Serotonin Enhances the Excitatory Acetylcholine Response in the RB Cell Cluster of *Aplysia californica*

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The RB cells, an identified cluster of serotonergic neurons in the abdominal ganglion of *Aplysia californica*, are excited by ACh. We have found that serotonin (5-HT) enhances the ACh responses recorded from the RB cells. The locus and specificity of the serotonergic interaction with the ACh response were analyzed using the conventional two-electrode voltage-clamp technique. We compared the current responses evoked in RB cells by constant-current iontophoretic pulses of ACh in control solutions and in solutions containing various concentrations of 5-HT. Serotonin consistently enhances the ACh evoked response in a dose-dependent manner while simultaneously directly activating the RB cells by turning on a small steady-state inward current. The dose-response characteristics for the 5-HT-mediated steady-state current differ, however, from those of the 5-HT-induced ACh response facilitation.

The ACh response enhancement appears to be an effect of 5-HT upon RB cell ACh receptors since (1) the enhancement is present under conditions that eliminate presynaptic input, and (2) 5-HT is still capable of enhancing the iontophoretically evoked response when the AChE-resistant agonist carbachol is substituted for ACh.

We tested other neurons in the *Aplysia* CNS exhibiting ACh evoked responses qualitatively similar to the RB cell ACh responses; 5-HT does not enhance the iontophoretically evoked ACh responses of these cells. Also, although the RB cells are responsive to dopamine and histamine, these putative neurotransmitters do not enhance the ACh response.

Serotonin (5-HT) has an important function as a conventional, nonconventional, and modulatory neurotransmitter in *Aplysia californica*. Gerschenfeld and his colleagues have described a multiplicity of serotonergic receptor types in molluscan cells, including *Aplysia* (for a review, see Gerschenfeld et al., 1981). Some of these 5-HT receptors behave like conventional transmitter-gated channels: 5-HT-binding to receptors initiates a conductance increase to sodium (A and A' responses), potassium (B response), and chloride (C response) (Gerschenfeld and Paupardin-Tritsch, 1974a, b). Two nonconventional synaptic responses to 5-HT application, conductance decreases to potassium (α response) and to potassium and sodium (β response) were also initially described by Gerschenfeld and Paupardin-

Tritsch (1974a, b). Recently, Walsh and Byrne (1985) analyzed a 5-HT-initiated decreased conductance to potassium in *Aplysia* L14 motor neurons, which may be mediated by cyclic AMP.

Serotonin also modulates several different types of voltage-gated channels in the *Aplysia* nervous system. Kandel, Schwartz, and their colleagues have demonstrated that 5-HT induces a long-lasting closure of a unique type of potassium channel in abdominal ganglion sensory cells (Klein et al., 1982; Siegelbaum et al., 1982) that is mediated by a cyclic AMP-dependent phosphorylation cascade (Bernier et al., 1982; Brunelli et al., 1976; Castellucci et al., 1980, 1982; Shuster et al., 1985). Pollock et al. (1985) have recently shown that 5-HT closes a similar type of potassium channel in pleural ganglion sensory cells. This effect is also mediated by a cyclic AMP-dependent process. Levitan and his colleagues have described a 5-HT-induced increase in membrane potassium conductance in cell R15, which is mediated by a cyclic AMP-dependent protein phosphorylation (Adams and Levitan, 1982; Drummond et al., 1980). Finally, Pellmar and Carpenter have described a 5-HT-dependent opening of voltage-gated calcium channels in the RB and LB cell clusters (Pellmar, 1984; Pellmar and Carpenter, 1980) that does not appear to be mediated by a cyclic AMP-dependent mechanism (Pellmar, 1981).

Serotonin also modulates the activity of buccal (Ram et al., 1981; Weiss et al., 1978) and heart (Liebeswar et al., 1975) muscle in *Aplysia*, with cyclic AMP mediating the effect of 5-HT in both muscle tissues (Mandelbaum et al., 1979; Sawada et al., 1984; Weiss et al., 1979).

