Serotonin Receptor Antagonists Discriminate Between PKA- and PKC-Mediated Plasticity in *Aplysia* Sensory Neurons

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Dumitriu, Bogdan, Jonathan E. Cohen, Qin Wan, Andreea M. Negroiu, and Thomas W. Abrams. Serotonin receptor antagonists discriminate between PKA- and PKC-mediated plasticity in *Aplysia* sensory neurons. *J Neurophysiol* 95: 2713–2720, 2006. First published October 19, 2005; doi:10.1152/jn.00642.2005. Highly selective serotonin (5-hydroxytryptamine, 5-HT) receptor antagonists developed for mammals are ineffective in *Aplysia* due to the evolutionary divergence of neurotransmitter receptors and because the higher ionic strength of physiological saline for marine invertebrates reduces antagonist affinity. It has therefore been difficult to identify antagonists that specifically block individual signaling cascades initiated by 5-HT. We studied two broad-spectrum 5-HT receptor antagonists that have been characterized biochemically in *Aplysia* CNS: methiothepin and spiperone. Methiothepin is highly effective in inhibiting adenyl cyclase (AC)-coupled 5-HT receptors in *Aplysia*. Spiperone, which blocks phospholipase C (PLC)-coupled 5-HT receptors in mammals, does not block AC-coupled 5-HT receptors in *Aplysia*. In electrophysiological studies, we explored whether methiothepin and spiperone can be used in parallel to distinguish between the AC-cAMP and PLC-protein kinase C (PKC) modulatory cascades that are initiated by 5-HT. 5-HT-induced broadening of the sensory neuron action potential in the presence of tetraethylammonium/nifedipine, which is mediated by modulation of the S-K currents, was used as an assay for the AC-cAMP cascade. Spike broadening initiated by 5 μM 5-HT was unaffected by 100 μM spiperone, whereas it was effectively blocked by 100 μM methiothepin. Facilitation of highly depressed sensory neuron-to-motor neuron synapses by 5-HT was used as an assay for the PLC-PKC cascade. Spiperone completely blocked facilitation of highly depressed synapses by 5 μM 5-HT. In contrast, methiothepin produced a modest, nonsignificant, reduction in the facilitation of depressed synapses. Interestingly, these experiments revealed that the PLC-PKC cascade undergoes desensitization during exposure to 5-HT.

**INTRODUCTION**

Serotonin (5-hydroxytryptamine, 5-HT) is an important modulatory neurotransmitter in the gastropod mollusk *Aplysia californica*, playing roles in cardiac regulation (Liebeswar et al. 1975; Sawada et al. 1984) and in behaviors such as feeding (Rosen et al. 1989), locomotion (Mackey and Carew 1983; Marinesco et al. 2004; McPherson and Blankenship 1991, 1992; Parsons and Pinsker 1989), and the modulation of the defensive withdrawal reflexes. Release of 5-HT contributes to strengthening of the synaptic connections in the afferent limb of the defensive withdrawal reflexes during both nonassociative and associative learning (Barbas et al. 2003; Glanzman et al. 1989; Marinesco and Carew 2002). 5-HT-induced facilitation of the connections from sensory neurons (SNs) to motor neurons (MNs) is initiated by two signaling cascades that involve cAMP and protein kinase C (PKC) (Byrne and Kandel 1996). In the SNs, increases in cAMP and activation of protein kinase A (PKA) result in short-term enhancement of release at nondepressed or moderately depressed synapses and reduction in the S-K currents and modulation of *I*<sub>KV</sub>-early, (Bråha et al. 1990; Ghiardi et al. 1992; Goldsmith and Abrams 1991, 1992; Hochner and Kandel 1992; Klein 1993; Siegelbaum et al. 1982). Cyclic AMP and PKA also initiate transcription cascades via activation of the transcription factor cyclic AMP response element-binding protein (CREB), which in turn contributes to long-term increases in synaptic strength (Bailey et al. 2004; Kaang et al. 1993; Martin et al. 1997; Michael et al. 1998). Activation of PKC in the SNs results in short-term facilitation of highly depressed synapses and intermediate-term enhancement of transmitter release (Ghiardi et al. 1992; Manseau et al. 2001). PKC also contributes to long-term modulatory changes in these neurons (Manseau et al. 1998; Sutton and Carew 2000; Sutton et al. 2004; Upadhyya et al. 2004).

