Proc. Natl. Acad. Sci. USA
Vol. 77, No. 8, pp. 5013–5017, August 1980
Neurobiology

Serotonin-induced hyperpolarization of an identified *Aplysia* neuron is mediated by cyclic AMP

(K^+ conductance/neurotransmitter/voltage clamp/serotonin receptors)

ALAN H. DRUMMOND*, JACK A. BENSON, AND IRWIN B. LEVITAN†

Friedrich Miescher-Institute, Post Office Box 273, CH-4002 Basel, Switzerland

Communicated by V. Prelog, May 27, 1980

ABSTRACT **Addition of serotonin to the medium bathing an *Aplysia* abdominal ganglion causes a change in the endogenous bursting activity of the identified neuron R15. At serotonin concentrations in the micromolar range, the predominant effect is an increase in depth and duration of the interburst hyperpolarization and consequent decrease in burst rate. At higher concentrations (10 μM) serotonin can inhibit bursting completely. We have shown previously that these changes can be mimicked by bath application or intracellular injection of several cyclic AMP analogs substituted at the 8 position. Voltage clamp analysis indicates that serotonin and cyclic AMP analogs both cause an increase in membrane slope conductance in R15, with reversal potentials for the responses between −75 and −80 mV, close to the K^+ equilibrium potential. When the K^+ concentration in the bathing medium is changed, the reversal potentials change in a manner suggesting that serotonin and cyclic AMP analogs are causing an increase in K^+ conductance. The effects of saturating concentrations of serotonin and cyclic AMP analogs on K^+ conductance are not additive. Furthermore, the effects of low concentrations of serotonin can be potentiated by the phosphodiesterase inhibitor Ro 20-1724. A pharmacological analysis indicates that the serotonin receptor that mediates hyperpolarization in R15 is similar to the serotonin receptor that we have shown to be coupled to adenylate cyclase. The present electrophysiological and pharmacological observations, together with our previous biochemical and pharmacological results, demonstrate that the serotonin-induced hyperpolarization of neuron R15 is mediated by cyclic AMP.**

Cyclic AMP is an intracellular second messenger in the action of a number of hormones on a variety of target tissues (1). There has been much speculation as to whether cyclic AMP plays a similar role in the action of neurotransmitters on nerve cells (2–4). There is evidence that cyclic AMP may mediate the effects of norepinephrine on cerebellar Purkinje neurons (2). In addition, recent reports from several laboratories suggest that cyclic AMP may be involved in the effects of serotonin on various *Aplysia* nerve and muscle cells (5–9). However, in none of these cases have all the criteria necessary to establish that cyclic AMP plays a role in a physiological response (1, 4) been satisfied.

We have been studying the effects of changes in cyclic AMP levels on the activity of the identified *Aplysia* neuron R15. This cell exhibits rhythmic activity consisting of bursts of action potentials separated by an interburst hyperpolarization phase (10). Cyclic AMP levels have been increased within R15 by activation of adenylate cyclase (11), application of phosphodiesterase inhibitors (12–14), and intracellular injection or bath application of cyclic AMP analogs (12–14). For each case, the predominant effect has been to extend the amplitude and duration of the interburst hyperpolarization, in some cases with complete inhibition of bursting. Until now it has not been clear whether these pharmacological changes reflect mechanisms involved in normal physiological responses of the neuron. In the present communication we demonstrate that serotonin applied at low concentrations causes neuron R15 to hyperpolarize and that this response is due to activation of a K^+ conductance and is mediated by cyclic AMP.

MATERIALS AND METHODS

Aplysia californica weighing 200–300 g were obtained from Pacific Bio-Marine Laboratories (Venice, CA) and were kept at 18–21°C in an Instant Ocean (Aquarium Systems, Eastlake, OH) artificial sea water aquarium. Lights in the animal room were on from 08:00 to 20:00, and experiments were usually begun between 10:00 and 14:00. The abdominal ganglion was pinned dorsal side up in a Sylgard dish (Dow Corning), and neuron R15 was penetrated with one or two microelectrodes (3–10 MΩ) for recording and voltage clamping. In most of the experiments described here, a single-electrode voltage clamp was used (ref. 15; design modified by W. B. Adams, unpublished). The ganglion was continuously perfused with *Aplysia* medium (16) at 20–22°C, with additions or modifications as described for individual experiments. Steady-state current–voltage (I–V) curves were obtained by stepping the voltage from a holding potential (usually −70 mV) and measuring the current 5 or 10 sec after the beginning of the step. The voltage was then stepped back to the holding potential and kept there for at least 5 sec before the next step. Identical results were obtained when the membrane voltage was continuously swept over the voltage range −30 to −130 mV, using a triangular pulse of 100 sec duration (50 sec up and 50 sec down). This pulse is long relative to the membrane time constant, and short relative to the duration of the effects of serotonin and cyclic AMP analogs reported below. All voltages shown have been corrected for changes in electrode tip potential during the experiment.

