antagonist (without a change in fold-stimulation) is reflected as a reduction in stimulation. Transmitter stimulation of AC in the presence of antagonist as a percent of control stimulation was calculated as

\[
\frac{\text{AC activity with antagonist & transmitter}}{\text{AC activity with transmitter} - \text{basal AC activity}} \times 100 \quad (3)
\]

(i.e., the ratio of Eq. 2 to Eq. 1). The inhibition of transmitter stimulation by antagonist is equal to this value subtracted from 100%.

**Data analysis**

Dose-response and dose-inhibition data were first normalized to basal activity and then normalized to maximal response within each assay. The assumption of simple competition giving a slope of one was confirmed with a Schild plot (Arunlakshana and Schild 1959). Statistical tests were performed using SPSS software (SPSS, Chicago, IL). Multivariate ANOVA, using a repeated-measures design for comparisons within preparations, was followed by post hoc pairwise comparisons with Bonferroni adjustment for multiple comparisons. Pearson correlation analysis for 5-HT receptors were performed using published inhibition data for mammalian 5-HT receptors.

**RESULTS**

**Comparison of the effectiveness of diverse 5-HT receptor antagonists at the AC-coupled 5-HT receptor in Aplysia CNS**

We began this study by examining how 5-HT stimulation of AC in Aplysia CNS was affected by antagonists selective for subtypes of mammalian 5-HT receptors. Although Aplysia hemolymph has a very high ionic strength, these AC assays were conducted in buffer with 100 mM ionic strength; the use of conventional biochemistry buffer enabled the pharmacological sensitivity of the Aplysia receptor to be compared with the pharmacology of mammalian 5-HT receptors. Stimulation of AC in CNS membranes was 117 ± 7% with 1 μM 5-HT and 217 ± 13% with 25 μM 5-HT (n = 33; means ± SE; stimulation expressed as percent above AC activity in the absence of exogenous transmitter). One micromolar 5-HT produces approximately half-maximal activation of AC, whereas
25 μM 5-HT produces near maximal activation. All selective antagonists tested were inactive: the selective 5-HT₁A receptor antagonist NAN-190 (Glennon et al. 1988), the selective 5-HT₂C receptor antagonist RS-102221 (Bonhaus et al. 1997), the selective 5-HT₄ receptor antagonists, GR-113808 and SB-204070 (Grossman et al. 1993; Wardle et al. 1994), and the selective 5-HT₆ antagonist Ro-04-6790 (Sleight et al. 1998) (Fig. 1). Olanzapine, a high-affinity antagonist for 5-HT₂ and 5-HT₃ receptors (Fuller and Snoddy 1992; Roth et al. 1994), was also inactive. All compounds were tested at 10 μM, which is at least three orders of magnitude above the Kᵢ’s for the corresponding most sensitive mammalian 5-HT receptor subtype.

We next tested a number of less specific antagonists. Spiperone, which blocks the PLC-coupled 5-HT receptor from Aplysia CNS, Ap5-HTB₂, had no affect on 5-HT stimulation of AC. Clozapine, cyproheptadine, ritanserin (Ocorr and Byrne 1986), and risperidone were somewhat effective in inhibiting 5-HT stimulation of AC (Fig. 1). This antagonism was partially or fully surmountable with 25 μM 5-HT. Metergoline, fluphenazine, and methiothepin were the most effective antagonists. Metergoline produced 84 ± 7 and 66 ± 10% (n = 4) inhibition of AC stimulation by 1 and 25 μM 5-HT, respectively (Fig. 1). Fluphenazine produced 91 ± 1% inhibition of AC stimulation by 1 μM 5-HT and 58 ± 4% inhibition of AC stimulation by 25 μM 5-HT (n = 5). Methiothepin was the most active antagonist tested, producing 99.9 ± 1.0 and 95 ± 1% inhibition of AC stimulation by 1 and 25 μM 5-HT, respectively (n = 19; see figure legends for these and most other statistical results.)

Five of the active antagonists, risperidone, ritanserin, clozapine, fluphenazine, and methiothepin caused a decrease in AC activity in the absence of exogenous 5-HT (reducing AC activity on average by 39 ± 5% (n = 3), 12 ± 2% (n = 5), 39% ± 2% (n = 3), 26 ± 7% (n = 5), and 40 ± 4% (n = 19), respectively (Fig. 2A). Overall effects of antagonists on activity were highly significant; F(13,62) = 15.9, P < 0.001; individual probabilities from post hoc pairwise comparisons, with Bonferroni adjustment for multiple comparisons, P < 0.001 for each, except for ritanserin for which P < 0.05. Olanzapine, which produced a nonsignificant (22 ± 5%) reduction in AC stimulation by 5-HT (Fig. 1A), caused a significant reduction in AC activity in the absence of 5-HT (by 27 ± 5%, P < 0.001, n = 4). Cyproheptadine, at 10 μM, did not have a significant effect on AC activity in the absence of 5-HT; at 200 μM, cyproheptadine reduced AC activity in the absence of exogenous 5-HT by 19 ± 2% (n = 5, P < 0.01, paired t-test). The inactive compounds GR-113808, NAN-190, Ro-04-6790, RS-102221, SB-204070, and spiperone had no effect on activity in the absence of exogenous transmitter. In contrast to the other antagonists, metergoline was a partial agonist; 10 μM metergoline increased AC activity by 42 ± 10% (n = 4) in the absence of 5-HT (Fig. 2A).

To analyze the mechanism of the inhibition of basal AC activity, we focused on methiothepin because it had the largest effect. The decrease in basal AC activity could, in principle, be due to a nonspecific effect either on AC itself or on the stimulatory G protein, Gₛ. To determine whether methiothepin...
directly inhibited either AC or \( G_i \), independent of any effect on the 5-HT receptor, we examined whether methiothepin altered AC stimulation by small cardioactive peptide B (SCP\(_{B}\)), 5-HT, or SCP\(_{B}\) activate AC through independent receptors (Abrams et al. 1984; Ocorr and Byrne 1986). Whereas methiothepin and SCP\(_{B}\) activate AC through independent receptors (Abrams et al. 1984; Ocorr and Byrne 1986), 5-HT is plotted as a percent of basal activity (without either antagonists or 5-HT); activity is shown for control (without antagonists) and for methiothepin and metergoline. Methiothepin by itself significantly decreased, and metergoline by itself significantly increased, AC activity (\( P < 0.001 \) in each case). Antagonist concentration was 10 \( \mu \text{M} \). B: inhibition of AC activity by methiothepin in the absence of 5-HT was highly variable as compared with inhibition of 5-HT-stimulated AC activity. In each experiment, inhibition of AC activity in the absence of 5-HT is expressed as percent of control activity in the absence of exogenous transmitter, and inhibition of 5-HT-stimulated AC activity is expressed as percent of control 5-HT-stimulated activity. (Note that inhibition of activity in the absence of 5-HT or 5-HT-stimulated activity is expressed as a positive effect, (in contrast to Figs. 1 and 2A), such that 100% represents complete inhibition).