It would be attractive to be able to pharmacologically dissociate the 5-HT receptors coupled to phospholipase C (PLC) and PKC from the receptors coupled to adenyl cyclase (AC) and PKA. Evidence for multiple 5-HT receptor subtypes in *Aplysia* CNS has been obtained in pharmacological and molecular studies (Angers et al. 1998; Barbas et al. 2002; Gerschenfeld and Paupardin-Tritsch 1974). Two 5-HT receptors that inhibit AC have been cloned (Angers et al. 1998; Barbas et al. 2002). However, as yet a 5-HT receptor that is positively coupled to AC has not been molecularly characterized. A number of receptor antagonists have been used in physiological studies of the defensive withdrawal reflex, including cinar- serin, methiothepin, and cyproheptadine (Goldsmith and Abrams 1992; Mercer et al. 1991; Sun and Schacher 1996) with relatively limited analysis of their pharmacology. Based on AC assays, in combination with published studies of PKC activation (Sossin et al. 1994), Cohen et al. (2003b) concluded that these antagonists were nonselective. This was not surprising because in mammalian systems, these same compounds are broad-spectrum antagonists that act at multiple receptor subtypes. Given the lack of effect of spiperone on AC activation in *Aplysia* (Cohen et al. 2003b) and the efficacy of spiperone in antagonizing mammalian PLC-coupled 5-HT<sub>2</sub> receptors (Hoyer et al. 1994), we speculated that this antagonist might be a relatively selective blocker in physiological studies of 5-HT responses mediated by PLC (Cohen et al. 2003b). We were also influenced by the observation that spiperone antagonized...
recombinant putative PLC-coupled receptors from *Aplysia*; however, these cloned spiperone-sensitive receptors from *Aplysia* are no longer believed to be 5-HT receptors (Li et al. 1995) and, based on sequence homology, may be dopamine receptors (Barbas et al. 2006). Methiothepin was found to be the most effective antagonist of those tested for inhibiting 5-HT receptors that are positively coupled to AC. However, methiothepin also antagonizes mammalian PLC-coupled 5-HT receptors and may affect PLC-coupled 5-HT receptors in *Aplysia*. We therefore proposed that spiperone and methiothepin could be used in parallel experiments to distinguish AC- and PKC-mediated effects of 5-HT.

It is important to emphasize that biochemical assays do not accurately predict the efficacy of these receptor antagonists at physiological ionic strength because the high salt concentrations can cause substantial decreases in affinity compared with low ionic strength biochemical buffers (Cohen et al. 2003b). In the present study, we determined the efficacy and specificity of spiperone compared with methiothepin in electrophysiological experiments on neurons in intact ganglia at physiological ionic strength.

To test the efficacy of spiperone and methiothepin in blocking physiological effects of 5-HT mediated by PLC and AC, we measured two 5-HT-dependent processes, each of which is believed to be mediated selectively by a different signaling cascade: 5-HT-induced facilitation of highly depressed SN-to-MN synapses (“reversal of synaptic depression”), which requires phosphorylation by PKC (Brahra et al. 1990; Byrne and Kandel 1996; Ghirardi et al. 1992; Manseau et al. 2001), and 5-HT-induced prolongation of the SN action potential recorded in the presence of tetraethylammonium (TEA) and nifedipine, which block the PKC-modulated channels (Baxter and Byrne 1989; Brahra et al. 1993; Sugita et al. 1994); with this combination of channel blockers, 5-HT-induced spike broadening is due entirely to the PKA-mediated reduction in S-K+ currents (see RESULTS and DISCUSSION).

**METH O DS**

**Drugs**

Methiothepin mesylate and spiperone hydrochloride were obtained from RBI (Natick, MA). It is important to note that with both antagonists, the specific salt used affects solubility, as does pH in the case of methiothepin (Nelson et al. 1979). Subsequently, we have obtained spiperone hydrochloride from Tocris when this form was no longer available from RBI. 5-hydroxytryptamine creatine sulfate was purchased from Sigma (St. Louis, MO); stock solutions of 10 mM 5-HT were made in 0.1 M acetic acid and stored at 4°C. Spike-broadening measurements were conducted in the presence of 100 mM TEA chloride (JT Baker, Phillipsburg, NJ) and 20 μM nifedipine (Sigma). Spiperone and nifedipine were dissolved as 1,000X stocks in DMSO, aliquoted and stored at −20°C. Methiothepin was prepared fresh at 100 or 150 μM in saline.