RESULTS

Effect of Serotonin on R15 Bursting Activity. The effects of bath-applied serotonin on rhythmic bursting activity in neuron R15 are illustrated in Fig. 1. Threshold responses to serotonin are occasionally observed at concentrations as low as 0.01 μM, and usually at 0.1 μM. At these concentrations there is an increase in the depth and duration of the interburst hyperpolarization phase of the burst cycle (Fig. 1*B*). This response is enhanced at higher serotonin concentrations (Fig. 1*C*). In some cases bursting is abolished (Fig. 1*D*) and the membrane potential approaches the potassium equilibrium potential (estimated to be −75 mV in R15; see ref. 17). The cell can fire action potentials during this time in response to injections of

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviation: LSD, lysergic acid diethylamide.

* Present address: Department of Pharmacology, University of Glasgow, Glasgow G12 8QQ, Scotland.
† To whom reprint requests should be addressed.

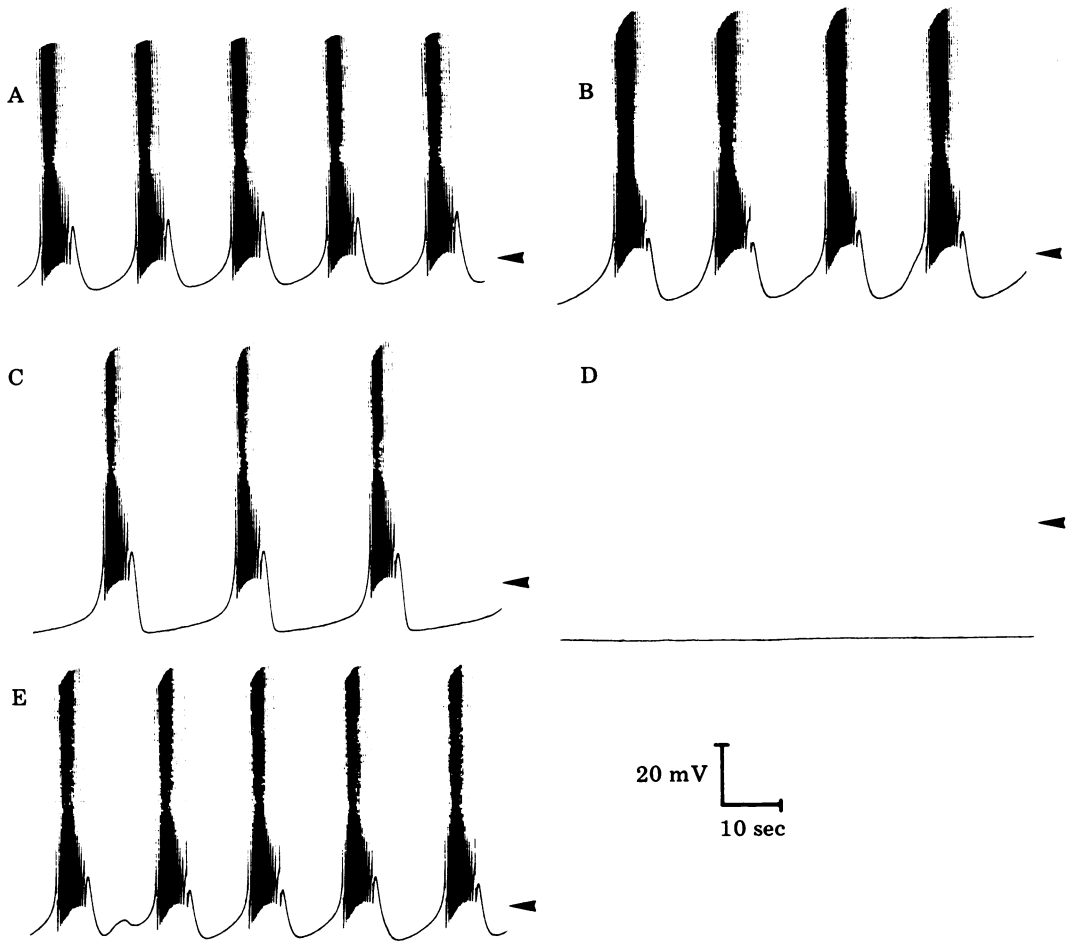

FIG. 1. Effect of serotonin on bursting activity in unclamped neuron R15. (*A*) Control. When the neuron is perfused with serotonin at low concentrations (0.1 μM, 30 min; *B*), the predominant effect is to increase the hyperpolarization phase of the oscillatory cycle. A further increase in the serotonin concentration (1 μM, 20 min; *C*) increases both the interburst hyperpolarization and the interval between bursts. At a high concentration (10 μM, 10 min; *D*) the bursting activity of the cell is abolished and the cell is hyperpolarized to approximately −75 mV. This and the other effects are readily reversed by perfusing the ganglion in serotonin-free medium for 30 min (*E*). The arrow to the right of each trace indicates −40 mV membrane potential.

depolarizing current, and the bursting pattern returns to normal after a prolonged wash with normal medium (Fig. 1*E*). In the presence of serotonin concentrations above 20–50 μM, the net effect on R15 is often excitatory (not shown) indicating that serotonin may have more than one effect.

Ionic Basis of Effects of Serotonin and Cyclic AMP Analogs. The response of neuron R15 to serotonin illustrated in Fig. 1 is mimicked when cyclic AMP analogs are bath-applied or injected directly into R15 (12–14). In order to investigate the ionic mechanism of these responses we have analyzed them under voltage clamp. Steady-state *I–V* curves for neuron R15 are shown in Fig. 2. In the presence of serotonin (Fig. 2*A*) or 8-benzylthio-cyclic AMP (Fig. 2*B*), the slope of the *I–V* curve at voltages more negative than −70 mV is increased, and the experimental curves intersect the controls between −75 and −80 mV. Identical results are obtained with 8-parachlorophenylthio-cyclic AMP (not shown). There is relatively little difference between the control and experimental curves in the negative slope region, between −70 and −40 mV (Fig. 2 *A* and *B*). The *I–V* curves return to normal after a wash with normal medium.

The net currents elicited by serotonin or 8-benzylthio-cyclic AMP at various membrane voltages can be determined by subtracting the control from the experimental curves in Fig. 2 *A* and *B*, respectively. The results, plotted in Fig. 2 *C* and *D*, demonstrate that the evoked currents reverse sign between −75 and −80 mV, very close to the K^+ equilibrium potential (17). This observation suggests that the hyperpolarizations evoked by both serotonin and 8-benzylthio-cyclic AMP are due to activation of a K^+ conductance. Note also that the net current elicited by both agents is linear with respect to membrane voltage at voltages more negative than −70 mV but deviates from linearity at less negative voltages (Fig. 2 *C* and *D*). When 5 μM serotonin and 700 μM 8-benzylthio-cyclic AMP are applied to R15 simultaneously, the net current is no different from that elicited by these saturating concentrations of serotonin or 8-benzylthio-cyclic AMP alone.

To further test the hypothesis that serotonin and cyclic AMP analogs activate a K^+ conductance, the reversal potential for the elicited currents was determined in the presence of different concentrations of extracellular K^+. In both cases, the reversal potential becomes more positive as extracellular K^+ concentration is increased (Fig. 3). The change is 55 mV per 10-fold change in K^+ concentration in the case of serotonin, and 49 mV in the case of 8-benzylthio- or 8-parachlorophenylthio-cyclic AMP. The Nernst equation predicts a change of 58 mV per 10-fold change in K^+ concentration if a K^+ conductance is being activated. The response to serotonin is unaffected by externally applied ouabain (0.1 mM), indicating that an electrogenic pump is unlikely to be involved.

Effects of Phosphodiesterase Inhibition. If the action of serotonin on R15 is mediated by cyclic AMP, it should be pos-

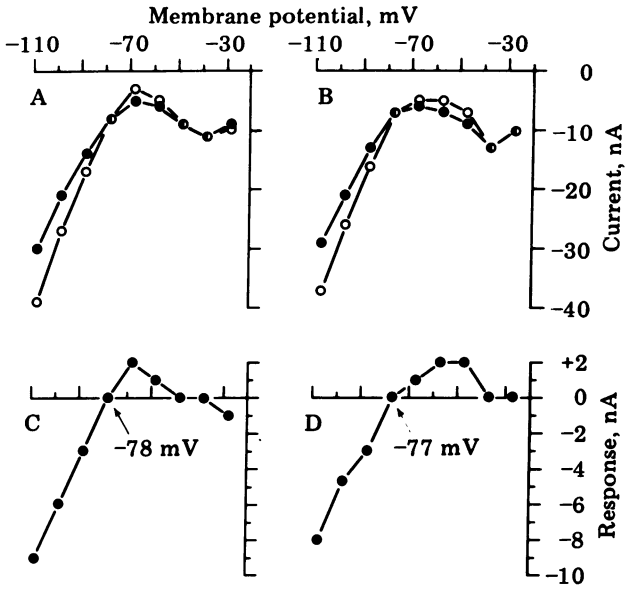

FIG. 2. Steady-state *I–V* curves, and net current elicited by serotonin and 8-benzylthio-cyclic AMP, in voltage-clamped neuron R15. (*A* and *B*) Membrane currents under control (●) and experimental (○) conditions for 1 μM serotonin (25-min perfusion; *A*) and extracellularly applied 700 μM 8-benzylthio-cyclic AMP (60 min; *B*). The reversal potentials for the evoked currents are the membrane potentials at which the control and experimental *I–V* curves intersect. By subtracting the experimental *I–V* curves from the controls, the net evoked currents are obtained for serotonin (*C*) and 8-benzylthio-cyclic AMP (*D*). These elicited currents reverse sign, in the examples illustrated, at −78 mV for serotonin and −77 mV for 8-benzylthio-cyclic AMP, close to the K^+ equilibrium potential for R15 (ref. 17).

sible to potentiate the effects of low concentrations of serotonin by inhibiting phosphodiesterase, the enzyme responsible for hydrolyzing cyclic AMP. We used the phosphodiesterase inhibitor Ro 20-1724 (provided by W. Burkard, Hoffmann–La Roche, Basel, Switzerland), because at low concentrations it selectively inhibits *Aplysia* cyclic AMP phosphodiesterase and has relatively little effect on cyclic GMP phosphodiesterase (13). Low concentrations of either serotonin (Fig. 4*B*) or Ro 20-1724 (Fig. 4*C*), added separately to the bathing medium, have little effect on the activity of R15 in this experiment. However, when the two are added together to the bathing medium, the cell hyperpolarizes and bursting is almost abolished (Fig. 4*D*). If the cell is washed with normal medium (Fig. 4*E*) and the experiment is repeated, the results are the same (Fig. 4 *F* and *G*). Similar data can be obtained under voltage clamp; serotonin or Ro 20-1724 alone at low concentrations has little effect on the steady-state *I–V* curve, whereas together they cause an increase in the membrane slope conductance similar to that illustrated in Fig. 2.

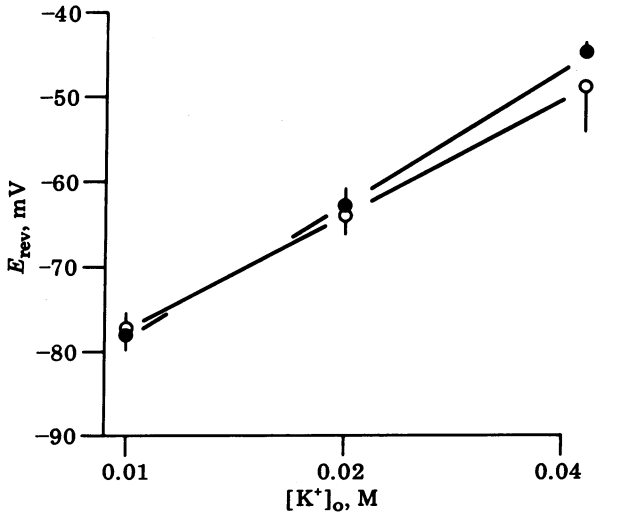

FIG. 3. Variation in the reversal potential (E_{rev}) of currents elicited by serotonin and cyclic AMP analogs as a function of extracellular K^+ concentration ($[K^+]_o$). ●, Reversal potential of current evoked by 1 μM serotonin; ○, reversal potential of current evoked by extracellular application of either 700 μM 8-benzylthio- or 8-parachlorophenylthio-cyclic AMP. Each point is the mean ± SD for between three and eight experiments.

Serotonin Receptor Pharmacology. We have previously characterized in detail the pharmacology of serotonin and dopamine receptors in molluscan ganglia and muscle, by [^{3}H]lysergic acid diethylamide (LSD) binding (18–20) and adenylate cyclase stimulation (19, 20). A more limited pharmacological analysis has been carried out on the serotonin receptor that mediates hyperpolarization in R15, by examining the effects of various serotonin agonists and antagonists on bursting activity and on the steady-state *I–V* curves. An example of such an experiment is shown in Fig. 5, which illustrates that *d*-LSD causes an increase in the membrane slope conductance of R15 (Fig. 5*A*) whereas the pharmacologically inactive stereoisomer *l*-LSD is without effect at the same concentration (Fig. 5*B*). No further increase in membrane slope conductance can be elicited by serotonin in the presence of *d*-LSD (Fig. 5*A*), whereas *l*-LSD leaves the response to serotonin unaffected (Fig. 5*B*). It is important to note that this blocking action of *d*-LSD is complete even when its agonist effect is small relative to that of serotonin. *d*-Butaclamol (but not *l*-butaclamol) antagonizes the effect of serotonin but shows no agonist effect. The action of 8-parachlorophenylthio-cyclic AMP is not blocked by *d*-butaclamol.

The concentrations at which a series of pharmacological agents alter R15's electrical activity are summarized in Table 1, and they are compared with the concentrations required to affect serotonin-sensitive adenylate cyclase activity in a

Table 1. Pharmacological analysis of serotonin-induced hyperpolarization of neuron R15 and comparison with serotonin-sensitive adenylate cylase

Agent tested	Threshold conc. for hyperpolarization in R15, μM	EC_{50} or K_i in adenylate cyclase assay,* μM
Agonists		
Serotonin	0.02–0.10	1.65
Bufotenine	0.05–0.10	1.25
N-Methylserotonin	0.05–0.10	1.55
5-Methoxytryptamine	0.10–0.30	6.20
Tryptamine	0.50–2.00	18.00
6-Hydroxytryptamine	1.00–3.00	26.00
d-LSD	0.50–10.00	0.01
l-LSD	50.00	10.00
Antagonists (measured in the presence of 1 μM serotonin)		
d-Butaclamol	2.00–10.00	0.02
l-Butaclamol	100.00	13.00

All electrophysiological experiments were carried out at room temperature (20–22°C). The drugs were dissolved directly in *Aplysia* medium and perfused over the abdominal ganglion. With the exception of the LSD and butaclamol isomers, effects were observed within 30 min of the start of drug perfusion. For *d*-LSD and *d*-butaclamol, at least 1–2 hr of perfusion was necessary to detect significant agonist or antagonist activity.

* Data from ref. 20. EC_{50}, 50%-effective concentration; K_i, inhibitor constant.

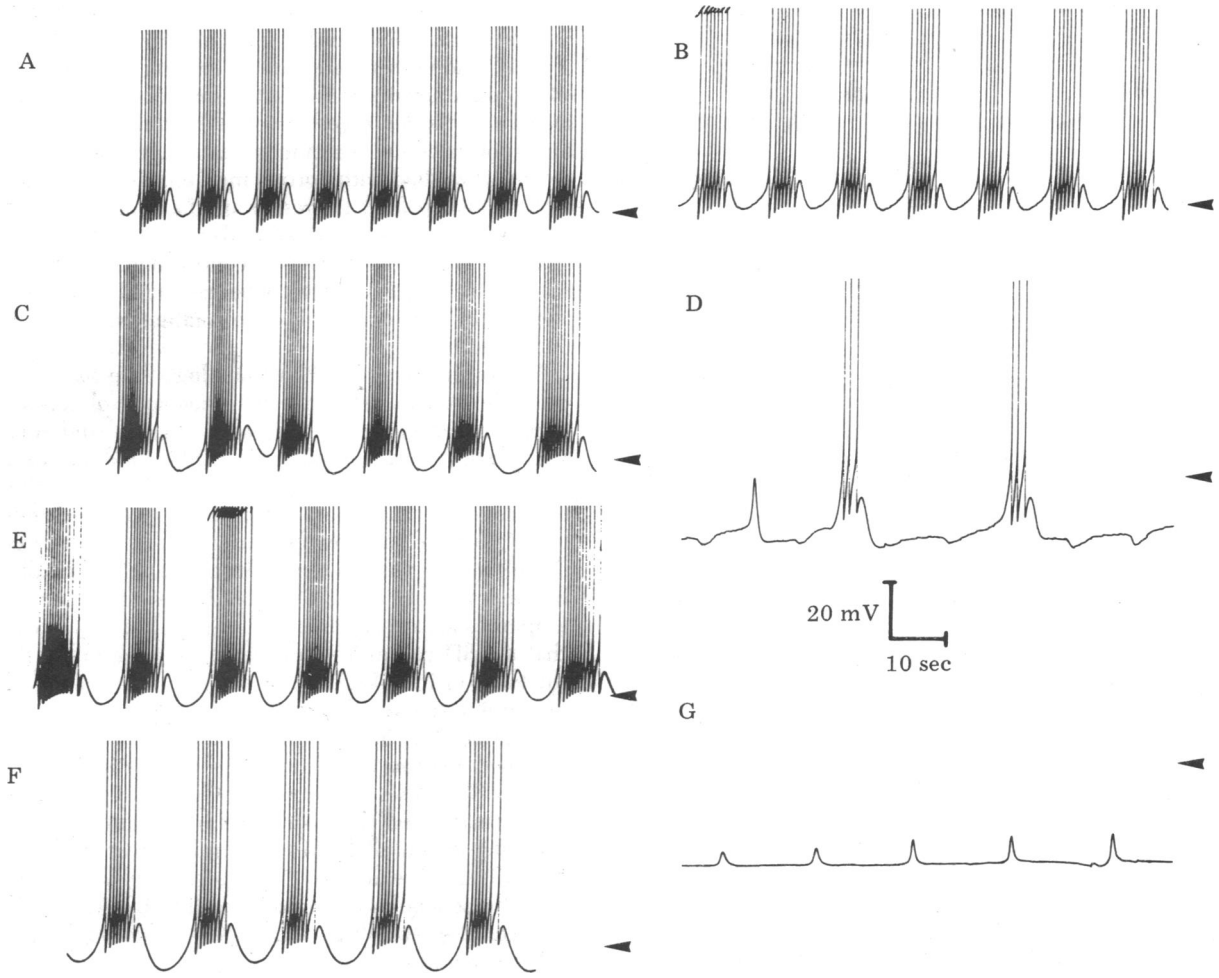

FIG. 4. Potentiation of serotonin effects on unclamped neuron R15 by a phosphodiesterase inhibitor (Ro 20-1724). (*A*) Control. In this cell 0.1 μM serotonin (*B*), and 1 μM Ro 20-1724 (*C*) in a 30-min perfusion do not by themselves elicit marked responses. However, when they are used simultaneously for 30-min perfusion, a large increase in both the interburst interval and the interburst hyperpolarization is noted (*D*). This effect, which is readily reversed by washing for 30 min (*E*), was duplicated 3.5 hr later in the same cell (*F*: serotonin, 30 min; *G*: serotonin and Ro 20-1724, 30 min) and the effect was, if anything, more marked. The small depolarizing responses in *G* are spontaneous excitatory synaptic potentials. The arrow to the right of each trace indicates −40 mV membrane potential. The tops of the action potentials have been truncated by the chart recorder.

membrane preparation (data from ref. 20). With the exception of *d*-LSD and *d*-butaclamol, the rank order of potency of compounds on the electrical response is similar to that on the cyclase. Furthermore, *d*-LSD is a stereospecific agonist, and *d*-butaclamol is a stereospecific antagonist on both the electrical response and the cyclase. Even at high concentrations, *d*-LSD and *d*-butaclamol affect R15 only after 1–2 hr of application. We have no good explanation for this time course, or for the observation that high concentrations of *d*-LSD and *d*-butaclamol are required to affect responses in the intact cell. However, our finding that these compounds act stereospecifically indicates that their effects are receptor-mediated.

DISCUSSION

There appear to be serotonin receptors present on many molluscan neuronal and muscle cells (5–9, 21, 22). Serotonin may have any of several effects, depending on the target cell, and often may produce more than one response in a single neuron (21). We show here that the predominant effect of serotonin on *Aplysia* neuron R15 is an increase in the duration and depth of the interburst hyperpolarization, with further hyperpolarization and inhibition of bursting at higher serotonin concentrations. The hyperpolarization results from activation of a K^+ conductance. In addition there appears to be an excitatory effect, which is most apparent at high concentrations of serotonin (20–50 μM). This may be due to activation of a voltage-dependent inward ion conductance carried by Na^+, Ca^{2+}, or both, as has been suggested for the action of serotonin on some other *Aplysia* neurons (22). The nonlinearity of the current elicited by serotonin in the depolarized range of membrane potentials (Fig. 2*C*) is consistent with such a suggestion. These possibilities have not yet been investigated in detail. Another possible explanation for the nonlinearity is that the K^+ conductance is voltage-dependent and inactivates at depolarized potentials.

The activation of a K^+ conductance and the resulting hyperpolarization produced by serotonin are mimicked by bath application of 8-benzylthio- or 8-parachlorophenylthio-cyclic AMP. Furthermore, S. Treistman and P. Drake have carried out voltage clamp analysis after intracellular injection of cyclic AMP analogs, and they have studied activation of adenylate cyclase within R15 by intracellular injection of the GTP analog guanylylimidodiphosphate. In both cases they found that the predominant effect is an increase in the membrane slope conductance, although guanylylimidodiphosphate also appears to decrease the negative slope of the *I–V* curve (23). It seems likely that the K^+ conductances activated by serotonin and cyclic AMP analogs are identical, because the currents elicited by saturating concentrations of these agents applied simultaneously are nonadditive.

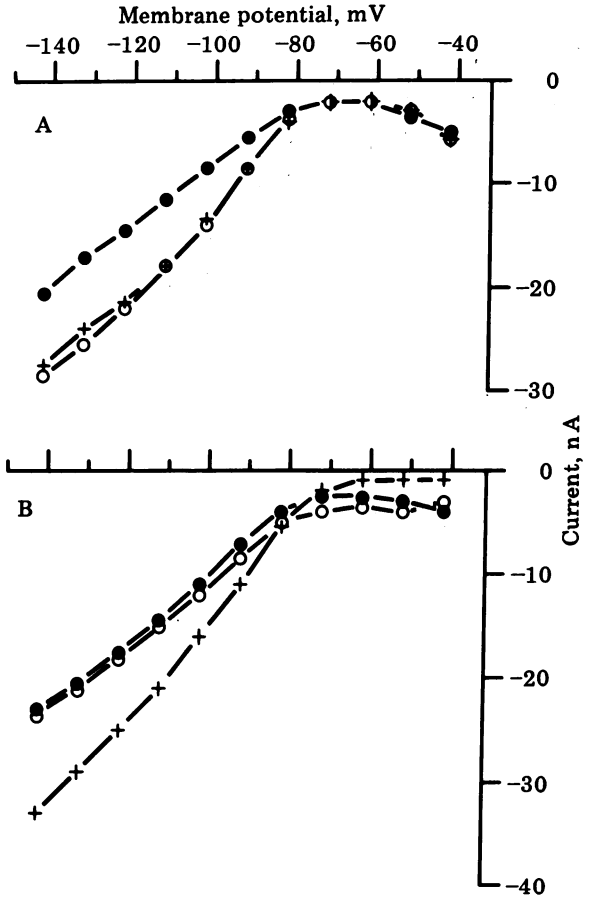

FIG. 5. Steady-state *I–V* curves from voltage-clamped neuron R15, showing the effects of 1 μM serotonin (25-min perfusion) in the presence of 10 μM *d*-LSD (*A*) or 10 μM *l*-LSD (*B*). (*A*) After 90 min, 10 μM *d*-LSD alone (○) increases membrane conductance in comparison with the control (●), and there is no effect of 1 μM serotonin applied for 25 min in the continued presence of *d*-LSD (+). (*B*) Membrane conductance of R15 after 90 min in 10 μM *l*-LSD (○) remains similar to the control (●); 25-min perfusion with 1 μM serotonin (in the continued presence of *l*-LSD) causes a marked increase in membrane conductance (+).

It is worthy of note that the net current evoked by cyclic AMP analogs (Fig. 2*D*) shows the same nonlinearity as that produced by serotonin. This suggests that the K^+ conductance increase and the other component of the response may both be mediated by the occupation of a single receptor, or perhaps the occupation of two different receptors, both of which are coupled to adenylate cyclase. Gerschenfeld and Paupardin-Tritsch (21) have provided considerable evidence for the existence of more than one serotonin receptor on a single neuron. It is also possible that serotonin and cyclic AMP analogs trigger a single primary response, for example entry of Ca^{2+}, which in turn leads to activation of several ion conductances.

The pharmacological analysis presented here demonstrates that the serotonin receptor that mediates hyperpolarization in R15 is very probably coupled to adenylate cyclase. The present data, together with those we have obtained previously, fulfill all the criteria (1, 4) that must be satisfied before one can demonstrate a role for cyclic AMP in the response to a hormone or neurotransmitter: (*i*) the serotonin-evoked increase in K^+ conductance is mimicked by application of phosphodiesterase-resistant cyclic AMP analogs and by adenylate cyclase activation after guanylylimidodiphosphate injection; (*ii*) the effects of low concentrations of serotonin are potentiated by a phosphodiesterase inhibitor; (*iii*) serotonin causes cyclic AMP to accumulate within neuron R15 (refs. 24, 25); (*iv*) there is a serotonin-sensitive adenylate cyclase in membranes from neuron R15 (ref. 26); and (*v*) the pharmacological characteristics of the serotonin receptors mediating electrical changes in neuron R15 and adenylate cyclase stimulation are very similar. These findings lead us to conclude that the serotonin-induced increase in a K^+ conductance in neuron R15 is mediated by cyclic AMP.

We are grateful to Dr. W. B. Adams for designing and helping to build the single-electrode voltage clamp, and for his comments throughout the course of this work. We are also indebted to Dr. W. Burkard for the generous gift of Ro 20-1724.

1. Robison, A., Butcher, R. & Sutherland, E. (1971) *Cyclic AMP* (Academic, New York).
2. Bloom, F. E. (1975) *Rev. Physiol. Biochem. Pharmacol.* **74**, 1–103.
3. Nathanson, J. A. (1977) *Physiol. Rev.* **57**, 157–256.
4. Greengard, P. (1976) *Nature (London)* **260**, 101–108.
5. Kaczmarek, L. K., Jennings, K. & Strumwasser, F. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 5200–5204.
6. Klein, M. & Kandel, E. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 3512–3516.
7. Shimahara, T. & Tauc, L. (1977) *Brain Res.* **127**, 168–172.
8. Weiss, K., Schonberg, M., Mandelbaum, D. & Kupfermann, I. (1978) *Nature (London)* **272**, 727–728.
9. Mandelbaum, D., Koester, J., Schonberg, M. & Weiss, K. (1979) *Brain Res.* **177**, 388–394.
10. Frazier, W. T., Kandel, E. R., Kupfermann, I., Waziri, R. & Coggeshall, R. E. (1967) *J. Neurophysiol.* **30**, 1288–1351.
11. Treistman, S. N. & Levitan, I. B. (1976) *Proc. Natl. Acad. Sci. USA* **73**, 4689–4692.
12. Treistman, S. N. & Levitan, I. B. (1976) *Nature (London)* **261**, 62–64.
13 Levitan, I. B. & Norman, J. A. (1980) *Brain Res.* **187**, 415–429.
14. Treistman, S. N. & Drake, P. F. (1979) *Brain Res.* **168**, 643–647.
15. Wilson, W. A. & Goldner, M. M. (1975) *J. Neurobiol.* **6**, 411–422.
16. Levitan, I. B. & Barondes, S. H. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 1145–1148.
17. Kunze, D., Walker, J. & Brown, H. (1971) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **30**, 255.
18. Drummond, A. H., Bucher, F. & Levitan, I. B. (1978) *Nature (London)* **272**, 368–370.
19. Drummond, A. H., Bucher, F. & Levitan, I. B. (1980) *Brain Res.* **184**, 163–177.
20. