

Serotonin Acting via Cyclic AMP Enhances Both the Hyperpolarizing and Depolarizing Phases of Bursting Pacemaker Activity in the *Aplysia* Neuron R15

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Bath application of 5-HT, at concentrations below 10 μ M, enhances the amplitude of the interburst hyperpolarization in the *Aplysia* bursting pacemaker neuron R15. It is known that 5-HT acts via cyclic AMP to produce this effect by increasing the inwardly rectifying potassium current (I_K). Here, we report that further elevating the concentration of 5-HT produces an enhancement of the depolarizing phase of the burst cycle that can eventually lead to tonic spiking activity. Voltage-clamp studies reveal that high concentrations of 5-HT continue to increase I_K and, in addition enhance a voltage-gated inward current active near the action potential threshold. Pharmacological treatments and ion substitution experiments demonstrate that the inward current increased by high concentrations of 5-HT is a subthreshold calcium current (I_{Ca}).

The 5-HT-induced increase in I_{Ca} is mimicked by bath application of the adenylate cyclase activator forskolin or injection of 8-bromo-cyclic AMP and is potentiated by the phosphodiesterase inhibitor isobutylmethylxanthine. It is concluded that 5-HT, acting via the second messenger cyclic AMP, can increase both potassium and calcium currents in neuron R15. It is also shown that the 5-HT-induced increase in these 2 opposing voltage-gated currents not only produces complex changes in bursting activity, but also dramatically alters R15's response to inhibitory and excitatory stimuli.

The second messenger cyclic AMP mediates the actions of a wide variety of neurotransmitters and hormones on excitable cells (Siegelbaum and Tsien, 1983; Levitan, 1985). The actions of cyclic AMP vary between cell type and even within the nervous system of a single animal. In *Aplysia*, cyclic AMP produces excitatory effects in bag cell, sensory, RB, LB, and medial neurons, while it can inhibit spontaneous bursting in cell R15 (Kaczmarek et al., 1978; Klein and Kandel, 1978; Drummond et al., 1980; Pellmar, 1981; Kehoe, 1985a, b, 1986). The varying effects of cyclic AMP occur because cyclic AMP can modulate different ion channels in different neurons. Cyclic AMP has been shown to modulate 6 types of potassium channels, as well as

calcium, sodium, and chloride channels in molluscan neurons (Aldenhoff et al., 1983; Ewald and Eckert, 1983; Green and Gillette, 1983; Siegelbaum and Tsien, 1983; Connor and Hockberger, 1984; Hockberger and Connor, 1984; Kaczmarek and Strumwasser, 1984; Kehoe, 1985a, b, 1986; Lotshaw et al., 1986; Strong and Kaczmarek, 1986). In some cells, more than one ion channel is modulated. For example, cyclic AMP decreases 3 distinct potassium currents in *Aplysia* bag cell neurons to produce excitation (Kaczmarek and Strumwasser, 1984; Strong and Kaczmarek, 1986). In *Aplysia* medial neurons, 2 potassium currents are decreased and a sodium current increased (Kehoe, 1985a, b, 1986). Finally, in neuron R15, cyclic AMP increases the inwardly rectifying and calcium-activated potassium currents (Benson and Levitan, 1983; Ewald and Eckert, 1983). In all these cases, cyclic AMP produces its effects by modulating several ion channels which tend to act synergistically to produce either depolarization or hyperpolarization.

5-HT, at concentrations below 10 μ M, acts via cyclic AMP to enhance the interburst hyperpolarization produced by the bursting pacemaker *Aplysia* neuron R15 (Drummond et al., 1980). 5-HT causes this effect by increasing the inwardly (or anomalously) rectifying potassium current (I_K) (Benson and Levitan, 1983). Here, we report that higher concentrations of 5-HT also enhance the depolarizing phase of bursting pacemaker activity and can eventually lead to tonic spiking activity. This shift from bursting to beating activity is accompanied by an increase in the subthreshold calcium current (I_{Ca}). The 5-HT-induced increase in calcium current is shown to be mediated by cyclic AMP. These results demonstrate that 5-HT, acting via a single second messenger, cyclic AMP, can produce complex changes in bursting activity by increasing voltage-gated calcium and potassium currents. It is also shown that this modulation of opposing conductances alters the cell's responsiveness to inhibitory and excitatory stimuli.

Materials and Methods

Aplysia californica weighing between 100 and 500 gm were obtained from Alacritty Marine Biological Services (Redondo Beach, CA) and maintained at 16°C. Animals were injected with 50% of their body weight of 400 mM MgCl₂. The abdominal ganglion was then removed and the connective tissue sheath overlying cell R15 dissected away. In preliminary experiments, dissection of the sheath was facilitated by treating the ganglion for 1–10 min with 0.25% pronase (Calbiochem). All experiments were reproduced without enzyme treatment.

Cell R15 was identified by its location, pigmentation, and electrical activity (Frazier et al., 1967). R15 was impaled with 1–5 M Ω electrodes containing either 1.5 or 3 M KCl for measuring voltage and similar electrodes containing KCl or 0.5 M K₂SO₄ for passing current. Iontophoretic injection was performed under voltage clamp (Almost Perfect

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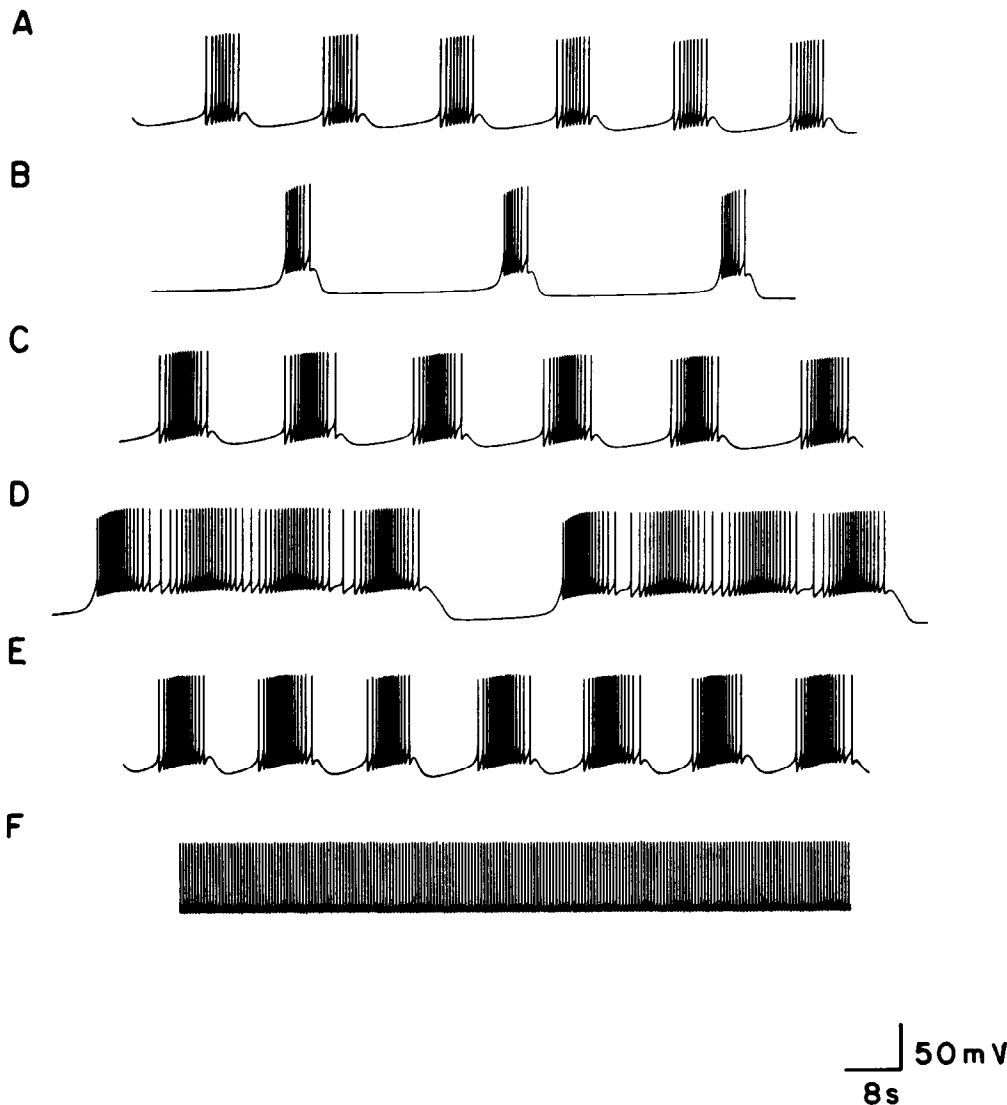


Figure 1. Effects of different concentrations of 5-HT on the bursting activity of cell R15. *A*, Control activity in 100 mM Mg SW. Peak interburst hyperpolarization was -46 mV. *B*, Same as *A* after 12 min exposure to $10 \mu\text{M}$ 5-HT. Peak interburst hyperpolarization was -68 mV. *C*, After washout of 5-HT. *D*, After 12 min exposure to $50 \mu\text{M}$ 5-HT. Peak interburst hyperpolarization was -64 mV. *E*, Control activity in another cell in ASW. *F*, Same as *E* after 15 min exposure to $25 \mu\text{M}$ 5-HT.

Electronics) from a third electrode containing 0.75 M potassium EGTA, pH 8, 2 M Cs_2SO_4 , or 0.1 M sodium 8-bromo-cyclic AMP. Iontophoresis of cesium and EGTA simultaneously was accomplished by using a Cs_2SO_4 current-passing electrode and an EGTA-filled iontophoresis electrode. Effectiveness of EGTA injections (typically 500 nA for 5 min) was verified by testing whether calcium-dependent tail currents and hyperpolarizations were blocked. Cesium was iontophored at 500 nA for 14 min.

The ganglion was superfused constantly at 1 ml/min in a 1 ml bath with artificial seawater (ASW) containing 460 mM NaCl, 10 mM KCl, 11 mM CaCl_2 , 55 mM MgCl_2 , 1% glucose, and 10 mM HEPES, pH 7.4. 5-HT was applied for 10 min unless otherwise stated. To decrease spontaneous synaptic activity, the concentration of MgCl_2 was raised to 100 mM at the expense of NaCl (100 mM Mg SW). No qualitative differences in the voltage-clamp data were observed in this solution. In other voltage-clamp experiments where maximal block of synaptic activity was desired without completely blocking subthreshold calcium channels, 2 solutions were used. One was similar to ASW except that the concentration of MgCl_2 was raised to 230 mM at the expense of NaCl (230 mM Mg SW). The other solution, which was similar to that used by Mayeri et al. (1985), contained 430 mM NaCl, 10 mM KCl, 75 mM MgCl_2 , 0.3 mM CaCl_2 , 3 mM MnCl_2 , 1% glucose, and 10 mM HEPES, pH 7.4 (0.3 mM Ca SW). The following solution (low Cl SW) was used when a lower external chloride concentration was desired: 392 mM sodium methylsulfate, 10 mM KCl, 50 mM MgCl_2 , 50 mM MgSO_4 , 11 mM CaCl_2 , 1% glucose, and 10 mM HEPES, pH 7.4. Tetraethylam-

monium bromide (TEA), 4-aminopyridine, manganese chloride, and cadmium chloride were added hypertonicity. Cobalt was substituted for calcium. Forskolin was added from a stock 30 mM solution in ethanol. Equivalent exposures of ethanol alone produced no effects. In some experiments, 0.5 mM hexamethonium bromide was included to block cholinergic synapses on R15.

5-HT (5-hydroxytryptamine creatinine sulfate), dopamine (3-hydroxytryptamine hydrochloride), sodium 8-bromoadenosine-3',5'-cyclic phosphate (8-Br-cyclic AMP), 3-isobutyl-1-methylxanthine (IBMX), *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES), and bistrispropane were obtained from Sigma. Forskolin was obtained from Calbiochem. Sodium methylsulfate was obtained from Kodak.

All experiments were replicated a minimum of 3 times, and the data shown are representative. The baseline bursting activity and voltage-clamp currents were stable over many hours, and all effects shown were readily reversible upon washing.

Results

5-HT can enhance both the depolarizing and hyperpolarizing phases of R15's bursting pacemaker activity

Bath application of $10 \mu\text{M}$ 5-HT leads to an enhancement of the depth and duration of the interburst hyperpolarization and can sometimes cause cessation of bursting activity in the *Aplysia* neuron R15 (Drummond et al., 1980). This is due to a cyclic

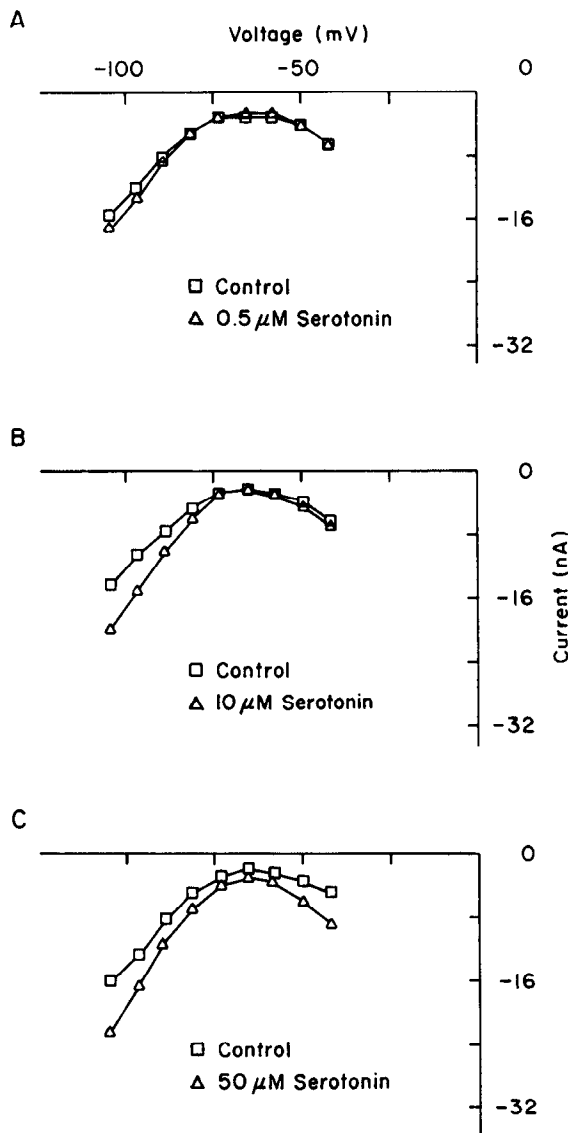


Figure 2. Effects of serotonin (5-HT) on the current versus voltage curve of neuron R15. The effects of $0.5 \mu\text{M}$ (A), $10 \mu\text{M}$ (B), and $50 \mu\text{M}$ (C) 5-HT on current evoked at the end of 1 sec pulses from a holding potential of -50 mV (\square , control; Δ , 5-HT). All data from a single cell.

AMP-dependent increase in I_R (Benson and Levitan, 1983). 5-HT at $500 \mu\text{M}$, on the other hand, has been found to depolarize R15 under current-clamp conditions (Tremblay et al., 1976). We have found that bath application of much lower concentrations of 5-HT (i.e., $25\text{--}50 \mu\text{M}$) can produce an enhancement of both the depolarizing and hyperpolarizing phases of the burst cycle. Figure 1A shows the basal bursting activity of R15 in the presence of elevated magnesium. Upon bath application of $10 \mu\text{M}$ 5-HT an enhancement of the interburst hyperpolarization is seen (Fig. 1B). After washing out the 5-HT and regaining basal bursting activity (Fig. 1C), $50 \mu\text{M}$ 5-HT was applied. Once again, an enhancement of the interburst is seen, this time accompanied by a large increase in the number of action potentials during the depolarizing phase (Fig. 1D). In normal ASW this depolarizing effect predominates so that the cell eventually ceases bursting and instead spikes tonically at a high frequency (Fig. 1F). Thus, increasing the concentration of 5-HT can convert its

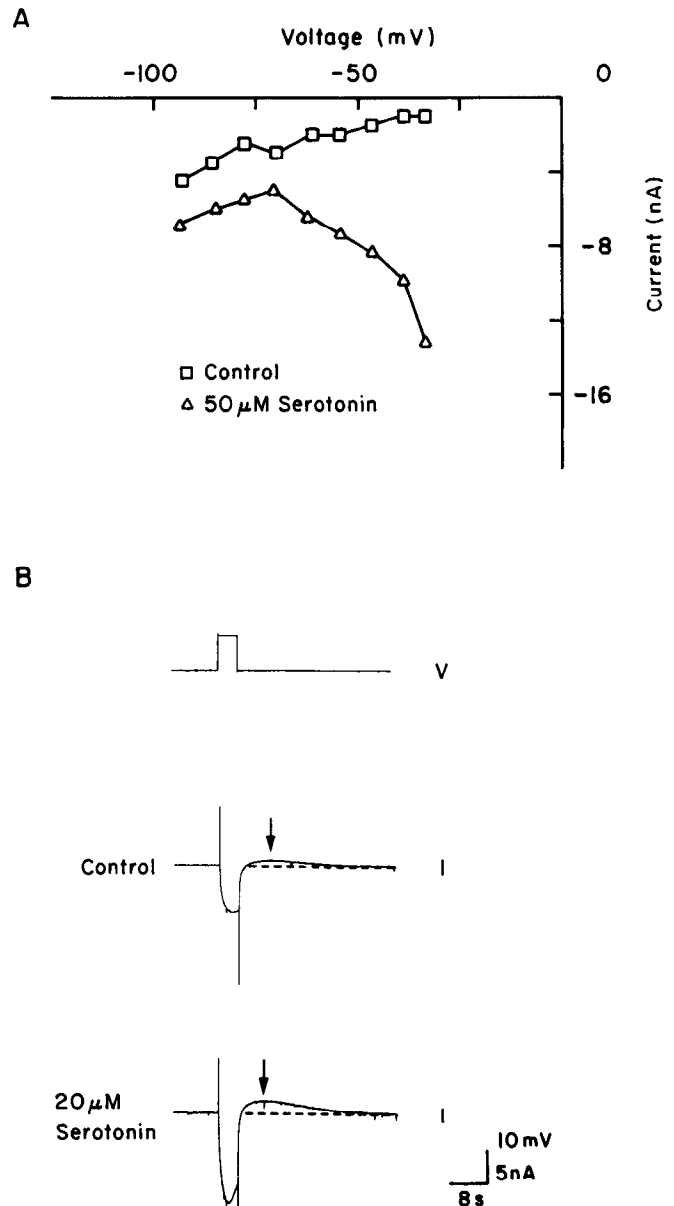


Figure 3. Effect of serotonin (5-HT) on the voltage-gated inward current and the calcium-dependent outward tail current. A, I_R was blocked by reducing external potassium to 2 mM in 0.3 mM Ca SW. Currents were evoked with 2 sec pulses from a holding potential of -40 mV : \square , control; Δ , after $50 \mu\text{M}$ 5-HT. B, Top trace shows a 10 mV depolarizing voltage pulse from -45 mV ; middle trace, the evoked current in the control; bottom trace, evoked current after 20 min exposure to $20 \mu\text{M}$ 5-HT. Note that 5-HT increases the voltage-gated inward current, as well as the slow tail current (arrows) that is outward relative to the holding current (dotted line).

effect from a predominantly inhibitory one to a predominantly excitatory one.

5-HT increases the subthreshold voltage-gated calcium and inwardly rectifying potassium currents

Voltage-clamp experiments were carried out to determine the ionic mechanism by which 5-HT modulates the bursting activity of R15. Bath application of $0.5 \mu\text{M}$ 5-HT produces an increase in a potassium current that activates with hyperpolarization [the inwardly rectifying potassium current (I_R); Benson and Levitan,

1983]. This change in current is most apparent at potentials more hyperpolarized than -75 mV, the potassium equilibrium potential (Fig. 2A). It is thought that increasing I_R leads to enhancement of the interburst hyperpolarization. Raising the concentration of 5-HT to 2 – 10 μM further increases I_R (Fig. 2B). In addition, this effect is accompanied by a small increase in the inward current at potentials more positive than -65 mV, the negative slope resistance region of the current versus voltage (I - V) curve (Fig. 2B). Increasing 5-HT to 50 μM produces little further change in I_R , indicating that the excitatory effects of 5-HT are not due to a reversal of the increase in that current. However, an additional increase in inward current is seen at depolarized potentials (Fig. 2C). This effect is also seen under conditions that dramatically lower synaptic activity but that leave calcium currents partially intact (Fig. 3A), as well as in totally isolated neuron R15 in culture (Lotshaw et al., 1986). Hence, it results from a direct effect of 5-HT on R15.

Blocking I_R with either low potassium (Fig. 3A) or barium (data not shown) reveals that 5-HT apparently increases 2 additional currents. One component is clearly voltage-gated and displays slow kinetics (Fig. 3B). It begins to activate between -70 and -60 mV and increases with depolarization. These characteristics are reminiscent of the subthreshold calcium current. A voltage-independent current is seen at more hyperpolarized potentials (Fig. 3A) and may be due to activation of a voltage-independent sodium current (Connor and Hockberger, 1984) or to activation of the same voltage-gated current described above but at sites remote from the cell body where voltage cannot be controlled. In favor of the latter interpretation is the finding that the voltage-independent current is not seen when R15 is maintained in primary cell culture and processes are removed prior to voltage clamping (D. P. Lotshaw and I. B. Levitan, unpublished observations). Furthermore, agents that reduce the voltage-gated current, such as cobalt, manganese, or dopamine (see below), also reduce the voltage-independent current induced more negative than -70 mV. Thus, it seems likely that 5-HT induces only one type of current in addition to I_R . We concentrated on studying the voltage-gated conductance at depolarized potentials which probably is responsible for producing 5-HT's excitatory effect.