We have extended the analysis of the modulatory role of 5-HT in the *Aplysia* CNS by describing a novel serotonergic modulation of the excitatory cholinergic receptors in the RB cell cluster. The RB cells are a homogeneous cluster of serotonergic cells (Eisenstadt et al., 1973; Goldstein et al., 1984; Kistler et al., 1985) located in the caudal right quadrant of the dorsal abdominal ganglion. One identified cell of this cluster, the RB_{HE} neuron, increases heart activity during food arousal (Koch et al., 1984). The RB cells receive direct input from the cholinergic neurons L10, L24, and Int XIII (Kandel et al., 1967; Segal and Koester, 1982). Iontophoretic application of ACh to RB somas elicits an excitatory response due to ACh receptor-gating of channels that are relatively nonselectively permeable to cations (Ascher et al., 1978; Simmons, 1983). In this paper we demonstrate that bath-applied 5-HT selectively enhances the response of the RB cells to iontophoretic application of ACh. A preliminary report of these results has already been presented (Simmons, 1984).

Materials and Methods

Animals

Aplysia californica, ranging from 20 to 100 gm, were obtained from various sources: Sea Life Supply (Sand City, CA), Marinus (Westchester,

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CA), Pacific Biomarine (Venice, CA), and from eggs raised at the mariculture facility at the Howard Hughes Medical Institute Marine Biological Laboratory (Woods Hole, MA). The results discussed in this paper were consistently observed in all *Aplysia*, regardless of their origin. The animals were maintained in commercially produced artificial seawater (ASW) (Instant Ocean, Aquarium Systems, Mentor, OH) at 15°C.

Dissection

Cholinergic receptors of the RB cell cluster, cell R15, unidentified cells in the LC cell cluster in the abdominal ganglion, and the anterior cell cluster of the left pleural ganglion were investigated in this study. The cells and cell clusters were identified using the criteria outlined by Frazier et al. (1967), Koester and Kandel (1977), and Ascher (1972). After pretreating the ganglia in 0.5% glutaraldehyde in ASW for 30–45 sec to inactivate the sheath muscle, the surface cells were exposed by desheathing. In some experiments, the RB cell clusters were tied off (using fiber strands of unwaxed dental floss) and separated from the rest of the ganglion in order to facilitate space-clamping and to eliminate spontaneous synaptic input.

Solutions and perfusion system

The desheathed ganglia were pinned in Sylgard- (Dow Corning, Midland, MI) containing dishes and maintained in an open, continuously perfusing system of ASW. Inflow was gravity controlled while the effluent was removed by suction. Temperatures ranged from 17 to 22°C across preparations. A Cambion (Cambridge, MA) temperature-controlled Peltier plate held the temperature variation within preparations to $\pm 0.5^\circ\text{C}$. The bath volume was 1.5 ml with a turnover rate between 1 ml/7 and 1 ml/30 sec (across preparations). In order to determine the turnover kinetics of the perfusion system, we conducted experiments introducing a dye into the perfusate. After infusing the dye, the perfusate was periodically sampled using a Beckman (Palo Alto, CA) spectrophotometer. The dye was first observed in the bath within the range of 20 sec (1 ml/7 sec) to 3.5 min (1 ml/30 sec) after the start of infusion. At the fastest fluid turnover rate, a 95% change of the bath volume required approximately 2 min, while the slowest turnover rate required 10 min. The majority of these experiments were conducted at an intermediate perfusion rate (1 ml/12–20 sec), with new solutions reaching the bath in 1–3 min, initiating a 95% change in bath volume in 4–8 min.

Normal ASW contained (in mM) NaCl, 460; KCl, 10; CaCl_2 , 11; MgCl_2 , 55; and HEPES buffer, 10 (titrated to pH 7.4 with NaOH). In some experiments we used a high-divalent cation ASW consisting of (in mM) NaCl, 317; KCl, 10; CaCl_2 , 33; MgCl_2 , 165; and HEPES buffer, 10 (pH 7.4). Immediately prior to each experiment, the various putative neurotransmitters and drugs (all from Sigma Chemical Co., St. Louis, MO) were mixed into normal ASW at the required concentrations for perfusion into the bath. These included 5-hydroxytryptamine creatinine sulfate (serotonin) (5-HT), 2(4-imidazolyl)ethylamine dihydrochloride (histamine), 3-hydroxytyramine (dopamine), and creatinine sulfate. Serotonin was maintained in an acidic stock solution (2×10^{-2} M 5-HT in 10^{-1} M acetic acid) and diluted to the desired concentration in ASW. For concentrations higher than 10^{-4} M 5-HT, the transmitter was directly dissolved in ASW. Acetylcholine chloride and the cholinergic agonist carbamylcholine chloride (carbachol) (both from Sigma Chemical Co.) were dissolved to a 1 M concentration in deionized water for iontophoresis.

Electrophysiology

The individual cells were voltage-clamped (Dagan, Minneapolis, MN) using two shielded electrodes containing 2.5 M KCl (2–5 M Ω). One electrode monitored the membrane potential of the cell, while the second electrode passed the current necessary to maintain the command holding potential. A third electrode, the iontophoretic micropipette containing ACh or carbachol (20–40 M Ω), was connected to a constant-current generator, which provided the constant, inward-bucking current (10–30 nA) needed to prevent ACh diffusion from the pipette. This pipette was visually positioned in close proximity to the cell body. Iontophoretic ejection of ACh was elicited by outward constant-current pulses of 2.5–4 sec duration (unless otherwise noted), with amplitudes ranging from 15 to 40 nA. The iontophoretic currents, clamp currents, and voltage traces were displayed on a Gould (Cleveland, OH) chart recorder and stored on an A. R. Vetter (Rebersburg, PA) FM tape recorder for further analysis.

Figure 1. Serotonin-enhanced ACh response in a representative RB cell. The RB cell was clamped to -60 mV and an iontophoretic pipette containing 1 M ACh positioned next to the cell body surface. Constant-current pulses of ACh were presented every 30 sec. The amplitudes of the ACh-induced clamp current responses were measured as the distance from the baseline current just before the response to the most negative value of current during the response. Each response, normalized to the mean of the control responses (defined as 100%), is plotted as a function of time for the initial control, experimental (10^{-5} M 5-HT), and washout periods. Insets, Current traces illustrating the response of the cells to iontophoretic ACh application in the different solutions (inward current is down). The slow downward trace is the ACh response. The fast inward current transients are due to spontaneous excitatory synaptic input. The ACh response almost doubles with 5-HT in the bath, and when returned to control ASW, reverses to control values.

Results

Serotonergic modulation of the ACh response in voltage-clamped RB cells

The RB cells were clamped at holding potentials between -50 and -60 mV (these cells normally exhibit a resting membrane potential of -40 to -45 mV in control ASW). Iontophoretic application of ACh onto voltage-clamped RB somas elicits a purely excitatory response, an inward current caused by the opening of ion channels that are permeable to cations. Constant-current pulses of ACh were presented every 30 sec while the preparation was being perfused with control and various experimental ASW solutions. Cells that did not display a stable ACh response in control ASW solutions were rejected. The slow exponential rise and decay of the ACh current responses in these experiments reflect the slow diffusion of ACh to and from cell body cholinergic receptors (see raw data insets of Fig. 1). In this paper we will use the term “receptor” to include both the ACh-binding component and the coupled ion channel.

Figure 1 depicts a representative experiment showing the time course and magnitude of the ACh response enhancement in response to 10^{-5} M 5-HT. The peak ACh-induced currents were measured from the holding current baseline recorded prior to each ACh pulse. The ACh responses have been normalized to the ACh responses evoked during the initial control period (defined as 100%). In this experiment, with a bath turnover rate of 1 ml/20 sec, approximately 2.5 min after the addition of 5-HT there is an augmentation in the amplitude of the response to ACh. The enhancement becomes maximal 6–7 min after infusing 5-HT/ASW, with the ACh response returning to control values after 14–15 min in control ASW. The insets show raw data current records observed in the initial control, experimental, and washout conditions. The slow downward traces are the response of the cell to ACh; the fast activity superimposed upon the slow traces reflects spontaneous excitatory synaptic input.

Since a creatinine sulfate salt of 5-HT was used in these ex-

Figure 2. Dose-response characteristics of the 5-HT-mediated ACh response enhancement in an RB cell. This cell was voltage-clamped to -60 mV. The frequency of the ACh iontophoretic pulses was 0.033 Hz. Each data point is the mean of four time points after the ACh response had peaked in the respective 5-HT/ASW concentrations. SEM bars are within the filled circle symbols. The 5-HT-induced enhancement of the ACh response has a threshold between 10^{-8} and 10^{-7} M 5-HT, saturates around 10^{-4} M, and is depressed at 10^{-3} M 5-HT. The curve was fitted by eye.

periments, control experiments were required to determine whether the ACh response modulation was due solely or in part to the creatinine sulfate complex. We determined that creatinine sulfate does not produce the ACh response enhancement observed with 5-HT (data not shown).

The dependence of the enhancement of the ACh response on 5-HT concentration is illustrated in Figure 2. These results are from a representative experiment. Each point is the mean of four data points observed several minutes after the ACh response had stabilized in the different 5-HT/ASW concentrations. A higher 5-HT concentration was introduced every 20–30 min. The threshold for the 5-HT-induced enhancement of the ACh response occurs between 10^{-8} and 10^{-7} M 5-HT. The enhancement saturates around 10^{-4} M 5-HT and decreases as the 5-HT concentration increases. Obtaining dose-response curves by serially adding increasing concentrations of 5-HT was possible because, unlike the typical neuronal response to prolonged exposure of a neurotransmitter, the 5-HT-induced augmentation of the ACh response did not desensitize with low concentrations of 5-HT (less than 5×10^{-5} to 5×10^{-4} M across preparations). Preliminary experiments showed that, once the ACh response had attained its maximum enhancement in the experimental solution, there was no desensitization (or decrease in the 5-HT-induced enhancement) for periods up to 60 min. For higher concentrations of 5-HT, however, the initial enhancement of the ACh response was followed by a relative decline to a steady-state level of enhancement. In Figures 2 and 3, the enhancement of the ACh response at 10^{-3} M 5-HT was determined after the ACh response had stabilized for several minutes during the relatively depressed phase.

The perfusion rates utilized in these experiments were slow enough to blur the time boundary between a change in the bath concentration of 5-HT and the onset and development of ACh response modulation. Both the onset latency and the rate of 5-HT-induced ACh response enhancement could be explained by free diffusion and mixing of 5-HT in the bath. The recovery times necessary to reverse the ACh response enhancement during washout in control ASW depended upon the concentration of 5-HT: the higher the concentration, the longer the recovery rate. This slow reversal during washout could be a function of removal of 5-HT from inaccessible regions of the ganglion. If the washout of 5-HT were rate-limiting, this would explain the

Figure 3. Summary histograms with SEM bars illustrating the effect of various concentrations of 5-HT on RB cell responses to ACh across experiments. The numbers depict the number of cells studied at each 5-HT concentration.

longer time required for reversing the effects of higher concentrations of 5-HT.

Serotonin enhances the RB cell ACh response in a highly reproducible fashion; in 78 out of 79 RB cells (64 out of 65 *Aplysia*), 5-HT (10^{-7} – 10^{-3} M) significantly enhanced the iontophoretically evoked ACh response by a factor of 1.14–3.04 ($p < 0.001$; two-tailed t test). Figure 3 is a summary graph illustrating the modulatory effect of five concentrations (10^{-8} , 10^{-7} , 10^{-6} , 10^{-5} , and 10^{-3} M) of 5-HT upon the responses of RB cells to iontophoretic ACh across ganglia. Despite a large degree of variability across preparations, the enhancement at low concentrations of 5-HT and the relative decline of the enhancement at higher concentrations of 5-HT is still observed across ganglia.

Serotonin activates a small, steady-state inward current with dose-response characteristics different from those of the ACh response enhancement

In unclamped RB cells, 5-HT directly initiates a membrane-conductance change, producing a depolarization that leads to excitation. Pellmar (1984) has shown that one underlying component of this 5-HT-induced excitation is the opening of calcium channels. Using 2 sec command pulses in voltage-clamped RB cells, Pellmar demonstrated that the 5-HT-mediated conductance change is voltage-dependent, requiring that the cell membrane be depolarized to at least -40 mV.

In our voltage-clamp experiments, with clamp-holding potentials between -50 and -60 mV for the duration of the experiments (up to several hours), we sometimes observed a small net inward current during perfusion with 5-HT/ASW. The enhanced ACh response was superimposed upon this 5-HT-induced steady-state current. When compared with the 5-HT-sensitive ACh response modulation, however, the 5-HT-induced steady-state inward current displayed a different concentration dependence. The net inward current was observed only once in 26 experiments in response to 10^{-6} and/or 10^{-7} M 5-HT, concentrations that reliably produced an enhanced ACh response. In all 25 experiments in which the concentration of bath-applied 5-HT was 10^{-5} , 10^{-4} , and/or 10^{-3} M, a dose-dependent steady-state inward current (ranging from 0.5 to 6.0 nA) coincided with the ACh response modulation.

Figure 4 illustrates a representative long-term experiment

Figure 4. Long-term experiment illustrating the divergent concentration dependencies of the 5-HT-mediated steady-state inward current and the 5-HT-dependent ACh response enhancement in an RB cell. The holding potential was -60 mV. Constant-current ACh pulses were presented every 30 sec. The ACh response and the steady-state holding current were monitored simultaneously while infusing increasing concentrations of 5-HT into the bath. Each point is the mean of four time points (2 min), with the steady-state current being measured immediately prior to each ACh response. Inward current is depicted downward. The ACh response is facilitated at 10^{-7} and 10^{-6} M 5-HT, whereas the steady-state inward current does not appear until 10^{-5} M 5-HT. The two 5-HT-mediated processes diverge again in 10^{-4} M 5-HT (small arrow). The continued enhancement of the steady-state inward current coincides with a depression of the ACh response potentiation. The ACh response potentiation shows more depression in 10^{-3} M 5-HT. With the addition of control ASW, the ACh response becomes temporarily enhanced before returning toward control values (see large arrow), while the inward current demonstrates a smooth reversal towards control.

showing the lack of correlation between the amount of ACh response enhancement and the magnitude of the inward current as a function of the 5-HT concentration. The RB cell was clamped at -60 mV, and increasing concentrations of 5-HT were perfused into the bath. Both the steady-state holding current and the ACh response were monitored simultaneously. Each point is the mean of four time points (the steady-state current was measured immediately prior to each ACh response). The ACh response enhancement is observed exclusively at 10^{-7} and 10^{-6} M 5-HT. At 10^{-5} M 5-HT, the steady-state inward current appears. The ACh response enhancement and the inward current are still visible when 10^{-4} M 5-HT is perfused into the bath. During the incubation in 10^{-4} M 5-HT, however, the ACh response enhancement begins to show the characteristic decline, while the steady-state current continues to increase. During the initial phase of washout in control ASW, the ACh response is momentarily greater, presumably because of the removal of the depressant effects of high concentrations of 5-HT, before returning towards control values. Meanwhile, the steady-state current declines along a smooth time course towards control (see large arrow). Incubation in high concentrations of 5-HT for such long periods necessitated a very long washout period (on the order of hours) to return to original control values; the cell usually died before a complete return could be observed. The divergence between the steady-state current and the ACh response enhancement with high concentrations of 5-HT was not observed consistently. In 70% of the experiments, concentrations of 5-HT that initiated a decline in the ACh response enhancement also produced a smaller steady-state inward current. This inward current subsequently became temporarily larger during washout in control ASW before disappearing.

Serotonin modulates the ACh response by affecting the cholinergic receptor

With a protocol involving bath application of 5-HT, it is conceivable that 5-HT acts via an unknown interneuron, which in turn modulates the RB cell ACh response. We found, however, that 5-HT consistently enhanced ACh responses when (1) the

preparations were maintained in high-divalent-cation ASW to eliminate possible interneuronal input, and (2) the RB cell cluster was tied off and separated from the rest of the ganglion, thereby removing presynaptic input. Figure 5 illustrates the continued presence of the 5-HT-mediated enhancement of the ACh response in tied-off RB cells and in RB cells maintained in high-divalent-cation ASW for the duration of the experiment. In Figure 5A, addition of 10^{-5} M 5-HT produces the characteristic enhancement of the ACh response. The steady-state inward current (see Fig. 4) is present in high-divalent-cation ASW (the dashed line represents the current baseline of the voltage-clamped cell in control ASW). Figure 5B illustrates the enhancement observed when 10^{-6} M 5-HT is added to a representative tied-off RB cell. The constant-current ACh pulses were 61 sec in

Figure 5. Serotonin enhances the ACh response in RB cells maintained in a high-divalent ASW and in tied-off RB cells. *A*, Representative raw data for ACh responses evoked in control ASW (1) and in ASW containing 10^{-5} M 5-HT (2). The dashed line in (2) is the control holding-current baseline. The steady-state net inward current response to 5-HT is still present in cells incubated in a high-divalent ASW that depresses interneuronal activity. The representative raw data in *B* are ACh responses elicited by 61 sec duration iontophoretic current pulses in control ASW (1) and in 10^{-6} M 5-HT/ASW (2) in tied-off cells. The ACh response is enhanced, but 10^{-6} M 5-HT is below threshold for the steady-state inward current (see text).

Figure 6. Serotonin (10^{-5} M) enhances the excitatory response to carbachol iontophoresis in RB cells. In this experiment the RB cell was clamped at -60 mV, and the iontophoretic pulses of carbachol were presented every 30 sec.

duration. In this experiment, 10^{-6} M 5-HT is below threshold for the steady-state inward current (see Fig. 4). Using tied-off cells also reduces the possibility that the enhanced ACh response in 5-HT/ASW might be due to the interaction of ACh with membrane that is under poor clamp control.

Serotonin may enhance the ACh response by interacting with a molecular component that is closely associated with the ACh receptor. For example, rat brain AChE activity is inhibited by 5-HT (Fujimoto, 1976). Figure 6 shows a representative experiment demonstrating that the responses of RB cells to iontophoretic application of carbachol, an AChE-resistant cholinergic agonist, continue to show enhancement in the presence of bath-applied 5-HT.

Enhancement of the ACh response in RB cells is specific to 5-HT

RB cells inhibited by bath application of other putative neurotransmitters, such as dopamine (Ascher, 1972) and histamine (unpublished data). Infusing up to 10^{-4} M dopamine or histamine into the bath did not increase the response to iontophoretic

Figure 7. Dopamine and histamine do not mimic the 5-HT-induced facilitation of the ACh response in RB cells. The membrane potential of this RB cell was held at -60 mV. Iontophoretic pulses of ACh were presented every 30 sec. Each point is the mean of two data points (1 min). The ACh responses are normalized to current responses evoked by ACh in control ASW (Con). Dopamine (10^{-5} M) and histamine (10^{-5} M) do not change the ACh response, but 2×10^{-6} M 5-HT produces the characteristic facilitation of the ACh response.

Figure 8. Serotonin does not modulate the excitatory ACh response in R15 cells. This summary figure shows the effect of 10^{-5} – 5×10^{-5} M 5-HT on the ACh response in cell R15. The membrane potentials were held between -50 and -60 mV. Iontophoretic pulses of ACh were presented every 30 sec. Each point represents the mean and SEM of five R15 cells in control and experimental conditions.

pulses of ACh. Figure 7 illustrates a representative experiment showing the effects of 10^{-5} M dopamine and 10^{-5} M histamine on an RB cell that is voltage-clamped at -60 mV. At this holding potential, both transmitters elicit a steady-state outward current that inhibits the unclamped cell (data not shown), but the ACh response is unchanged. Subsequent addition of 2×10^{-6} M 5-HT produces the characteristic enhancement of the ACh response.

Serotonergic enhancement of the ACh response is not a universal feature of *Aplysia* excitatory ACh receptors

In the *Aplysia* CNS, there are cells other than RB cells that exhibit the same type of pure excitatory response to somatic ACh iontophoresis, presumably because the same type of receptor is being activated. One such cell is R15, a large peptidergic neurosecretory cell located in the dorsal abdominal ganglion, and usually partially surrounded by the RB cell cluster (Frazier et al., 1967). Levitan and his colleagues have demonstrated that 5-HT increases a distinct potassium conductance, thereby changing the bursting characteristics of the cell (Drummond et al., 1980). Figure 8 is a summary figure illustrating the effects of 10^{-5} M to 5×10^{-5} M 5-HT on the evoked responses to iontophoretically applied ACh in five R15 cells. As was previously reported by Tremblay et al. (1976), 5-HT does not change the response of the R15 cells to iontophoretically applied ACh.

We tested other *Aplysia* neurons with excitatory ACh responses for possible serotonergic modulation. Cells in the left anterior pleural ganglion display a pure excitatory response to iontophoretic ACh application (Ascher, 1972). In our experiments, with the holding-clamp potential between -55 and -60 mV, up to 10^{-4} M 5-HT did not change the response of the cells to iontophoretic application of ACh. There was, however, variation in the direct responses of the anterior cells to 5-HT. In some instances, 5-HT turned on an inward current; on other occasions, 5-HT initiated a steady-state outward current. These results are consistent with other evidence that cells of the anterior pleural cell cluster are somewhat heterogeneous in their properties (Kehoe, 1972). Unidentified cells in the abdominal ganglion LC cell cluster with excitatory responses to iontophoretic ACh application were also tested for possible 5-HT modulation of the ACh response. As with the RB cells, serotonin consistently activated an inward current (clamp potential at -60 mV), which elicited firing in the unclamped cell. But, unlike its effect on the RB cells, 5-HT did not modulate the ACh response of the LC neurons (data not shown).

Discussion

In vertebrates and invertebrates, 5-HT modulates the efficacy of CNS and PNS cholinergic synapses in a variety of ways. Studies show that 5-HT can inhibit or facilitate ganglionic transmission by depressing or enhancing presynaptic release (Dun and Karczmar, 1981; North et al., 1980; Wallis and Woodward, 1975). Serotonin can also indirectly modulate sympathetic ganglion transmission by increasing the membrane conductance of the postsynaptic cell to sodium and potassium, thereby "shunting" the cholinergic potential (Dun and Karczmar, 1981), or facilitating the ACh potential by decreasing membrane conductance (probably to potassium) (Dun and Ma, 1984; Dun et al., 1984).

A third means of modulation involves a 5-HT-dependent depression of subsynaptic cholinergic receptor sensitivities in the endplate and nonsynaptic regions of voltage-clamped frog sartorius muscle (Akasu et al., 1983) and on voltage-clamped frog sympathetic ganglion cells (Akasu et al., 1981; Koketsu et al., 1982). Akopyan et al. (1980) have demonstrated that 5-HT depresses the chloride-dependent responses elicited by bath-applied ACh in isolated, voltage-clamped neurons of the mollusk, *Limnaea stagnalis*. In this paper we show that 5-HT selectively enhances the cation-dependent response of voltage-clamped *Aplysia* RB cells to iontophoretic ACh application.

ACh activates three pharmacologically distinct receptors in *Aplysia*. The "fast" excitatory and inhibitory receptors are permeable to cations and chloride, respectively. The "slow" inhibitory receptors are selective for potassium (Kehoe, 1972). The cells of the RB cluster express the "fast" excitatory cholinergic receptors on their somas and on their physically distant synaptic regions (Segal and Koester, 1982). Bath-applied 5-HT facilitates the excitatory response of the RB cells to ACh. This potentiation is specific for 5-HT; other putative transmitters such as dopamine and histamine activate RB cell dopaminergic and histaminergic receptors without producing a concomitant increase in ACh response. Serotonin appears to be producing its effect by modulating the ACh receptor, since (1) the modulation is present both in cells bathed in high-divalent cation ASW and in tied-off cells, thereby ruling out the presence of an unidentified 5-HT-sensitive interneuron that might enhance RB cell cholinergic receptor activity, and (2) potentiation of carbachol-evoked excitatory responses also occurs with bath-applied 5-HT, eliminating the possibility that 5-HT is indirectly facilitating the ACh response by inactivating AChE.

This serotonergic modulation of ACh receptor activity is not a common property of all *Aplysia* cells with excitatory cholinergic receptors. The other cells tested—R15, unidentified anterior pleural cells, and unidentified LC cells—have excitatory ACh receptors that are not modulated by bath-applied 5-HT. If there is only one type of excitatory cholinergic receptor, as the pharmacological studies suggest, these results imply that there is something intrinsic to RB cells that mediates the 5-HT-dependent enhancement of the ACh response. Possibly, however, there is more than one type of excitatory ACh receptor in *Aplysia* only, one of which is modulated by 5-HT. A quantitative analysis of the conductance and kinetic characteristics of excitatory ACh-sensitive channels in different cells might unmask the different types.

The dose-response curves demonstrate a well-behaved relationship between 5-HT concentration and degree of ACh response facilitation. Drawing any conclusions regarding the shape of the dose-response curve is impossible, however, without knowledge of certain parameters, i.e., distance of the iontophoretic pipette from the cell surface, geometric configuration of the cell body, topographic receptor distribution, the ACh concentration profile, etc. (see, for example, Dreyer et al., 1978). At high 5-HT concentrations (5×10^{-5} to 10^{-3} M), the dose-

response curve saturates and shows a downward trend (see Fig. 2). The relative depression of the ACh response enhancement that occurs with increasing 5-HT concentrations may signal the introduction of a second, specific inhibitory process. A likely alternative, given these nonphysiological concentrations, is that 5-HT alters cholinergic receptor activity nonspecifically, e.g., by blocking the ACh-sensitive channels.

With the relatively slow methods of 5-HT bath application used in these experiments, the perfusion rate appears to determine the onset latency of the modulated ACh response and the rate of enhancement in 5-HT/ASW. This does not eliminate the possibility that a time-dependent second messenger is involved. For example, activation of the abdominal ganglion facilitator neurons induces a threefold increase in sensory cell cytosolic second-messenger concentrations within 30 sec (Bernier et al., 1982), a time period well below the resolution of our perfusion system. Cyclic AMP is a second messenger that, in at least some cases, mediates the serotonergic modulation of voltage-gated channels in *Aplysia*. Preliminary experiments to ascertain whether cyclic AMP is the second messenger for the 5-HT-mediated augmentation of the ACh response indicate that cyclic AMP is not involved (work in progress). Another common second messenger for numerous cell functions is calcium. Kumamoto and Kuba (1983) have described a long-lasting enhancement of sympathetic ganglion cell ACh receptor sensitivity that is dependent upon cytosolic levels of calcium. Preliminary experiments in our laboratory do not implicate cytosolic calcium as a second messenger for the 5-HT-mediated enhancement of RB cell ACh responses (work in progress).

We do not know the cellular mechanism(s) underlying the 5-HT-induced enhancement of the RB cell ACh responses. Whether 5-HT is interacting with RB cell ACh receptors directly to facilitate the ACh response, or via a second messenger, remains to be resolved. We also do not know if 5-HT is (1) modulating the receptor-binding affinity of the channels for ACh; (2) influencing ACh-channel kinetic and/or conductance parameters; and/or (3) modulating a secondary factor, i.e., rate of desensitization. Recent studies suggest that substance P accelerates the desensitization process of ACh receptors in ganglion cells (Role, 1984) and chromaffin cells (Clapham and Neher, 1984). We have begun to address such questions using the "cell-attached" patch clamp technique.

We observe a 5-HT-induced inward current at holding potentials more hyperpolarized (below -40 mV) than the voltage-dependent current described by Pellmar (1984). This small inward current appears to be a direct effect of 5-HT, since it was present when the preparation was maintained in a high-divalent cation ASW, as well as when the RB cell cluster was separated from the rest of the ganglion. Preliminary work indicates that this steady-state inward current is also voltage-dependent and requires 20–40 sec to peak during a command pulse. Such a slow 5-HT current would not have been observed using Pellmar's protocol of presenting 2 sec command pulses to voltage-clamped RB cells.

With low concentrations (less than 10^{-5} M) of 5-HT, we can observe facilitation of the ACh response without observing the steady-state inward current. This suggests that there is some degree of independence between the mechanisms underlying the modulated ACh response and the inward current; the ACh response enhancement is not dependent upon net current flow through the ionic channels mediating the steady-state inward current.

Until recently, all molluscan synaptic contact was thought to occur in the neuropile, distant from the cell body. The transmitter-sensitive receptors on molluscan somas were considered an extrasynaptic curiosity. Now immunocytochemical studies at the light-microscopic level show serotonergic fibers in close proximity to various *Aplysia* cell bodies, including RB somas

(Bailey et al., 1981; Goldstein et al., 1984; Kistler et al., 1985; Ono and McCaman, 1984; Schwartz and Shkolnik, 1981). These recent anatomical data suggest that somal receptors might have an important functional synaptic role in controlling overall cell activity.

In this paper, we have provided pharmacological evidence that 5-HT can modulate RB cell body excitatory ACh receptors. If this effect of 5-HT is physiological, it should be possible to identify serotonergic neurons that are presynaptic to the RB cells. One possible source of serotonergic input are the cells of the RB cluster, either via collaterals from one RB cell to another, or via RB cell collaterals onto themselves. We are also, at present, using the 5-HT-specific immunocytochemical map of Kistler et al. (1985) to test other possible candidate cells for direct serotonergic input onto RB cells. Whatever the modulatory role of 5-HT receptors on RB cells, 5-HT may have an important integrative function in *Aplysia* that goes beyond the direct activation of 5-HT-sensitive ionic channels. Recently, Shozushima (1984) demonstrated that 5-HT can depress the response of dopaminergic receptors in RB cells. Serotonin, therefore, appears to interact with channels sensitive to other putative neurotransmitters, and, by enhancing the excitatory ACh input and depressing the inhibitory dopaminergic input, to effectively increase RB cell excitability.

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