To directly test whether methiothepin was inhibiting AC activity stimulated by contaminating endogenous 5-HT, we performed a perfused-membrane assay on CNS membranes (Jarrard et al. 1993; Yovell et al. 1987). In this assay, homogenized CNS membranes are trapped on a filter and continuously perfused with large volumes of AC assay solution; because the filter chamber volume changes every 1.5 s, any endogenous 5-HT should be rapidly removed. With the perfused membrane assay, there was no detectable inhibition of activity in the absence of endogenous 5-HT by methiothepin (Fig. 4B). In contrast, in test-tube assays on this same CNS preparation, there was 40 \( \pm \) 2% (mean \( \pm \) SD, \( n = 5 \)) inhibition of AC activity (Fig. 4A). This lack of effect of methiothepin on basal AC activity in extensively washed membranes argues that contaminating 5-HT accounts for the inhibition of activity in the absence of endogenous 5-HT in the test-tube assays. These perfused membrane results also rule out the possibility that the inhibition of AC activity in the absence of exogenous 5-HT is due to activation of a \( G_i \)-coupled receptor by methiothepin.

If we assume that the AC activity in the presence of methiothepin represents the “true basal” activity in Aplysia CNS membranes, then total AC stimulation by 5-HT would actually be greater than the observed stimulation. When the minimum AC activity in each experiment determined in the presence of methiothepin is used as a measure of true basal activity (in Eq. 1), the total stimulation is 202 \( \pm \) 15% with 1 \( \mu \text{M} \) 5-HT and 366 \( \pm \) 24% with 25 \( \mu \text{M} \) 5-HT (\( n = 19 \)). The hypothesized contaminating endogenous 5-HT would at least partly explain why 5-HT stimulation is greater in perfused membrane assays.
that cyproheptadine inhibits the AC-coupled 5-HT receptor (Fig. 1). In dose-inhibition experiments, cyproheptadine inhibited 5-HT stimulation of AC with an IC$_{50}$ of 16 \( \mu \)M. Methiothepin was 31-fold more effective than cyproheptadine, inhibiting AC activity with an IC$_{50}$ of 510 nM \((n = 3;\) Fig. 5A). We also tested 200 \( \mu \)M cyproheptadine, which is the concentration that was used in the earlier electrophysiological studies; at 200 \( \mu \)M, cyproheptadine completely blocked AC stimulation by 1 \( \mu \)M 5-HT and inhibited by 85 \pm 1% AC stimulation by 25 \( \mu \)M 5-HT (Fig. 5B).

We performed a Schild analysis to determine the affinity of methiothepin for the AC-coupled 5-HT receptor. In dose-response experiments, methiothepin behaved as a competitive antagonist, with a \( K_b \) of 18 nM (Fig. 6). This value for the \( K_b \) than in steady-state test-tube assays (e.g., Figs. 4 and 5 in Jarrard et al. 1993).

Both methiothepin and cyproheptadine block all of the 5-HT receptors in CNS that activate AC

Cyproheptadine has been used in several electrophysiological studies in \textit{Aplysia} as an antagonist intended to be selective for specific 5-HT receptor subtypes in CNS (Emptage and Carew 1993; Mercer et al. 1991; Sun and Schacher 1996). However, published biochemical studies indicate there is no specificity. Sossin et al. (1994) found that cyproheptadine inhibited 5-HT-stimulated translocation of PKC, suggesting that it inhibits the PLC-coupled 5-HT receptor(s). Goldsmith and Abrams (1992) found that cyproheptadine inhibited 5-HT stimulation of AC in CNS and SN membranes. We confirmed

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**FIG. 4.** Extensive washing of CNS membranes eliminates inhibition of AC activity in the absence of 5-HT by methiothepin. AC activity in a single membrane preparation was assayed with 2 techniques: a conventional steady-state assay in test tubes and a perfused-membrane assay. \textit{A}: methiothepin at 10 \( \mu \)M produced 40 \pm 2% reduction in AC activity in steady-state assays on membranes conducted in test tubes. Data are the means \pm SD of 5 replicate assays. AC activity is expressed as pmoles cAMP synthesized \( \cdot \) mg protein \(^{-1} \) \( \cdot \) min \(^{-1} \). \textit{B}: methiothepin did not affect AC activity in a continuously perfused membrane preparation. AC activity is plotted before and during exposure to 10 \( \mu \)M methiothepin. Data are the means \pm SD of 3 replicate assays from the same membrane preparation as in \textit{A}. Membranes were trapped on a filter, washed with 30 ml of buffer to remove cytosol, and continuously perfused with \[^{32}\text{P}]-\text{ATP}-\text{containing assay solution; } 6-\text{s fractions (250} \mu\text{l}) were collected and the synthesized cAMP was chromatographically separated from the precursor ATP. AC activity is expressed as pmoles cAMP synthesized \( \cdot \) mg protein \(^{-1} \) \( \cdot \) min \(^{-1} \), adjusted for steady deterioration of AC activity in membranes on the filter measured prior to the onset of methiothepin exposure (\( \sim \)a 2.3% decrease in activity/6-\text{s interval; see Fig. 6 of Jarrard et al., 1993, for an example). For the assays in \textit{A} and \textit{B}, homogenized membranes homogenate from 6 CNSs was separated into 2 aliquots. One aliquot was centrifuged, and the membrane pellet washed to remove cytosol (as described in METHODS). The second aliquot, equivalent to the membranes from 3 CNSs, was divided into 3 portions, each of which was injected onto a filter and used for a single perfused-membrane assay.

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**FIG. 5.** Effects of methiothepin and cyproheptadine on 5-HT stimulation of AC in \textit{Aplysia} CNS. \textit{A}: dose-dependence of effects of methiothepin and cyproheptadine. Stimulation of AC activity by 5 \( \mu \)M 5-HT in the presence of antagonist is expressed as percent of 5-HT stimulation in the absence of antagonist. Data for methiothepin (\( \diamond \)) and cyproheptadine (\( \square \)) were obtained in separate experiments; in each experiment all concentrations of one antagonist were tested with a single membrane preparation. Each data point is the mean \pm SE for 3 experiments, each of which was conducted in quadruplicate. (Some of the error bars are smaller than the symbols.) Dose-inhibition data were fit with a logistic equation of the form

\[
\text{Response} = \frac{(R_{\max} - R_{\min})[1 + ([B]IC_{50})^n]}{1 + ([B]IC_{50})^n} + R_{\min}
\]

where \([B]\) is the concentration of antagonist, \( R_{\max} \) is the stimulation of AC in the absence of antagonist, \( R_{\min} \) is the stimulation of AC in the presence of the maximally effective concentration of antagonist, IC$_{50}$ is the concentration of antagonist giving half-maximal inhibition, and \( n \) is the Hill coefficient. \textit{B}: effect of 200 \( \mu \)M cyproheptadine on 5-HT stimulation of AC. 5-HT stimulation of AC activity was calculated according to Eqs. 1 and 2 (see METHODS, \( n = 5 \)).
for methiothepin agrees well with the value obtained by determining the shift in the dose-response relationship for 5-HT produced by 10 μM methiothepin, (Kᵦ = 23 ± 6 nM, mean of 15 experiments). Although we cannot be certain this represents a single AC-coupled receptor, we call this class of receptor 5-HT₅<sub>apAC</sub>.

Comparisons of the pharmacological sensitivities of the 5-HT₅<sub>apAC</sub> receptor and mammalian 5-HT receptors

We compared the antagonist profile of the 5-HT₅<sub>apAC</sub> class of receptors with those of mammalian 5-HT receptors by calculating Pearson correlations. Competitive dose-response curves were fitted to inhibition data from AC assays for 5-HT₅<sub>apAC</sub> and dissociation constants were estimated for each antagonist. We used published Kᵦ and Kᵦ<sub>d</sub> values for mammalian receptors (Table 1). Because all of the selective antagonists tested (GR-113808, NAN-190, olanzapine, Ro-04-6790, RS-102221, and SB-204070) had minimal affinity for the 5-HT₅<sub>apAC</sub> class of receptor, they would bias the correlation analysis against those five receptors for which highly selective antagonists existed; therefore we excluded the data for these selective antagonists. Instead we used data for those eight nonselective antagonists for which binding data are available for most receptor subtypes: clozapine, cyproheptadine, fluoxetine, mepatoline, mefoxthepin, risperidone, ritanserin, and spiperone. The Pearson correlation values between the 5-HT₅<sub>apAC</sub> receptor and the 5-HT₂<sub>A</sub>, 5-HT₂<sub>C</sub>, 5-HT₆, and 5-HT₇ receptors, were 0.29, 0.02, 0.49, 0.92, and 0.53, respectively. Thus the strongest correlation was observed between the 5-HT₅<sub>apAC</sub> receptor and the mammalian 5-HT₆ receptor. The correlations with the 5-HT₄ and 5-HT₅ receptors were not analyzed because for these receptors, information is not available about the affinity of many of these eight antagonists. However, the sensitivity of the 5-HT₅<sub>apAC</sub> receptor to the two most potent antagonists, methiothepin and metergoline, is substantially different from the sensitivities of the 5-HT₄ and 5-HT₅ receptors. Both methiothepin and metergoline are inactive at the 5-HT₄ receptor. At the 5-HT₅<sub>₂</sub> and 5-HT₅<sub>B</sub> receptors, methiothepin is active (with Kᵦ’s of 100 and 16 nM, respectively); however, metergoline is inactive at these receptors. To further assess whether the class of 5-HT₅<sub>apAC</sub> receptors resembles the 5-HT₄ receptor, we examined the relative sensitivity to the agonist 5-CT. At the 5-HT₄, 5-HT₂<sub>C</sub>, 5-HT₅<sup>a</sup>, and 5-HT₇ receptors, 5-CT is a more active agonist than 5-HT, whereas at the 5-HT₆ receptor, 5-HT is more active than 5-CT (Hirst et al. 1997; Hoyer et al. 1994). At the 5-HT₄ receptor, 5-CT is inactive. 5-CT was less potent than 5-HT in stimulating AC in Aplysia CNS; EC₅₀ values were 1.1 and 13 μM for 5-HT and 5-CT, respectively (Fig. 7). Thus in its sensitivity to both agonists and antagonists, the 5-HT₅<sub>apAC</sub> class of receptor most closely resembles the mammalian 5-HT₆ receptor.

**Table 1. 5-HT receptor antagonist affinity values (pKᵦ) used for Pearson correlations**

<table>
<thead>
<tr>
<th>Antagonist</th>
<th>Ap5-HT₅&lt;sub&gt;apAC&lt;/sub&gt;</th>
<th>5-HT₂&lt;sub&gt;A&lt;/sub&gt;</th>
<th>5-HT₂&lt;sub&gt;C&lt;/sub&gt;</th>
<th>5-HT₆</th>
<th>5-HT₇</th>
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</thead>
<tbody>
<tr>
<td>Spiperone</td>
<td>4.3</td>
<td>7.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.37&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.06&lt;sup&gt;b&lt;/sup&gt;</td>
<td>5.8&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Risperidone</td>
<td>5.3</td>
<td>6.4&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.49&lt;sup&gt;e&lt;/sup&gt;</td>
<td>6.37&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Ritanserin</td>
<td>5.5</td>
<td>&lt;5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.53&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.57&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.36&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
<tr>
<td>Cyproheptadine</td>
<td>5.5</td>
<td>&lt;5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9.1&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>6.87&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>Clozapine</td>
<td>6.0</td>
<td>6.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.96&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.76&lt;sup&gt;a&lt;/sup&gt;</td>
<td>7.89&lt;sup&gt;b&lt;/sup&gt;</td>
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<td>6.5</td>
<td>&lt;5&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>6.18&lt;sup&gt;b&lt;/sup&gt;</td>
<td>7.7&lt;sup&gt;a&lt;/sup&gt;</td>
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<td>7.52&lt;sup&gt;b&lt;/sup&gt;</td>
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<tr>
<td>Methiothepin</td>
<td>7.8</td>
<td>7.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>9.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8.61&lt;sup&gt;b&lt;/sup&gt;</td>
<td>8.74&lt;sup&gt;b&lt;/sup&gt;</td>
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</table>

<sup>a</sup>Values from Bonhaus et al. (1997); <sup>b</sup>values from Hoyer et al. (1994); <sup>c</sup>values from Leyson et al. (1994); <sup>d</sup>values from Roth et al. (1992); <sup>e</sup>values from Roth et al. (1994); <sup>f</sup>values from Wander et al. (1987). Experimental Kᵦ values for Ap5-HT₅<sub>apAC</sub> were determined by first fitting 5-HT antagonists data obtained at 0, 1, and 25 μM 5-HT with a 4-parameter logistic equation

\[
\text{Response} = \frac{R_{\text{max}} - R_{\text{min}}}{1 + ([A]/EC_{50})^n} + R_{\text{min}}
\]

where \(R_{\text{max}}\) = maximal response in the presence agonist, \(R_{\text{min}}\) = minimal response in the absence of agonist, EC₅₀ = concentration of agonist giving half-maximal response, \(n\) = Hill coefficient, and \([A]\) = concentration of agonist. EC₅₀ values were then used to calculate Kᵦ values according to the following equation

\[\log K_a = \log (DR - 1) - \log [B]\]

where DR = dose ratio, the ratio of the EC₅₀ in the absence and presence of antagonist and \([B]\) = concentration of antagonist.
ganglion VC cluster. Stimulation of AC by 5-HT and 5-carboxyamidotryptamine maleate (5-CT). Stimulation of AC activity by 5-HT (○) or 5-CT (□) is expressed as percent of maximal AC stimulation by 5-HT. Data are means ± SE for 3 experiments, each of which was conducted in quadruplicate; within each experiment, both agonists were tested in a CNS membrane preparation from 4 animals. (Some of the error bars are smaller than the symbols.) Dose-response data were fit with a logistic equation of the form

\[
\text{Response} = \frac{R_{\text{max}} - R_{\text{min}}}{1 + \left(\frac{[A]}{EC_{50}}\right)^n} + R_{\text{min}}
\]

where \([A]\) is the concentration of agonist, \(R_{\text{max}}\) is the maximal stimulation of AC by 5-HT, \(R_{\text{min}}\) is AC activity in the absence of agonist, \(EC_{50}\) is the concentration of agonist giving half-maximal response, and \(n\) is the Hill coefficient.

**Methiothepin inhibits AC-coupled DA receptors**

Methiothepin is a nonselective antagonist that affects a wide variety of 5-HT and DA receptors (Hoyer et al. 1994). We tested whether it affects AC-coupled DA receptors in *Aplysia* CNS. Because DA produces relatively weak AC stimulation, these experiments were carried out with a saturating DA concentration of 200 μM. Methiothepin effectively blocked DA stimulation of AC in CNS membranes; DA stimulation was decreased from 24 ± 2% without methiothepin to 2 ± 1% with methiothepin (stimulation expressed as a percentage above basal activity, without either methiothepin or DA; \(n = 3\)). Within these same experiments, fluphenazine caused a more modest and significantly smaller decrease in DA stimulation of AC; stimulation by DA in the presence of fluphenazine was 17 ± 1%. [Both antagonists inhibited DA stimulation significantly; \(F(1,4) = 34.8, P = 0.004\), repeated-measures ANOVA, testing stimulation × antagonist interaction. For post hoc pairwise comparisons, \(P < 0.001\) for methiothepin vs. control and \(P < 0.001\) for fluphenazine vs. control; the effects of the 2 antagonists were significantly different, \(P < 0.001\) for methiothepin vs. fluphenazine.]

**Inhibition by methiothepin of the AC-coupled 5-HT receptor in Aplysia SNs**

We tested whether methiothepin also blocked 5-HT stimulation of AC in membranes from somata in the pleural ganglion VC cluster. Stimulation of AC by 5 μM 5-HT (as a percent of basal activity) was 1.0 ± 0.3% in the presence of 20 μM methiothepin versus 160 ± 24% in the absence of antagonist (Fig. 8A). A previous study of 5-HT effects on co-cultured SNs and postsynaptic motoneurons suggested that in SNs, there may be methiothepin-insensitive 5-HT receptors that activate AC (Sun and Schacher 1996). It seemed possible that SNs could have a second type of AC-coupled 5-HT receptor localized to their presynaptic processes in the neuropil. We therefore tested the effect of methiothepin on desheathed pleural ganglia, which contain presynaptic neuropilar processes of the VC cluster SNs. In pleural ganglion membranes, 10 μM methiothepin completely inhibited AC stimulation by 25 μM 5-HT (Fig. 8B). Thus in preparations enriched for SNs, no detectable 5-HT stimulation of AC was mediated by a methiothepin-insensitive receptor.

**Inhibition of the AC-coupled 5-HT receptor in SNs measured under physiological conditions**

It has been observed that in intact SNs, methiothepin did not effectively block modulatory effects of 5-HT that are believed to be mediated by cAMP (Sun and Schacher 1996). It seemed possible that the efficacy of methiothepin might be reduced in

![Graph showing dose dependence of AC stimulation by 5-HT and 5-carboxyamidotryptamine maleate (5-CT).](Image)

![Graph showing effect of methiothepin on AC in sensory neuron (SN) and pleural ganglion membranes. A: effect of methiothepin on AC in SN membranes. Membranes from pleural ganglion SN clusters were assayed with and without 5 μM 5-HT, in the presence and absence of 20 μM methiothepin. Data are the means ± SE of results from 3 experiments on separate membrane preparations, each from the pleural ganglion SN clusters of 9 animals. Methiothepin significantly inhibited AC stimulation by 5-HT (\(P < 0.05\), 2-tailed \(t\)-test). B: effect of methiothepin on AC in pleural ganglion membranes. Membranes from 4 desheathed pleural ganglia, each from a separate animal, were assayed with and without 25 μM 5-HT, in the presence and absence of 10 μM methiothepin. Data are the means ± SD of 5 replicate assays. In both A and B, AC activity is expressed as percent of activity without exogenous 5-HT or methiothepin (basal activity). Note that in contrast to experiments on CNS membranes, in experiments on SN clusters or on desheathed pleural ganglia, methiothepin had no effect on AC activity in the absence of exogenous 5-HT.](Image)
physiological saline. To assess methiothepin inhibition of 5-HT stimulation of AC in intact SNs, we recorded the broadening of the SN action potential produced by 5-HT in the presence of 100 mM TEA and 20 μM nifedipine (Goldsmith and Abrams 1992; Jarrard et al. 1993). Together these two compounds block the 5-HT-modulated currents except for the two S-K⁺ currents, IKS,slow and IKS,steady state (Baxter and Byrne 1989, 1990; Edmonds et al. 1990; Goldsmith and Abrams 1992; Sugita et al. 1994). These two remaining 5-HT-sensitive currents are modulated via protein kinase A (PKA); therefore in TEA/nifedipine, spike broadening by 5-HT should be mediated exclusively by cAMP. We measured the effect of methiothepin using a protocol in which 5 μM 5-HT was applied initially in the absence of antagonist and then in the presence of first 20 μM methiothepin and finally 100 μM methiothepin. This approach with sequential comparisons within each SN enables more accurate quantification of the effects of antagonists (Goldsmith and Abrams 1992). In principle, prolonged exposure to 5-HT could result in desensitization, causing spike broadening to decrease during the late phase of the 5-HT exposure, at the time that the antagonist is applied. However, Jarrard et al. (1993) found that in TEA/nifedipine, after 10 min of exposure to 50 μM 5-HT, SNs exhibited no desensitization of the spike broadening response (see also Abrams et al. 1984).

Spike broadening by 5-HT was decreased 55 ± 10% by 20 μM methiothepin and 82 ± 8% by 100 μM methiothepin (P = 0.002 and P = 0.001, respectively). At 100 μM, methiothepin was also significantly more effective than at 20 μM (Fig. 9). We were unable to test higher concentrations to see whether spike broadening could be completely blocked because at concentrations >100 μM, methiothepin precipitates out of physiological saline at pH 7.6 (see METHODS for pH dependence of the solubility of methiothepin).

Methiothepin is lipophilic; in our experiments its effects did not reverse rapidly. Lukyanetz and Kostyuk (1996) and Kostyuk et al. (1992) similarly observed incomplete recovery of 5-HT sensitivity in Helix neurons after washout of methiothepin. In contrast, in studies of dissociated neurons in culture, Sun and Schacher (1996) found that 1 h after washout of methiothepin, the 5-HT response recovered.

**Effects of physiological saline on antagonist binding**

The partial block of 5-HT-induced spike broadening by methiothepin contrasted with the complete inhibition observed in AC assays on membranes from CNS or SN clusters. Because the cellular electrophysiological studies were conducted in Aplysia physiological saline, which has a high ionic strength (>675 mM), and the biochemical assays were conducted in low-ionic-strength buffer, we hypothesized that high ionic strength contributed to the reduced efficacy of methiothepin in the physiological experiments. We were unable to study directly the effect of physiological salt concentrations on methiothepin inhibition of AC stimulation in homogenized membranes because 460 mM NaCl resulted in a large reduction (approximately sixfold) in AC activity as compared with 100 mM ionic-strength buffer. In principle, this inhibition of basal AC activity by high-ionic-strength physiological saline could be due to an effect on the extracellular surface of the membrane; however, the EC₅₀ for the 5-HT-dependent increase in excitability in intact SNs in physiological saline is in the same range (~1 μM) (Stark et al. 1996) as the EC₅₀ for 5-HT stimulation of AC in membrane homogenates in 100 mM ionic-strength buffer (Fig. 7). Therefore the inhibition by NaCl of basal AC activity is most likely due to an effect on the cytoplasmic surface of the membrane. In several experiments, we examined the effects of methiothepin on 5-HT stimulation of AC in intact SNs using an RIA for cAMP. In contrast to the complete inhibition of AC stimulation by 10 μM methiothepin observed in 100 mM ionic-strength buffer (e.g., Fig. 8A), in physiological saline in the presence of 20 μM methiothepin, 5 μM 5-HT still increased intracellular cAMP in SNs (Fig. 10A). 5-HT stimulation of cAMP levels was 29.6 ± 14.7 fmole/cluster (n = 10) for methiothepin versus 47.5 ± 22.3 fmole/cluster for control saline (in both cases the 5-HT stimulation was significant, P < 0.05, 1-tailed t-test for paired comparisons with contralateral control clusters). With 20 μM methiothepin,
5-HT stimulation of cAMP levels both with methiothepin produced significant physiological saline, there was not effective inhibition by methiothepin; 5-HT SN membranes conducted in low-ionic-strength assay buffer, in intact SNs in 5-HT-treated and control clusters. Note that in contrast to AC assays on physiological saline can decrease the affinity of methiothepin. Nevertheless, these [125I]–LSD results directly demonstrate that high-ionic-strength saline can dramatically reduce the affinity of a ligand for 5-HT receptors; a similar reduction in affinity in physiological saline may also occur with methiothepin, as our RIA measurements suggest.

Effects of methiothepin and cyproheptadine on the 5-HT-induced increase in SN excitability

_Aplysia_ SNs normally exhibit dramatic spike frequency adaptation; when stimulated with prolonged depolarizing current pulses, these neurons typically stop firing within the first 100 ms. 5-HT produces an increase in excitability due substantially to a reduction in this spike frequency adaptation (Klein et al. 1986). This 5-HT modulation of excitability is mediated by effects on two K$^+$ currents: a reduction in the slowly activating current $I_{K,\text{slow}}$ decreases accommodation (Goldsmith and Abrams 1992; Klein et al. 1982, 1986) and a reduction in the tonically activated, time-independent current $I_{K,\text{steady state}}$ decreases current threshold (Goldsmith and Abrams 1992; Siegelbaum et al. 1982). Although this modulation of SN excitability in the short term is mediated by cAMP (Goldsmith and Abrams 1992; Hochner and Kandel 1992),1 cyproheptadine has been found not to affect or only partially affect the 5-HT-induced increase in excitability in pleural ganglion SNs (Morgan et al. 1991; Sun and Schacher 1996). This was puzzling because cyproheptadine blocks the AC-coupled 5-HT receptor in biochemical studies (Figs. 1 and 5) (Goldsmith and Abrams 1992). Furthermore, spike broadening in TEA/nifedipine is mediated by modulation of the same two 5-HT-sensitive S–K$^+$ currents that are involved in the excitability increase, and this modulatory effect in intact SNs is completely blocked by cyproheptadine (Goldsmith and Abrams 1992).

We considered the possibility that these inconsistent results may be explained by the ionic-strength-dependent decrease in the efficacy of these antagonists. Would cyproheptadine block the increase in excitability produced by a lower concentration of 5-HT than previously tested? We reexamined the effect of cyproheptadine on the excitability increase by making within-cell sequential comparisons, exposing pleural ganglion SNs to 1 μM 5-HT, followed by 5-HT plus antagonist. This sequential comparison procedure enables more precise quantification of

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1 Although phorbol esters, which activate PKC have been shown to increase excitability in SNs, this effect is substantially more modest than that of 5-HT or cAMP (compare Sugita et al. 1997, Figs. 2 and 3) and it requires long exposures to phorbols (Brahall et al. 1990).
an antagonist’s partial inhibitory effects. When SNs were exposed to 1 μM 5-HT in the absence of antagonist, the increase in excitability was maintained for approximately 20 min, indicating that there was no desensitization of the AC-coupled 5-HT receptor (Fig. 11D). Cyproheptadine effectively reverses the 5-HT-induced increase in excitability. We observed that the extent of this reversal of the increase in excitability was dependent both on the duration of the exposure to cyproheptadine and on the amplitude of the test current (Fig. 11, A and B). Early during the exposure to cyproheptadine (within 2–4 min), the excitability tested with a current 1.25 × threshold was substantially reduced (P = 0.049), whereas the excitability tested with a current 2.5 × threshold was not significantly affected (P = 0.332, post hoc pairwise comparisons). Later, when the cyproheptadine effect had reached a maximum (after 4–8 min), the increased excitability at both current intensities was completely reversed (P = 0.033 and P = 0.003 for the low and high current intensities, respectively; Fig. 11, A and B, see legend for ANOVA). Thus depending both on the duration of cyproheptadine exposure and on the test current, the block by cyproheptadine of the excitability increase by 5-HT was either partial or complete. The slower block of the 5-HT-induced excitability increase with the larger test current suggests that cyproheptadine is less effective in physiological saline than in the AC assays.

We next examined whether methiothepin would act like cyproheptadine to inhibit the excitability increase produced by 1 μM 5-HT in SNs. Methiothepin at 100 μM completely blocked the increase in excitability produced by 1 μM 5-HT at a low stimulus intensity (1.25 × threshold), and significantly reduced the excitability increase at a higher stimulus intensity.
(2.5 × threshold) by 48 ± 4% (Fig. 11C). This excitability change remaining in the presence of 100 μM methiothepin may be explained by the decrease in the affinity of the antagonist in high-ionic-strength saline.

**Discussion**

**5-HT antagonists as functional probes**

The goals of this study were to pharmacologically characterize the 5-HT receptor or receptors that activate AC in *Aplysia* CNS. It would be useful to have potent, high-affinity antagonists that selectively block the 5-HT receptors that are coupled to either AC or PLC in *Aplysia* CNS to study the contribution of each of these signal transduction pathways to neural plasticity. Unfortunately, methiothepin, the most effective antagonist of 5-HT\textsubscript{apAC} that we identified, blocks multiple *Aplysia* 5-HT receptors, including the PLC-coupled 5-HT receptors (Angers et al. 1998; Li et al. 1995); we also observed it blocked AC-coupled DA receptors. In contrast, spiperone blocks the known PLC-coupled 5-HT receptors, Ap\textsubscript{5-HT\textsubscript{B1}} and Ap\textsubscript{5-HT\textsubscript{B2}}, as effectively as methiothepin (Li et al. 1995) but was inactive at the 5-HT\textsubscript{apAC} receptor (Fig. 1). Spiperone is also inactive at the 5-HT\textsubscript{ap1} receptor, which inhibits AC (Angers et al. 1998). Using methiothepin and spiperone in parallel experiments, one can distinguish AC-mediated responses from PLC-mediated responses (B. Dumitriu, J. E. Cohen, and T. W. Abrams, unpublished results). If one can exclude the involvement of DA (e.g., if DA does not mimic the physiological response), methiothepin may be a useful tool in the analysis of the roles of the AC-coupled 5-HT receptor in behavioral plasticity. For example, recently, using methiothepin, Liao et al. (1999) were able to demonstrate that various components of long-term plasticity in *Aplysia* SNs were differentially dependent on 5-HT.

**Effects of high-ionic-strength physiological saline**

We observed a decrease in the inhibition by methiothepin in cellular electrophysiological assays conducted on intact SNs compared with the inhibition by methiothepin in biochemical assays. Our results suggest that the high ionic strength of the saline used for cellular studies (or the high concentration of either Na\textsuperscript{+}, Mg\textsuperscript{2+}, or Cl\textsuperscript{−}) weakens the binding of methiothepin to 5-HT receptors. Consistent with this possibility, 5-HT stimulation of cAMP levels in intact SNs in high-ionic-strength saline was not effectively blocked by a concentration of methiothepin (20 μM) that produced maximal inhibition of AC activity in low-ionic-strength buffer. A similar difference has been observed in the inhibition by methiothepin of 5-HT stimulation of AC in buccal muscle membranes and in intact *Aplysia* buccal muscle assayed in low salt buffer and physiological saline, respectively (L. E. Fox, P. E. Lloyd, J. E. Cohen, and T. W. Abrams, unpublished results). Also consistent with this hypothesis was change in antagonist affinity, we found in *Aplysia* CNS membranes that physiological saline produced a threefold decrease in the binding of a radiolabeled antagonist (Fig. 10B). This concept that the high ionic strength of the physiological saline of marine animals can significantly alter the inhibition produced by an antagonist is supported by radioligand binding results demonstrating that ionic-strength differentially affects the affinity of various ligands (Hou et al. 1996).

Recently investigators have expressed recombinant *Aplysia* 5-HT receptors in mammalian cell lines and measured ligand binding and second-messenger stimulation under low-ionic-strength conditions (e.g., Angers et al. 1998; Li et al. 1995). Our results suggest that pharmacological assays of recombinant receptors performed at low ionic strength may not accurately reflect the efficacy of antagonists in the high-ionic-strength physiological saline of marine invertebrates.

**Comparison of the 5-HT\textsubscript{apAC} receptor with other *Aplysia* 5-HT receptors**

Several *Aplysia* 5-HT receptor subtypes have been characterized using electrophysiological analysis, photoaffinity labeling, and molecular techniques. Gerschenfeld and Paupardin-Tritsch (1974) distinguished six different 5-HT responses in *Aplysia* neurons based on distinct pharmacologies that presumably are mediated by different receptor subtypes. Photoaffinity labeling of 5-HT receptors in *Aplysia* CNS suggested the existence of at least five serotonin receptor subtypes (Saitoh and Shih 1987). To date, five *Aplysia* receptors have been cloned. Li et al. (1995) cloned two PLC-coupled receptors: Ap\textsubscript{5-HT\textsubscript{B1}}, which is expressed in the ovotestis and spermatheca, and Ap\textsubscript{5-HT\textsubscript{B2}}, which is expressed in the CNS. Unlike the 5-HT\textsubscript{apAC} class of receptor, these two PLC-coupled receptors were highly sensitive to spiperone. Angers et al. (1998) and Barbas et al. (2002) cloned two 5-HT receptors expressed in CNS, which inhibit AC: the 5-HT\textsubscript{ap1} and 5-HT\textsubscript{ap2} receptors. These two receptors display a pharmacological profile distinct from that of the 5-HT\textsubscript{apAC} receptor. A fifth *Aplysia* 5-HT receptor has been partially cloned, which is expressed at high levels in peripheral tissues and at a low level in CNS (Williams et al. 1997). Methiothepin is an effective antagonist against all four recombinant *Aplysia* 5-HT receptors: Ap\textsubscript{5-HT\textsubscript{B1}}, Ap\textsubscript{5-HT\textsubscript{B2}}, 5-HT\textsubscript{ap1}, and 5-HT\textsubscript{ap2} as well as against 5-HT\textsubscript{apAC}. Similarly, methiothepin is an effective antagonist at all mammalian G-protein-coupled 5-HT receptor subtypes, except the 5-HT\textsubscript{4} receptor (Hoyer et al. 1994).

**Which 5-HT receptors are blocked by cyproheptadine?**

Several studies have used cyproheptadine to selectively interfere with specific forms of 5-HT-dependent plasticity in SNs (Emptage and Carew 1993; Mercer et al. 1991; Sun and Schacher 1996). Mercer et al. (1991) found that 200 μM cyproheptadine blocked spike broadening induced by 5 μM 5-HT without affecting the accompanying increase in excitability. Emptage and Carew (1993) used cyproheptadine to block 5-HT-induced short-term synaptic facilitation without interfering with long-term facilitation. Based on these dissociations, it was proposed that broadening of the SN action potential and short-term facilitation are triggered via cyproheptadine-sensitive receptors, at least some of which activate PLC, whereas enhanced excitability and long-term facilitation are triggered via cyproheptadine-insensitive receptors that activate AC. In contrast, the biochemical evidence indicates that cyproheptadine does not differentiate among 5-HT receptors in *Aplysia* CNS. In pleural-pedal-ganglia, cyproheptadine (at 200 μM) inhibits translocation of PKC by 5-HT, presumably by block-
ing PLC-coupled receptors (Sossin et al. 1994). In SN membranes in low-ionic-strength buffer, AC stimulation by 5-HT is also blocked by 200 μM cyproheptadine (Goldsmith and Abrams 1992).

If there were a cyproheptadine-insensitive, AC-coupled 5-HT receptor that primarily mediates the increase in excitability, then one would expect to see residual AC stimulation by 5-HT in the presence of cyproheptadine. Although with the 5 μM 5-HT concentration used in the earlier electrophysiological experiments (Emptage and Carew 1993; Mercer et al. 1991), cyproheptadine never inhibited >92% of AC stimulation in biochemical assays (Fig. 5A), with 1 μM 5-HT, AC stimulation was completely blocked by 200 μM cyproheptadine (Fig. 5B). It could be argued that a second AC-coupled 5-HT receptor present in intact SNs does not contribute detectably to 5-HT stimulation of AC in homogenized membranes. It is unlikely that a 5-HT receptor that activates AC went undetected in our AC assays for several reasons. In a wide range of receptor systems, it is possible to study agonist stimulation of AC after homogenizing membranes; therefore it is not likely that the hypothesized cyproheptadine-insensitive receptor became non-functional. It is possible that a less abundant, regionally restricted, AC-coupled 5-HT receptor contributes importantly to functional. It is possible that a less abundant, regionally restricted, AC-coupled 5-HT receptor contributes importantly to SN physiology but was missed in these biochemical experiments. However, electrophysiological experiments also indicate that there is not a cyproheptadine-insensitive (or methiothepin-insensitive) 5-HT receptor in SNs that activates AC. Goldsmith and Abrams (1992) found that 200 μM cyproheptadine inhibited >98% the spike broadening in TEA/nifedipine produced by 1 μM 5-HT; because TEA and nifedipine block the other 5-HT-modulated currents, spike broadening in the presence of these two compounds is almost entirely due to cAMP-dependent reduction in the two S-K⁺ currents.

If cyproheptadine is a broad spectrum 5-HT antagonist, how can it selectively block a subset of the responses to 5-HT? It is possible that 200 μM cyproheptadine only partially blocked the 5-HT-stimulated increase in cAMP levels in electrophysiological experiments, in contrast to the complete inhibition by cyproheptadine of 5-HT stimulation of AC in biochemical experiments. Such incomplete inhibition of AC stimulation could have resulted from a reduction in the affinity of cyproheptadine for the 5-HT₆AC receptor in high-ionic-strength physiological saline as we observed occurs with methiothepin and LSD. Thus a residual increase in cAMP levels that persists with cyproheptadine may have mediated the 5-HT-induced increase in excitability previously seen in the presence of this antagonist (Mercer et al. 1991). Consistent with the possibility that cyproheptadine at 200 μM is blocking the AC-coupled 5-HT receptor, though only partially, we observed that cyproheptadine was effective in blocking the excitability increase initiated by a fivefold lower concentration of 5-HT than tested in the studies of Mercer et al. (1991) (Fig. 11A). Also consistent with the possibility that 200 μM cyproheptadine only partially blocks the AC-coupled 5-HT receptor in physiological saline, we observed that the efficacy of cyproheptadine in blocking 5-HT modulation of SN excitability depended on the precise test conditions (Fig. 11, A and B). Differential inhibition of increased excitability depending on the test current was also observed with methiothepin (Fig. 11C). The observation that the efficacy of an antagonist varies substantially within a single neuron, depending on the intensity of the test current or the duration of exposure to the antagonist, suggests that the antagonist concentration is within the steep region of the dose-response curve; in this concentration range, small shifts in parameters can produce large changes in the physiological effect of the antagonist. To optimally detect inhibitory effects of an antagonist, it is important that the agonist (e.g., 5-HT) be used at concentrations that are substantially below saturating for the response being studied.

Increased excitability is particularly prone to being insensitive to antagonists that only partially block a population of receptors. The increase in SN excitability has a strikingly nonlinear dependence on the underlying decrease in K⁺ current, due, at least in part, to the discrete threshold for spike initiation. When measuring excitability, a partial inhibition of the 5-HT-induced reduction in K⁺ current may not be detected if the remaining K⁺ current is not sufficient to prevent the initiation of action potentials. Moreover, the slowly activating S-K⁺ current (I_{KS,slow}) is modulated by relatively low cAMP concentrations (Goldsmith and Abrams 1992); perhaps as a consequence, increased excitability occurs at relatively low concentrations of 5-HT (Stark et al. 1996) and may require the activation of only a modest percentage of receptors. This would make the increase in excitability difficult to block, particularly when the 5-HT concentration is substantially above the EC₅₀ for activation of AC. (The hypothesis that phosphorylation of S-K⁺ channels occurs at a high rate and is difficult to block is illustrated in Fig. 12A.)

Sun and Schacher (1996) found in cultured SNs that the inhibition by cyproheptadine of the 5-HT-stimulated increase in excitability varied substantially depending on the specific culture conditions. These authors interpreted this variation in the efficacy of cyproheptadine as indicating that under some culture conditions, the SNs express a cyproheptadine-insensitive (and methiothepin-insensitive) receptor that activates AC. Because our biochemical results indicate that the AC-coupled 5-HT receptors in CNS were all sensitive to both cyproheptadine and methiothepin, we interpret the variable block by these antagonists in electrophysiological studies as indicating that the antagonist concentration used was insufficient to completely block cAMP increases and that the modulatory cascade initiated by the antagonist concentration used was insufficient to completely block cAMP increases and that the modulatory cascade is expressed more powerfully at certain times as synapses are forming in culture (see cartoon in Fig. 12B). Given that in our experiments, the efficacy of both methiothepin and cyproheptadine varied with the precise experimental parameters, it is not surprising that the blockade of the excitability increase by these antagonists can change substantially as synapses develop in culture.

It is important to emphasize that our finding that cyproheptadine does not discriminate among 5-HT receptors or among the second-messenger cascades that 5-HT initiates does not weaken the two central conclusions of the early studies that used cyproheptadine: 1) long-term facilitation can be initiated without short-term facilitation and 2) during short-term facilitation, presynaptic spike broadening and increased transmitter release are independent of changes in excitability (Mercer et al. 1991). It is also important to point...
out that our analysis does not address the issue of whether there are multiple AC-coupled 5-HT receptors in *Aplysia* CNS. We believe, however, that the predominant receptors that activate AC are sensitive to both cyproheptadine and methiothepin at concentrations of 100 μM.

In summary, our results indicate that it is possible to observe selective blockade of a subset of effects produced by a single modulatory cascade when there is incomplete inhibition of that cascade. Indeed, even a single modulatory effect may be differently inhibited depending on test conditions. Thus dissoci-
atation of different effects due to partial inhibition of a cascade may not demonstrate mediation by distinct signaling cascades. In general, caution is warranted in using receptor antagonists to dissociate second-messenger cascades when monitoring downstream physiological changes. Dose-inhibition curves in combination with the use of nonsaturating concentrations of agonists reduce the likelihood of being misled by selective inhibition of a subset of responses.

Comparison with classes of mammalian 5-HT receptor

None of the antagonists with high selectivity for mammalian 5-HT receptors effectively blocked the 5-HT$_{apAC}$ receptor. Specific antagonists targeted against mammalian 5-HT receptor subtypes may be inactive against homologous molluscan 5-HT receptors because of the large evolutionary distance between these two phyla (Peroutka and Howell 1994); thus small numbers of amino acid substitutions near the binding site might substantially decrease the affinity of these highly specific antagonists and yet have more modest effects on the affinity of broad spectrum antagonists. We therefore compared the sensitivity of the 5-HT$_{apAC}$ class of receptor to the less specific antagonists with the sensitivities of various mammalian 5-HT receptor subtypes to these same antagonists. Our Pearson correlation analysis suggested that the pharmacological profile of the 5-HT$_{apAC}$ receptor was most similar to the profile of the 5-HT$_6$ receptor, with a correlation of $>$0.9, compared with correlations of $<$0.5 for the next two most similar 5-HT receptor subtypes. Moreover, both the 5-HT$_{apAC}$ and 5-HT$_6$ receptors differ from the 5-HT$_{4}$, 5-HT$_{5}$, and 5-HT$_{7}$ receptors in that, at the 5-HT$_{apAC}$ and 5-HT$_6$ receptors, 5-HT has both higher potency and efficacy than the agonist 5-CT. Therefore we predict that 5-HT$_{apAC}$ receptor is most homologous to the 5-HT$_6$ receptor. Interestingly, the 5-HT$_6$ class of receptors evolved relatively early, ~750 million years ago, well before the divergence of vertebrates from invertebrates (Peroutka and Howell 1994). The selective 5-HT$_6$ antagonist that we tested, Ro-04-6790, was completely inactive at the 5-HT$_{apAC}$ receptor; however, this is consistent with the perspective that highly specific antagonists against mammalian receptors are likely to be ineffective against phylogenetically distant, though homologous, invertebrate receptors. Cloning of the 5-HT$_{apAC}$ receptor or receptors will enable confirmation of the predicted homology between the 5-HT$_{apAC}$ receptor and the mammalian 5-HT$_6$ receptor.

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Two distinct receptors operate the cAMP


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