The subthreshold inward current active at potentials more positive than -75 mV has been shown to be due to voltage-gated calcium channels that are inactivated by elevated internal calcium (Gorman et al., 1982; Lewis et al., 1984; Adams and Levitan, 1985; Kramer and Zucker, 1985b). Action potentials or depolarizing voltage pulses that allow calcium influx produce a slow outward tail current, which has been shown to be due to calcium-dependent inactivation of resting calcium current normally active at subthreshold voltages (Adams and Levitan, 1985; Kramer and Zucker, 1985b). We found that this outward tail current is increased along with the voltage-gated inward current by 5-HT (arrows in Fig. 3B). This effect is also seen in high-magnesium seawater (data not shown), where synaptic input is minimized (Mayeri et al., 1985). This result is consistent with the hypothesis that 5-HT increases a calcium current (I_{Ca}).

In order to test further whether 5-HT increases subthreshold I_{Ca} , calcium currents were blocked with either manganese, cobalt, or cadmium. Shown in Figure 4A is the effect of 5-HT on the slow inward current elicited by depolarization in normal saline in a cesium-loaded cell. As in Figure 3B, 5-HT nearly doubles the voltage-gated inward current. After washing out the 5-HT, manganese was added. This blocks the voltage-gated in-

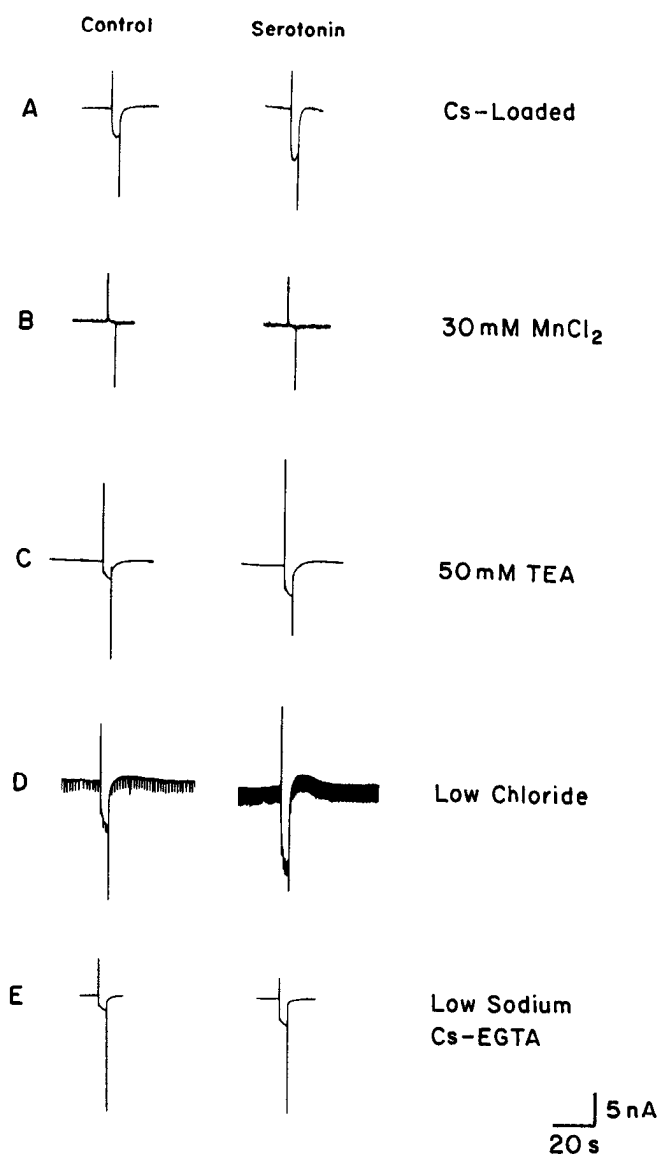


Figure 4. Pharmacology of the serotonin (5-HT)-induced voltage-gated inward current. In each case the current trace at left is the control; at right, after 3–12 min exposure to 50 μM 5-HT. *A*, Currents evoked by a 10 mV depolarizing pulse from -50 mV in a cesium-loaded cell. *B*, Currents in the same cell in the presence of 30 mM added MnCl_2 . Similar results were obtained when calcium was replaced with manganese or cobalt. *C*, Currents evoked in another cell by a 5 mV depolarizing pulse from -24 mV in 230 mM Mg SW containing 50 mM TEA. *D*, Currents evoked in another cell by a 5 mV depolarizing pulse from -50 mV in low-chloride SW. Downward rapid current deflections are due to a spontaneous cholinergic synaptic input. *E*, After iontophoretically loading R15 with cesium and EGTA, sodium was lowered to 50 mM by substitution with bistrispropane. Currents were then evoked by a 7 mV depolarizing pulse from -36 mV before and after exposure to 5-HT.

ward current (Fig. 4B, left). Application of 5-HT in the presence of manganese fails to produce any change in current (Fig. 4B, right). Similar results were obtained when calcium was replaced with manganese or cobalt in cells not loaded with cesium (data not shown). Addition of 1 mM cadmium was also found to block the 5-HT induced current (data not shown). These results suggest that 5-HT acts upon I_{Ca} and/or calcium-activated currents.

5-HT might increase the voltage-gated inward current by de-

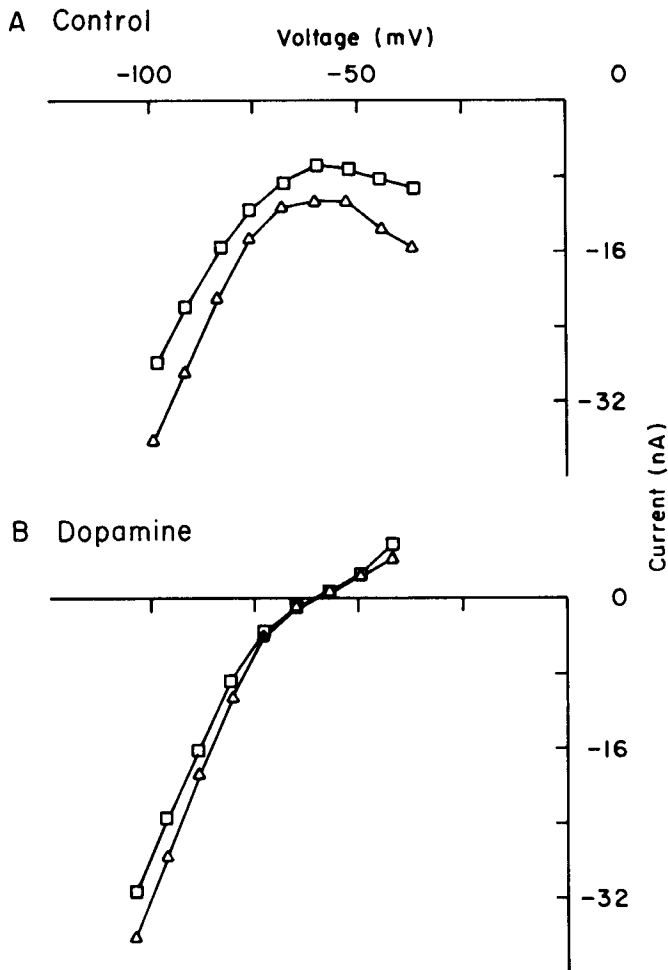


Figure 5. Dopamine attenuates the 5-HT-induced increase in the subthreshold inward current. *A*, Effect of $20\ \mu\text{M}$ 5-HT on R15's I - V curve; \square , control; \triangle , 5-HT. *B*, Effect of 5-HT in the presence of $250\ \mu\text{M}$ dopamine; \square , control; \triangle , 5-HT.

creasing outward potassium currents, although this would produce a decrease rather than the observed enhancement (Fig. 3*B*) of the outward tail current. External application of 50 mM TEA does not block the 5-HT-induced increase in the voltage-gated inward current (Fig. 4*C*) despite the fact that this concentration is sufficient to block 99% of the calcium-activated potassium current and 89% of the delayed rectifying potassium current in R15 (Hermann and Gorman, 1981). Application of 5 mM 4-AP also leaves the 5-HT effect intact (data not shown).

At least 2 potassium currents that are insensitive to TEA and 4-AP but that are blocked by substitution of external calcium with cobalt are known to exist in *Aplysia* neurons. One of these is a TEA-insensitive, calcium-activated potassium channel. Dietmer and Eckert (1985) reported that iontophoretic loading of cells with cesium blocks this current. Likewise, iontophoretically loading tail sensory neurons with cesium blocks a 5-HT-modulated potassium current that is sensitive to cobalt (Pollock et al., 1985). Cesium loading R15 to a similar extent, as judged by the measured block of the delayed rectifying potassium current (data not shown), does not block the 5-HT-induced increase in the subthreshold inward current (Fig. 4*A*). These results lead us to conclude that 5-HT does not act by decreasing potassium currents.

5-HT might produce an apparent increase in the subthreshold inward current by decreasing an opposing outward chloride current. In order to test this possibility, external chloride was reduced from 624 to 132 mM by substituting with methylsulfate, an ion that does not permeate acetylcholine-gated chloride ion channels in *Aplysia* (Kehoe, 1972). This reduction would be expected to shift the chloride equilibrium potential from approximately $-58\ \text{mV}$ (Russell and Brown, 1972; Ascher et al., 1976) to about $-19\ \text{mV}$, a potential well above the spike threshold. 5-HT still produces a robust increase in the inward current at $-45\ \text{mV}$ despite reversal of chloride currents (Fig. 4*D*), and thus cannot be acting by decreasing a chloride current.

Yet another possibility is that 5-HT activates a voltage-gated sodium current. Reduction of sodium 9-fold does indeed block 5-HT's effect on the inward current at potentials more positive than $-75\ \text{mV}$ (data not shown). However, it has been shown that removal of sodium also blocks the subthreshold calcium current unless the cell is loaded with EGTA and potassium currents are blocked (Gorman et al., 1982). This may occur because sodium removal might allow calcium entry (perhaps by inhibiting sodium-calcium exchange), which in turn leads to inactivation of I_{Ca} by internal calcium. We have confirmed that iontophoretic loading of R15 with cesium and EGTA, which buffers calcium and blocks potassium channels, dramatically slows the loss of the subthreshold inward current upon reduction of external sodium. Furthermore, under these conditions 5-HT still produces an increase in the subthreshold inward current in low sodium (Fig. 4*E*). This, together with the calcium channel blocker experiments, suggests that 5-HT does not activate a sodium current. Furthermore, 5-HT cannot be modulating some intracellular calcium-dependent process since these are blocked following EGTA loading while the 5-HT effect is still intact (Fig. 4*E*). These results leave the hypothesis that 5-HT increases I_{Ca} as the most viable one. A similar conclusion has been reached from studies of cell R15 maintained in primary culture where pharmacological isolation of calcium currents can be done more rigorously (D. P. Lotshaw and I. B. Levitan, unpublished observations). There it has been shown that 5-HT increases the subthreshold and suprathreshold I_{Ca} without changing its voltage dependence.

This conclusion is further reinforced by the finding that dopamine, which is known to decrease axonal I_{Ca} (Lewis et al., 1984), dramatically reduces the 5-HT-induced increase in inward current. Shown in Figure 5*A* are current versus voltage curves from a cell before and after exposure to 5-HT. The 5-HT-induced increases in I_{R} and I_{Ca} are evident. Application of a saturating concentration of dopamine blocks the axonal subthreshold I_{Ca} . This is seen in Figure 5*B* as a loss of the inward current at potentials positive to $-65\ \text{mV}$. Application of 5-HT in the continued presence of dopamine still produces some small increase in I_{Ca} (above $-50\ \text{mV}$) (Fig. 5*B*), but this effect is much less than in the absence of dopamine (Fig. 5*A*). The 5-HT-induced increase in slope conductance below $-75\ \text{mV}$, which is due to I_{R} , remains intact in the presence of dopamine. Thus, a transmitter known to decrease I_{Ca} in R15 reduces the ability of 5-HT to increase the subthreshold inward current. These data not only support the conclusion that 5-HT modulates calcium channels but also suggest that some of the calcium channels activated by 5-HT are inhibited by dopamine. These channels presumably reside in the axon or axon hillock regions of R15 where dopamine is known to act (Ascher, 1972; Lewis et al., 1984).

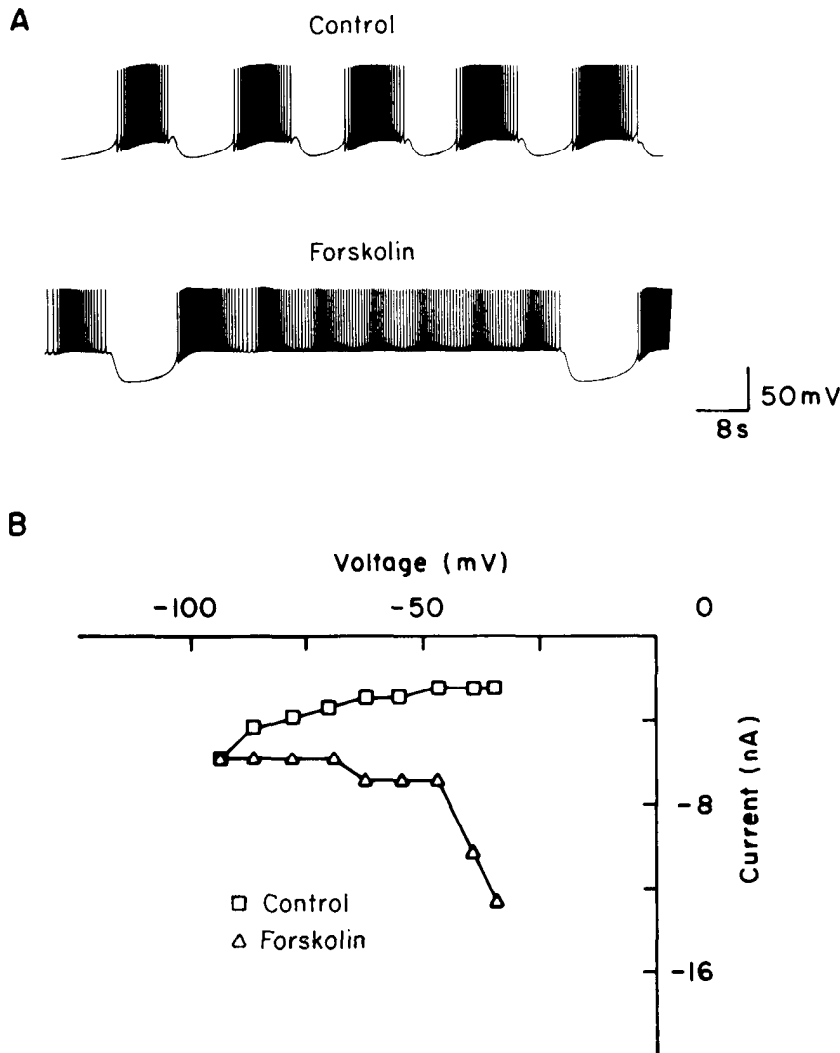


Figure 6. Effect of forskolin on bursting activity and on I_{Ca} . *A*, Top trace shows control bursting activity; bottom trace, bursting activity after 30 min in 80 μ M forskolin. *B*, Effect of forskolin on the I - V curve in 0.3 mM Ca SW containing 2 mM potassium to reduce I_R ; □, control; △, after 10 min exposure to 90 μ M forskolin.

5-HT acts via cyclic AMP to increase calcium current in R15

It has been demonstrated that 5-HT activates adenylate cyclase and elevates cyclic AMP in R15 (Cedar and Schwartz, 1972; Levitan, 1978). This, in turn, mediates 5-HT's effects on I_R (Drummond et al., 1980; Lemos and Levitan, 1984) via activation of cyclic AMP-dependent protein kinase (Adams and Levitan, 1982). A number of experiments were performed to test whether activation of adenylate cyclase is also responsible for the increase in I_{Ca} seen with higher levels of 5-HT. Forskolin, an activator of adenylate cyclase in many tissues, including *Aplysia* neurons (Seamon et al., 1981; Kauer and Kaczmarek, 1985), was bath applied to R15. Enhancement of bursting activity (Fig. 6*A*) and increases in I_R (data not shown) and subthreshold I_{Ca} (Fig. 6*B*) were seen even under conditions that minimized synaptic activity. Furthermore, the effects of 5-HT and forskolin occlude each other, suggesting that activating adenylate cyclase modulates the same ion channels as 5-HT (data not shown). The possibility that cyclic AMP could mediate 5-HT's effects on R15 was further tested by injecting 8-Br-cyclic AMP into R15. Once again, I_R and I_{Ca} were increased (Fig. 7). These results lead us to conclude that activation of adenylate cyclase and elevation of cyclic AMP can increase subthreshold I_{Ca} in cell R15.

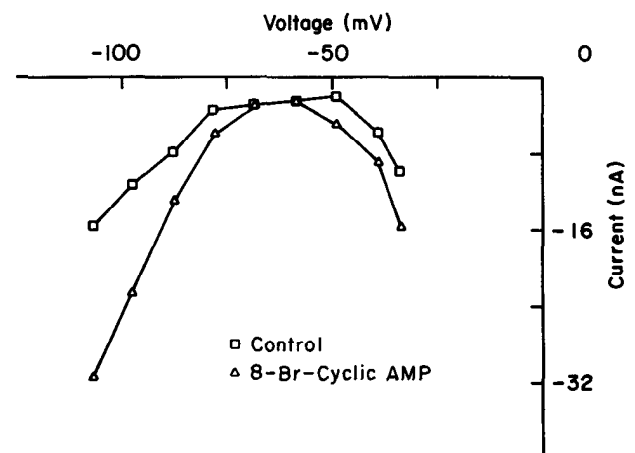


Figure 7. Effect of 8-Br-cyclic AMP injection on the I - V curve of R15. Currents evoked by 2 sec pulses from -40 mV before (□) and 4 min after (△) pressure injection of 8-Br-cyclic AMP. The injection electrode contained 10 mM 8-Br-cyclic AMP. Similar results were obtained with iontophoretic injections.

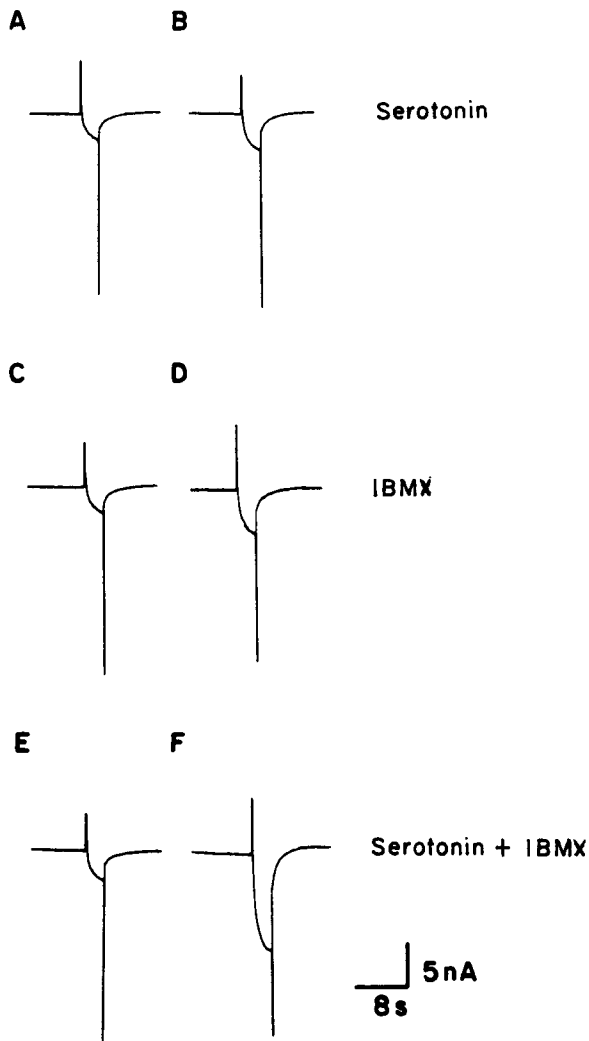


Figure 8. Phosphodiesterase inhibitor potentiates the serotonin (5-HT)-induced increase in I_{Ca} . In each case the current evoked by an 8 mV depolarizing voltage pulse from -48 mV is shown. *A*, Control. *B*, After addition of $1 \mu\text{M}$ 5-HT. *C*, After washing out the 5-HT. *D*, After addition of 0.2 mM IBMX. *E*, After washing out the IBMX. *F*, After exposure to $1 \mu\text{M}$ 5-HT together with 0.2 mM IBMX. Note that the increase in current produced by the combined application of 5-HT and IBMX is much larger than the sum of the increases produced by 5-HT and IBMX separately.

It remains possible, however, that 5-HT increases I_{Ca} by some mechanism independent of cyclic AMP. To address this possibility, we conducted experiments with the phosphodiesterase inhibitor IBMX. Figure 8, *A* and *B*, shows the effect of $1 \mu\text{M}$ 5-HT on I_{Ca} ; Figure 8, *C* and *D*, shows the effect of bath application of 0.2 mM IBMX. In both cases, only very small increases in I_{Ca} occurred. Application of 5-HT and IBMX together, however, produced a large increase (Fig. 8, *E*, *F*). Thus, a phosphodiesterase inhibitor potentiates the 5-HT-induced increase in I_{Ca} . This result, taken together with the facts that 5-HT elevates cyclic AMP in R15 and that forskolin and 8-Br-cyclic AMP mimic the effects of 5-HT, leads us to conclude that 5-HT acts via a single second messenger, cyclic AMP, to activate at least 2 ion channels, I_R and I_{Ca} . It may be the balance between these 2 conductances, which have opposing effects on cell excitability, that determines whether a given concentration of 5-HT produces profound inhibition, tonic excitation, or enhancement

of both the depolarizing and hyperpolarizing phases of the burst cycle.

R15 responses to inhibitory and excitatory input are altered by 5-HT

R15 often does not exhibit robust bursting activity in the intact nervous system; rather, its activity is sometimes found to be dominated by a constant stream of synaptic input (see Adams and Benson, 1985). Therefore, in order to determine the possible physiological importance of modulating 2 voltage-gated conductances with opposing effects on cell excitability, one must determine how 5-HT alters the sensitivity of R15 to synaptic currents. As a preliminary approach to this question, current pulses were applied to R15 in the hope that these would mimic the effects of synaptic currents. While these stimuli are not physiological, passing current directly into R15 insures that any effects seen arise in R15 and not in a presynaptic cell.

Short hyperpolarizing (Fig. 9, *A*, *B*) and depolarizing (Levitan and Levitan, 1988) current pulses produce little effect on the normal bursting activity in R15. Application of $25 \mu\text{M}$ 5-HT, however, not only produces tonic firing activity but also dramatically alters R15's response to transient currents. A brief hyperpolarizing current pulse in the presence of excitatory concentrations of 5-HT triggers a long-lasting hyperpolarizing plateau potential (Fig. 9*C*). Increasing the magnitude of the current pulse produces almost no change in the depth and duration of the evoked hyperpolarization. These data suggest that the current pulse serves merely to trigger the activation (and/or inactivation) of voltage-gated currents, which, in turn, regeneratively produce the hyperpolarization.

If this is the case, then any outward current, if sufficiently large, may trigger a long hyperpolarizing plateau potential. Depolarizing current pulses that increase spike frequency produce a slow outward current (Levitan and Levitan, 1988), and this outward current can also trigger a long-lasting hyperpolarizing plateau potential in the presence of high concentrations of 5-HT (Fig. 9*E*; the increase in spike frequency is substantial but is not discernible on this time scale).

5-HT can also hyperpolarize and terminate bursting activity in R15 (Drummond et al., 1980), and under these conditions, depolarizing current pulses can trigger a burst of action potentials (Fig. 9*D*). Once again, this is reminiscent of "plateau potential" cells (Russell and Hartline, 1978; Selverston and Moulins, 1985). These results reinforce the conclusion that 5-HT does not produce simple excitation or inhibition, but rather alters R15's bursting activity and sensitivity to hyperpolarizing or depolarizing input. Effectively, 5-HT converts R15 from an autonomous burster into a plateau potential cell. These properties most likely arise because 5-HT increases 2 discrete voltage-gated currents having opposite effects on cell excitability.

Discussion

We report here that the role of cyclic AMP is more complicated than previously suspected in the bursting pacemaker neuron R15. Application of $10 \mu\text{M}$ 5-HT, a transmitter known to elevate cyclic AMP in R15 (Cedar and Schwartz, 1972), enhances the interburst hyperpolarization of the bursting pacemaker cycle (Drummond et al., 1980). At higher concentrations, 5-HT induces enhancement of both the depolarizing and hyperpolarizing phases of the burst cycle and often produces tonic spiking activity. Voltage-clamp experiments reveal that the increase in I_R underlying the inhibition of bursting at low concentrations

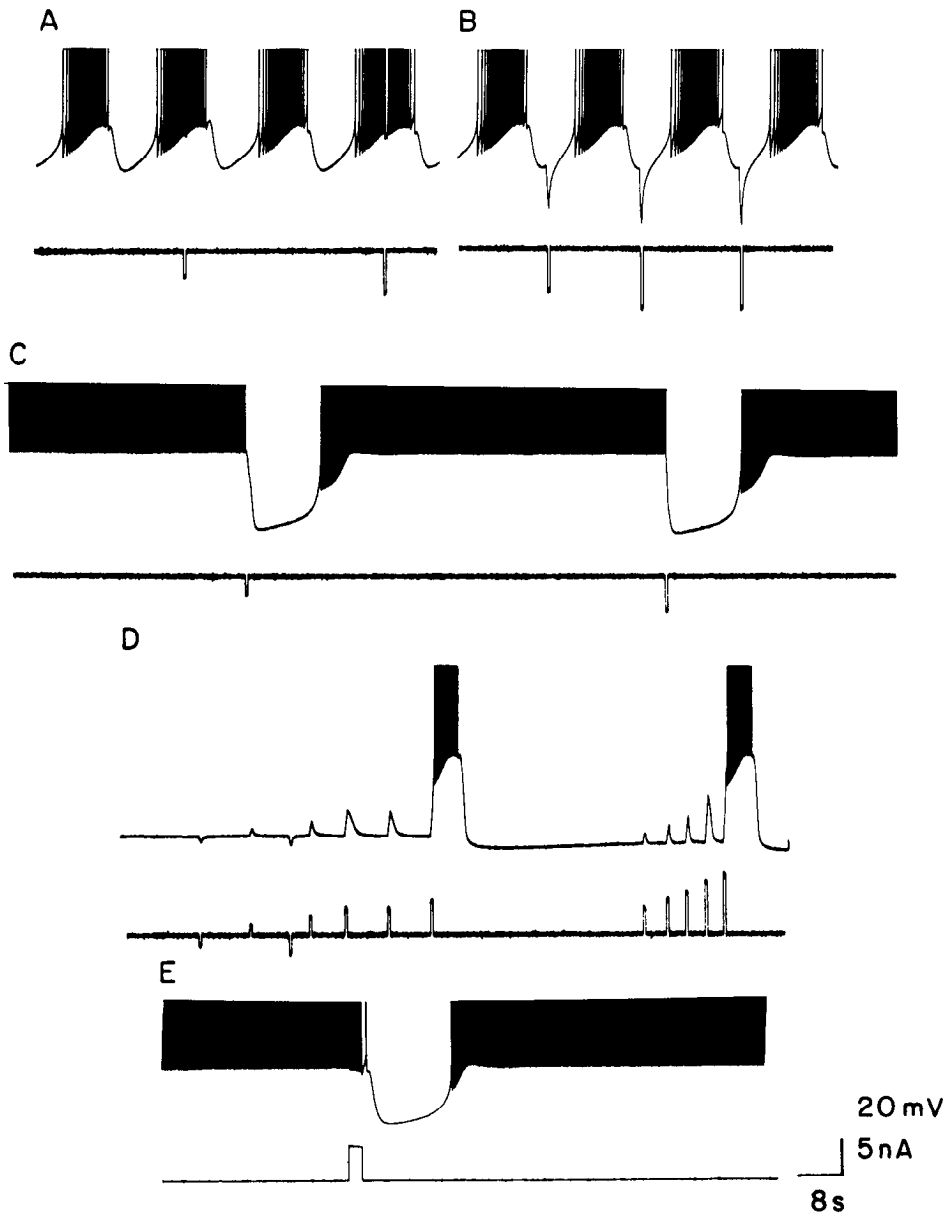


Figure 9. 5-HT alters the effects of hyperpolarizing and depolarizing current pulses on R15's activity. Brief hyperpolarizing current pulses (lower traces) applied to a control cell during a burst (*A*) or during the interburst (*B*) produce no long-term changes in R15's bursting activity. *C*, After inducing tonic spiking activity with 25 μ M 5-HT, small hyperpolarizing current pulses trigger long-lasting hyperpolarizations. Increasing the size of the pulse has little effect. *D*, While washing out the 5-HT, this cell hyperpolarized, and during this period depolarizing current pulses can trigger a burst. Bursts cannot be triggered in cells hyperpolarized with dopamine. *E*, In another cell, tonic spiking was induced with 30 μ M 5-HT. Long-lasting hyperpolarizations could be induced with depolarizing pulses that increase spike frequency. The change in spike frequency is not apparent with the time scale shown. A similar depolarizing pulse has little effect on basal bursting activity (not shown; see Levitan and Levitan, 1988). The tops of the spikes were cut off by the chart recorder in all records.

of 5-HT (Benson and Levitan, 1983) remains intact at higher concentrations. Enhancement of the depolarizing phase of the burst cycle, therefore, is not due to a loss of the cyclic AMP-mediated increase in I_R ; rather, 5-HT's depolarizing effect is accompanied by an increase in subthreshold I_{Ca} .

The modulation of I_{Ca} by 5-HT is mimicked by either the adenylylase activator forskolin or 8-Br-cyclic AMP, and it is potentiated by the phosphodiesterase inhibitor IBMX. These data, together with the fact that 5-HT increases cyclic AMP in R15 (Cedar and Schwartz, 1972), lead us to conclude that 5-HT's hyperpolarizing and depolarizing effects are both mediated by cyclic AMP. Thus, 5-HT, acting via a single second messenger (cyclic AMP), can produce complex changes in bursting activity by modulating voltage-gated ionic currents (I_{Ca} and I_R) with opposing effects on excitability.

The suggestion that cyclic nucleotides can be excitatory in R15 is not novel. Bath application of a membrane-permeable cyclic AMP analog can produce excitation in R15. However, its effects were attributed to the fact that it indirectly elevates

cyclic GMP by inhibiting phosphodiesterases (Levitan et al., 1979). Likewise IBMX has been reported to cause excitatory effects and an increase in the slow inward current, and it was hypothesized that these excitatory effects were also mediated by cyclic GMP, rather than cyclic AMP (Drake and Triestman, 1981). We have found that a membrane-permeable cyclic GMP analog in fact *decreases* I_{Ca} (Levitan and Levitan, 1988), and thus the excitatory effects of IBMX are probably mediated by cyclic AMP, not by cyclic GMP. This conclusion is reinforced by the fact that R020-1724, an inhibitor specific for cyclic AMP phosphodiesterase, also increases I_R and I_{Ca} (unpublished observations).

We have shown here that 5-HT, acting via cyclic AMP, can increase a potassium and a calcium current, and that these 2 currents are modulated at different concentrations of 5-HT. However, when R15s from juvenile *Aplysia* are maintained in primary cell culture, 1 μ M 5-HT is sufficient to produce large increases in both I_R and I_{Ca} . A chloride current, which is evident only when internal chloride is elevated, is also modulated by

5-HT in these cultured cells (Lotshaw et al., 1986). The fact that $1 \mu\text{M}$ 5-HT activates the different currents equally in cultured cells may be due to a greater sensitivity to 5-HT or, in part, to the fact that cultured cells are completely accessible to 5-HT, whereas *in situ* R15's soma is exposed while the axon is buried in the neuropil. If 5-HT-sensitive potassium channels are preferentially located on the soma, whereas 5-HT-sensitive calcium channels are located on the axon, then the unequal accessibility of the soma and axon *in situ* might produce an apparent difference in sensitivity to 5-HT. Such compartmentation of cyclic AMP-sensitive channels could be tested by locally applying 5-HT to R15's axon and soma.

The question arises of whether the cyclic AMP-induced increases in opposing currents, seen with high concentrations of 5-HT, are physiologically relevant. No known serotonergic neurons synapse on R15, and the levels of circulating 5-HT in *Aplysia* are not well documented. It is known, however, that excitation of the bag cell neurons leads to secretion of a peptide, egg-laying hormone (ELH), that augments both the hyperpolarizing and depolarizing phases of R15's burst cycle (Branton et al., 1978; Mayeri et al., 1985). We have recently found that bag cell discharge or application of pure ELH increases I_R and I_{Ca} , and that these increases are mediated by an increase in intracellular cyclic AMP in R15 (Levitan et al., 1987). Thus, a cyclic AMP-mediated increase in opposing currents represents a physiological situation.

The effects of ELH suggest a possible reason to modulate opposing currents, which in a normally silent cell makes little intuitive sense. I_R activates regeneratively with hyperpolarization at potentials more negative than the action potential threshold (i.e., -35 mV). Therefore, increasing I_R will tend to enhance the interburst hyperpolarization. The subthreshold I_{Ca} activates regeneratively with depolarization near the action potential threshold, and along with a spike-evoked, Ca^{2+} -activated inward current (Lewis et al., 1984; Adams, 1985; Adams and Levitan, 1985; Kramer and Zucker, 1985a), provides the depolarizing drive to maintain a burst. Since I_R and I_{Ca} gate at different voltages, and since the regenerative activation of one inactivates the other, increasing both of these voltage-gated currents tends to enhance both the hyperpolarizing and depolarizing phases of the bursting pacemaker cycle. Because bursting activity is thought to be optimal for producing secretion and synaptic release (Gillary and Kennedy, 1969; Dutton and Dyball, 1979), the enhancement of oscillation produced by increasing opposing currents may lead to an enhancement of secretion by R15, a cell thought to have a neurosecretory function (see Adams and Benson, 1985).

We suggest that another consequence of increasing I_{Ca} and I_R is to amplify R15's sensitivity to synaptic potentials. Normally, small hyperpolarizing current pulses have little effect on bursting activity. However, 5-HT increases I_R , which regeneratively activates with hyperpolarization and drives the cell towards the potassium equilibrium potential (-75 mV). Thus, in the presence of 5-HT, there is a larger amount of I_R to be activated by hyperpolarization [evoked by experimentally injecting current (Fig. 9C) or by increasing spike frequency (Fig. 9E)], and hence long hyperpolarizations are triggered by these small stimuli. These hyperpolarizations finally end because steady-state inactivation of I_{Ca} is slowly removed at the hyperpolarized potential, causing depolarization; this, in turn, inactivates I_R . These results suggest that the 5-HT-induced increases in 2 voltage-gated currents that have opposite effects on cell excitability may

enhance R15's sensitivity to excitatory and inhibitory synapses. In fact, the primary physiological effect of elevating cyclic AMP in R15 may be to convert R15 from a bursting cell to a plateau potential cell (Russell and Hartline, 1978; Selverston and Moulins, 1985).

It is of interest to compare the effects of dopamine and 5-HT. Like $10 \mu\text{M}$ 5-HT, dopamine enhances the interburst hyperpolarization and can terminate bursting activity (Ascher, 1972). In contrast to 5-HT, dopamine acts by decreasing I_{Ca} (Lewis et al., 1984). Furthermore, R15 cannot be induced to burst with depolarizing current in the presence of high concentrations of dopamine (Wilson, 1980). Hyperpolarizing concentrations of 5-HT, however, do not prevent the triggering of bursts by depolarizing current pulses (Fig. 9D). Thus, while 5-HT and dopamine can both inhibit R15's intrinsic bursting activity, they produce opposite effects on R15's responsiveness to excitatory stimuli.

Dopamine can interact with 5-HT by reducing the stimulation of I_{Ca} by 5-HT. This implies that both transmitters can modulate the same calcium channels. It also suggests that 5-HT must elevate cyclic AMP and modulate calcium channels in the axon where dopamine exerts its actions (Ascher, 1972; Lewis et al., 1984). Finally, the fact that dopamine reduces the excitatory effects of 5-HT suggests that small concentrations of dopamine might be sufficient to convert a net excitatory effect of 5-HT and cyclic AMP into a potent inhibitory one. This possibility is of physiological interest since stimulation of the branchial nerve can lead to long-term synaptic inhibition in R15, which appears to consist of a combination of 5-HT-like and dopamine-like effects (Adams et al., 1980).

The actions of cyclic AMP on cell R15 cannot simply be classified as excitatory or inhibitory. This is not wholly surprising since the basal activity of R15 is much more complex than that of some other neurons in which the actions of second messengers have been studied. It will be of interest to determine whether the modulation of opposing conductances by a single second messenger will be found to be a typical, rather than a unique, situation in bursting pacemaker neurons. If so, the fact that bursting pacemaker neurons play a central role in controlling neurosecretion and pattern generation (Rapp, 1979; Carpenter, 1982; Selverston and Moulins, 1985) suggests that this phenomenon may be of wide importance in the operation of the nervous system.

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