**Electrophysiology**

*Aplysia californica* (Alacrity, Redondo Beach, CA), weighing 80–120 g, were anesthetized by injection of isotonic MgCl2, and their abdominal and pleural-pedal ganglia were removed. Prior to desheathing, ganglia were treated with 0.5% glutaraldehyde for 50 s to prevent contraction of muscle cells in the remaining sheath. Ganglia were secured with minuten pins on wax in a recording chamber and desheathed in a 1:1 mixture of MgCl2 and artificial sea water. In experiments on facilitation of depressed synapses, synaptic connections between LE siphon SNs and LFS MNs in the abdominal ganglion were recorded after the left ventral surface of the ganglion was desheathed. In experiments on spike broadening in SNs, pleural ganglia were desheathed to expose the SNs in the ventraloccipital (VC) cluster.

Experiments were performed at room temperature. Ganglia were superfused with high-Mg2+/high-Ca2+ culture medium (6 × normal Ca2+; 1.6 × normal Mg2+) (Goldsmith and Abrams 1991) to reduce polysynaptic input and spontaneous activity from modulatory interneurons; (in mM) 328 NaCl, 10 KCl, 66 CaCl2, 88 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, Grand Island, NY), and MEM vitamin solution (0.7 × normal concentration, GIBCO, Invitrogen)]. This 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). Neurons were penetrated with 10- to 20-MΩ glass microelectrodes filled with 2 M K-acetate and 400 mM KCl. Data were acquired digitally and analyzed using Spike software (Hilal Associates, Englewood Cliffs, NJ).

For reversal of synaptic depression experiments, during penetration, 0.5- to 1.0-nA hyperpolarizing current was injected to prevent SN firing. SN action potentials were elicited by injection of 2-ms depolarizing current pulses. The membrane potential of postsynaptic MNs was hyperpolarized 50 or 60 mV below the resting potential to prevent action potentials. After a synaptic connection was identified, the synapse was rested for a minimum of 15 min. During the synaptic depression protocol, action potentials in SNs were elicited at 15-s interstimulus interval (ISI); synapses were depressed with 30 stimuli before superfusion with 5 μM 5-HT during 30 additional stimuli. We observed that, during the 7.5-min 5-HT exposure in control saline, the facilitation was transient; data for the last 1.5 min, after the facilitation had already declined, were not included in either the graphs or the final statistical analysis. After exposure to 5-HT, the 5-HT was washed out for 15 min with 50 bath volumes, and a second synapse was then identified with the same MN. The ganglion was then superfused with 5-HT antagonist or control saline and the synaptic depression protocol was initiated after a 15-min rest. At the start of the second trial, there was no consistent change observed in the input resistance of the MN due either to a prior exposure to 5-HT or to the presence of an antagonist. An experiment was discontinued if there was a substantial decrease in the input resistance of the MN between the testing of the first and second synapses (a change of more than 30%). There was not a significant difference between the initial amplitude of the first and second synapses; a difference would be expected if the MN penetration deteriorated [excitatory postsynaptic potential (EPSP) in trial 1 = 6.79 ± 0.73 vs. 6.05 ± 0.75 mV, for the first and second synapses, respectively]. There was also not a significant effect of exposure to spiperone or methiothepin versus control saline on the amplitude of the second synapse [F(2,26) = 1.866, P = 0.175]. Data were not included if the first exposure to 5-HT did not result in facilitation >50%. Poor facilitation occurred in 2 of 31 ganglia, both of which were in control experiments where 5-HT was applied twice without antagonist; the exclusion of these experiments did not affect the assessment of the efficacy of the two antagonists.

In spike-broadening experiments, recordings were made in parallel from two SNs in the VC cluster, stimulated with 2-ms depolarizing current pulses at a 15-s ISI. Additional experiments on spike broadening in 100 mM TEA and 20 μM nifedipine were conducted on pairs of SNs in the LE cluster in the abdominal ganglion. Spike duration was measured from the peak to the time at which it had decayed to 33% of the maximum amplitude. The broadening responses of the two SNs in each ganglion were averaged. SNs were exposed to 5 μM 5-HT and antagonists in the following sequence: 5-HT for 3 min,
followed by 5-HT plus either methiothepin, spiperone or the vehicle for spiperone (0.1% DMSO) for 5 min.

Statistics

Statistical tests were conducted with SPSS software (SPSS, Chicago, IL). Multivariate ANOVA, using a repeated-measures design for comparisons within preparations (i.e., within ganglia, a single ganglion per animal), was followed by post hoc pairwise comparisons with Sidak adjustment for multiple comparisons. When spike durations were averaged for two SNs in a ganglion or when consecutive EPSPs were averaged, the averaged data were used for statistical analysis. In the case of spike duration experiments, the data were normalized and the ANOVA was conducted on arc sine transformed data. MMATRIX syntax was used for post hoc evaluations of custom hypotheses in which groups of time points were compared (evaluating completeness of reversal of spike broadening or efficacy of synaptic facilitation in the presence of antagonist).

RESULTS

Based on pharmacological studies of mammalian 5-HT receptors (Pedigo et al. 1981; Peroutka and Snyder 1979; Roth 1994), we had proposed that spiperone might block PKC-dependent modulatory effects of 5-HT (Cohen et al. 2003b). To explore this possibility, we examined PKC-mediated facilitation of highly depressed SN synapses. In testing the effect of spiperone or methiothepin on reversal of synaptic depression, within each ganglion, we began by verifying whether 5-HT produced effective facilitation. We first depressed one SN-to-MN synapse with 30 stimuli and then monitored the facilitation produced by exposure to 5-HT. After washout of 5-HT (see METHODS), the protocol was repeated in the same ganglion with a second SN-to-MN synapse in the presence of one of the antagonists or control saline. Depression of SN-to-MN synapses occurred at the same rate independently of whether there had been an earlier exposure to 5-HT (during a control test of facilitation) and independently of the presence of either 5-HT antagonist (Fig. 1).

FIG. 1. Synaptic depression is not affected by an earlier exposure to serotonin (5-HT) or the presence of spiperone or methiothepin. Each sensory neuron (SN) was stimulated 30 times at a 15-s interstimulus interval (ISI). Synaptic depression protocols for synapse 2 and for methiothepin and spiperone were done after 30 min of washout of 5-HT following the 7.5-min exposure to 5-HT for the first synapse. There was no effect on synaptic depression of either a prior 5-HT exposure or the presence of either antagonist [repeated-measures ANOVA testing treatment × trial interaction, \(F(87,66) = 1.088, P = 0.36\)] Sample sizes: for controls, synapse 1, \(n = 27\) and synapse 2, \(n = 10\); for spiperone \(n = 10\) and for methiothepin, \(n = 7\).

In the control experiments in which 5 μM 5-HT was applied twice to the same ganglion, we observed that the second exposure produced less facilitation than the first. The peak facilitation induced by the second 5-HT exposure decreased by more than 45% as compared with the response to the first 5-HT exposure (Fig. 2); the facilitation during the continued exposure to 5-HT also decayed earlier during the second exposure \([F(9,72) = 2.406; P = 0.019]\) (see figure legends for detailed statistics). Interestingly, these results provide evidence that the cascade mediating facilitation of highly depressed SN synapses undergoes desensitization during exposure to 5-HT. Thus it appears that the first 7.5 min exposure to 5-HT resulted in substantial desensitization of the PLC-PKC pathway.

Spiperone, but not methiothepin, completely blocks 5-HT-induced facilitation of highly depressed SN synapses

Because of this desensitization, in assessing the effect of each 5-HT antagonist, we compared the second 5-HT exposure in the presence of antagonist in one group of ganglia with the second 5-HT exposure without antagonist in another group of ganglia. Spiperone completely blocked facilitation of these depressed SN synapses (maximum facilitation = 7.3 ± 6.3%, \(P = 0.73\); Fig. 3). In contrast, there was significant facilitation by 5-HT in the presence of methiothepin (maximum facilitation = 62.1 ± 16.5%, \(P = 0.002\)), and in control saline (maximum facilitation = 110.7 ± 31.0%, \(P < 0.001\), pairwise comparisons with Sidak adjustment for multiple comparisons). Desensitization of the 5-HT pathway involved in facilitation of depressed SN synapses...
FIG. 3. Spiperone is highly effective in blocking 5-HT-induced reversal of synaptic depression. Using the synaptic depression protocol of Fig. 1, one SN-to-MN synapse was depressed and 5 μM 5-HT was applied. After 5-HT washout, a second SN-to-MN synapse within the same ganglion was identified and methiothepin or spiperone (100 μM) was superfused for 15 min. The synaptic depression protocol was then repeated in the presence of antagonist. A–C: examples of facilitation in ganglia in control saline (A1 and A2) spiperone (B), and methiothepin (C). A1: examples of EPSPs before and during a first application of 5-HT (in a ganglion that later was tested in the presence of spiperone); A2: facilitation by a second application of 5-HT in control saline (as in Fig. 1, i). [In each example in A–C, “Depressed” was immediately before the 5-HT and “5-HT” was at the peak of the facilitation response, 2–3 min after 5-HT onset.] D: group data for control (n = 7), spiperone (n = 10), and methiothepin (n = 9). Results are for the second exposure to 5-HT in the presence or absence of antagonist. (First exposure to 5-HT was always in the absence of antagonist.) Data are mean of experiments ± SE; within each experiment, 3 consecutive EPSPs were averaged. Spiperone completely blocked facilitation of these depressed synapses, whereas methiothepin did not significantly affect facilitation [repeated-measures ANOVA testing treatment × time interaction, **F(2,24) = 8.208, P = 0.002;** paired comparisons]. There were no differences between methiothepin-treated synapses and control at any time points; methiothepin-treated synapses were significantly different from spiperone-treated synapses only at the 158-s time point (P = 0.045). To test whether even minimal facilitation occurred in the presence of spiperone, we analyzed specific time points at which maximum facilitation was observed with pairwise comparisons; 5-HT in the presence of spiperone did not significantly facilitate these depressed synapses (P = 0.73), whereas 5-HT produced significant facilitation both in normal saline (P < 0.001) and with methiothepin (P = 0.002).

Methiothepin, but not spiperone, blocks cAMP-mediated spike broadening

Our previous assays on homogenized CNS membranes indicated that spiperone does not antagonize Aplysia AC-coupled 5-HT receptors (Cohen et al. 1997b). To measure the effects of spiperone on 5-HT stimulation of AC in intact neurons at physiological ionic strength, we assayed a modulatory effect of 5-HT that is mediated entirely by PKA: broadening of the SN action potential in the presence of 100 mM TEA and 20 μM nifedipine (Goldsmith and Abrams 1992; Jarrard et al. 1993; Sugita et al. 1997). TEA at 100 mM blocks I_{Ks,slow} (Baxter and Byrne 1989); the modulation of I_{Ks,slow} by 5-HT involves PKC as well as PKA (Goldsmith and Abrams 1992; Hochner and Kandel 1992; Sugita et al. 1992). Nifedipine at 20 μM blocks the dihydropyridine-sensitive Ca^{2+} current (Edmonds et al. 1990); the modulation of this Ca^{2+} current by 5-HT is mediated by PKC (Braha et al. 1993). TEA also blocks the Ca^{2+}-activated K^{+} current that is reduced by 5-HT, which may be modulated via either PKA or PKC (Critz and Byrne 1992; Walsh and Byrne 1989). Thus, in combination, TEA and nifedipine block the 5-HT-modulated currents except for the two S- K^{+} currents, I_{Ks,slow} and I_{Ks,steady state} (Baxter and Byrne 1989; Goldsmith and Abrams 1992). Direct modulation of these two K^{+} currents by 5-HT appears to occur exclusively via PKA. Therefore in TEA/nifedipine, 5-HT-induced spike broadening should be mediated entirely by cAMP. We used a protocol in which 5 μM 5-HT was applied initially in the absence of antagonist, and the 5-HT exposure was then continued in the presence of either spiperone, methiothepin, or vehicle. Sequential comparison within each SN enables more...
accurate assessment of the effects of antagonists (Goldsmith and Abrams 1992). These experiments were conducted on VC cluster SNs in the pleural ganglion. Confirming previous observations (Abrams et al. 1984; Jarrard et al. 1993), there was no desensitization of the spike broadening response to 5-HT in TEA over >7 min in the absence of antagonist (Fig. 4B).

Superfusion with spiperone in the presence of 5-HT did not reduce the spike broadening response (compared with the maintained spike broadening with 5-HT and vehicle without antagonist). In contrast, superfusion with methiothepin resulted in a 95.1 ± 13.0% reduction in the spike broadening response produced by 5-HT. In the presence of methiothepin, no significant spike broadening remained (Fig. 4, A and B). The lack of effect of spiperone confirms that in the short term, the spike broadening response to 5-HT in TEA/nifedipine is entirely dependent on PKA with no detectable contribution from PKC.

To exclude the possibility that differences in the efficacy of these two antagonists in blocking these two forms of modulation resulted from possible differences between groups of SNs, we also tested the efficacy of methiothepin in blocking 5-HT-induced spike broadening in LE SNs in the abdominal ganglion. In several pilot experiments, we found that 100 μM methiothepin did not completely inhibit the spike broadening response in TEA/nifedipine. At 150 μM, methiothepin effectively blocked spike broadening in the LE SNs by 92.4 ± 2.6% (Fig. 4C). We do not know whether this small difference in efficacy represents a difference in SN type or a difference between populations of animals (experiments on LE and VC cluster SNs were conducted at different periods). This difference need not reflect a difference in receptor affinity; when an antagonist only partially blocks a receptor population, the apparent efficacy of the antagonist may vary depending on how effectively the receptor activates a second messenger cascade (e.g., if there are differences in the level of expression of a kinase or other proteins in the cascade). As was observed with pleural ganglion SNs, in LE SNs, spiperone did not produce a reduction in the spike broadening response to 5-HT (Fig. 4C).

DISCUSSION

Spiperone is a selective antagonist of PLC-coupled 5-HT receptors in Aplysia

These electrophysiological experiments identified spiperone as an effective inhibitor of PLC-coupled 5-HT receptors in Aplysia SNs. Spiperone at 100 μM entirely blocked the reversal of depression produced by 5-HT at SN-to-MN synapses. This facilitation of highly depressed synapses has been found to be mediated by PKC, and not by PKA (Ghirardi et al. 1992; Manseau et al. 2001). Under the conditions tested (in high-divalent saline and with 5 μM 5-HT), spiperone is selective and does not affect the AC-coupled 5-HT receptor, as it did not inhibit the cAMP-mediated spike broadening initiated by 5-HT in TEA/nifedipine. Spiperone also does not bind with high affinity to a cloned 5-HT receptor, 5-HT_{ap1}, that is, negatively coupled to AC (Angers et al. 1998).

Modulation of the S-K^+ currents by 5-HT is independent of PKC

As described in RESULTS, the combination of TEA and nifedipine blocks the 5-HT-sensitive currents that are regulated
via PKC, the dihydropyridine-sensitive Ca\(^{2+}\) current, $I_{KV$-early}, and $I_{KCa}$. Therefore with TEA and nifedipine, 5-HT-induced spike broadening should be mediated entirely by modulation of the S-K\(^{+}\) currents ($I_{KS,slow}$ and $I_{KS,steady\ state}$). The observation that spiperone had no effect on spike broadening provides independent confirmation that the 5-HT-induced reduction in the S-K\(^{+}\) currents does not involve PKC, at least during 7 min of 5-HT exposure. There has been some confusion in the literature about the contribution of PKC to modulation of the S-K\(^{+}\) currents. Braha et al. (1993) found that activation of PKC with phorbol esters did not modulate the slowly activating S-K\(^{+}\) current ($I_{KS,slow}$). In addition, Goldsmith and Abrams (1992) found that Walsh inhibitor peptide, a specific antagonist of PKA, blocked the 5-HT-induced increase in excitability, which is mediated by modulation of the S-K\(^{+}\) currents. Reciprocally, Braha and colleagues (1993) observed that phorbol esters did not affect excitability in the short term. On the other hand, Sugita et al. (1992, 1997) observed a very modest increase in excitability with phorbol ester treatment for 9–15 min. However, blocking PKC with staurosporine did not significantly inhibit the excitability increase produced by 5-HT, suggesting PKC is not involved (Sugita et al. 1992). Sugita et al. (1997) also recorded SN spike durations using the same combination of TEA plus nifedipine as in the present study; however, because their experiments were intended to examine longer-term effects of phorbol esters on the response to 5-HT, they did not measure the effects of activating PKC on the TEA/nifedipine action potential. After prolonged phorbol ester treatment (30–60 min), they observed complex interactions between PKC and the cAMP cascade, making the modest effects of phorbol esters on excitability more difficult to interpret. Indeed, these authors suggested that the effect of phorbol esters on excitability at 9–15 min may have been mediated by an increase in cAMP levels. Manseau et al. (1998) found that a brief exposure to phorbol esters produced a persistent increase in excitability at both 3 h (which was independent of protein synthesis) and at 24 h (which required protein synthesis); early effects were not examined. Given the lack of a rapid effect of phorbol esters in the Braha et al. (1993) study, the intermediate-term change in excitability observed by Manseau et al. (1998) may have been due either to an indirect effect of PKC on ion channels or to a slowly developing effect, such as a modulation of ion channel trafficking. In contrast, effects of PKA and PKC can be much more rapid; photorelease of caged cAMP produces spike broadening within several seconds (Cohen et al. 2003a), and phorbol esters can facilitate depressed SN synapses within 30 s (Braha et al. 1990). In summary, our results confirm the original conclusion that short-term modulation of the S-K\(^{+}\) currents by 5-HT is mediated by cAMP, independently of PKC.

**Methiothepin blocks cAMP-mediated modulatory effects of 5-HT, with only minimal influence on PKC-mediated effects**

We replicated the observation of Cohen et al. (2003b) that methiothepin potently inhibits 5-HT-initiated spike broadening in TEA/nifedipine. We have now found that this block of AC activation by 5-HT is a relatively selective effect, in that methiothepin caused only a modest, nonsignificant decrease in the 5-HT-stimulated facilitation of highly depressed SN synapses (Fig. 3), which is largely or completely mediated by PKC (Ghirardi et al. 1992; Manseau et al. 2001). This modest effect of methiothepin could be a consequence of a lower affinity interaction between methiothepin and the PLC-coupled 5-HT receptor or of the contribution of PKA to facilitation of moderately depressed synapses (Ghirardi et al. 1992; Goldsmith and Abrams 1991). Methiothepin slowed the rise of the facilitation response. This is reminiscent of the slowing of the development of facilitation of depressed synapses observed in experiments where PKC activity was inhibited by presynaptic expression of dominant negative PKC (Manseau et al. 2001). The similarity of these effects on the facilitation time course suggests that activation of the PLC-PKC pathway may be reduced by methiothepin. With less powerful serotonergic input or with a different assay, this possible effect of methiothepin on the PLC-PKC-coupled 5-HT receptor could be more substantial; however the 5 $\mu$M bath concentration of 5-HT used in the present experiments has been estimated to produce a concentration of 5-HT in the neuropil that is at the low end of the range of concentrations measured after tail nerve shock (Marinesco and Carew 2002). Alternatively, once synaptic depression has been reversed, PKA-mediated effects may also contribute to facilitation, and these would be reduced by methiothepin. It is worth noting that using a PKA antagonist, Rp-cAMPS, Ghirardi et al. (1992) similarly observed a modest, nonsignificant reduction in 5-HT-induced facilitation of highly depressed SN synapses. In any case, methiothepin is not entirely selective as it also binds with high affinity to two cloned receptors that are negatively coupled to AC, 5-HT$_{A1}$, and 5-HT$_{ap2}$ (Angers et al. 1998; Barbas et al. 2002).

**5-HT activation of PKC-dependent reversal of synaptic depression shows rapid desensitization, in contrast to cAMP-mediated effects**

Typically, during continuous testing of a previously depressed synapse in the maintained presence of 5-HT, the synaptic connection gradually declines (e.g., Fig. 1) (Goldsmith and Abrams 1991). However, in previous experiments with a single synapse and a single 5-HT exposure, one could not distinguish whether this decrement of the facilitated EPSP was due to further synaptic depression or to desensitization of the 5-HT response. Our comparison of responses to two consecutive 5-HT exposures using two highly depressed SN synapses demonstrates that there is desensitization. Two of our other observations are consistent with the conclusion that desensitization during exposure to 5-HT is primarily responsible for the decrement of the facilitated SN-to-MN EPSP: 1) during 7 min of continued exposure to 5-HT, the amplitude of the EPSP decayed to the same level as the EPSP when facilitation by 5-HT was blocked with spiperone (Fig. 3B), suggesting that the facilitatory effect has largely desensitized. 2) Facilitation during the second exposure to 5-HT had a briefer duration than during the first exposure. In summary, these data provide clear evidence for desensitization of the PLC-PKC modulatory pathway that mediates reversal of synaptic depression at these SN-to-MN synapses. This was at first unexpected because it has been thought that PKC-mediated plasticity in SNs develops with a slower time course than cAMP-mediated plasticity (Byrne and Kandel 1996; Manseau et al. 1998). In contrast, the results in Fig. 3D suggest that the facilitation of depressed synapses that is blocked by spiperone, and presumably is
PLC-mediated, reaches a plateau within 2 min. In their studies of depressed SN-to-MN synapses in culture with dominant negative PKC, Manseau et al. (2001) similarly observed that the 5-HT-induced facilitation that was mediated by PKC ApII reached a peak rapidly, within 1 min. Differences in the time course of development of PKC-dependent modulation may depend on the type of modulatory effect being studied (i.e., increased excitability and spike broadening versus facilitation). Alternatively, a slower onset of modulation may occur when ganglia are exposed to phorbol esters because of slow penetration of these lipophilic compounds (compare Goldsmith 1991 with Braha et al. 1990). In considering the locus of desensitization, we cannot distinguish whether the decrement of the facilitation response we observed is due to downregulation of 5-HT activation of PKC or to downregulation of a step downstream from PKC in the intracellular cascade that mediates reversal of synaptic depression. However, consideration of the results of Manseau et al. (2001) suggests that desensitization is dependent on phosphorylation by PKC. In their experiments on synapses in culture, facilitation by 5-HT peaked early and then declined within ~2 min (even more rapidly than in Fig. 2); in contrast, in SNs expressing dominant negative PKC ApII, peak facilitation reached only approximately one third of the control level, but it rose much gradually, showing no sign of desensitization. If receptor occupancy resulted in desensitization independently of PKC activation, then the most likely facilitation in the presence of dominant negative PKC ApII should have peaked at the same early time point as in controls. The proposal that phosphorylation by PKC is required for the desensitization process is consistent with the interpretation that methiothepin produces a modest inhibition of the PLC-PKC pathway. In the presence of methiothepin, peak facilitation was not significantly reduced, but the rise of facilitation was slowed so that it peaked at a later time than in controls.

Summary

In conclusion, an effective analysis of the contribution of these two classes of 5-HT receptors to plasticity in Aplysia CNS could be achieved through parallel experiments with methiothepin and spiperone. This use of spiperone and methiothepin in comparative experiments permits a more thorough dissection of the contribution of AC- and PLC-coupled 5-HT receptors to a given phenomenon. This comparative approach is important because with endogenous release of 5-HT, where local concentrations may be higher (Marinisco and Carew 2002) or in individual preparations where the sensitivity to 5-HT may be greater than in the present study, the blockade by the antagonist that affects a particular process may be incomplete; only by examining the effects of both antagonists can one obtain an estimate of the relative contribution of the two classes of 5-HT receptors. It should be noted that methiothepin also affects dopamine receptors in gastropod mollusks (Drummond et al. 1978; Green and Cottrell 1997; Lukyanetz and Kostyuk 1996; Pechenik et al. 2002). This will not be a problem in cases where dopamine is found not to mimic the phenomenon being investigated (e.g., Abrams et al. 1984). It might seem that a more direct approach would be the use of protein kinase inhibitors. However, in experiments that involve intact ganglia, it is difficult to be certain that extracellularly applied inhibitors reach sufficient concentrations intracellularly to completely block a pathway. For example, inhibitors such as Rp-cAMPS have limited membrane permeability (Botelho et al. 1988). Therefore for electrophysiological experiments on intact ganglia or for behavioral experiments on semi-intact preparations, 5-HT receptor antagonists offer some advantages compared with the use of inhibitors of signaling cascades. Furthermore, inhibitors of signaling cascades do not address the involvement of 5-HT. The use of methiothepin and spiperone in parallel in experiments on intact ganglia or semi-intact preparations should enable the evaluation of the contribution of 5-HT both to the induction phase and to the maintenance phase of various forms of plasticity, something that has been previously possible only with chronic depletion of 5-HT stores (Glanzman et al. 1989). Indeed, as mentioned in the introduction, previously there has been substantial use of 5-HT receptor antagonists in Aplysia with relatively limited analysis of their pharmacology. The characterization of the sensitivity of the PLC- and AC-coupled receptors described in the present study and by Cohen et al. (2003b) should also provide a useful fingerprint in the future for the identification of cloned 5-HT receptors.

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References


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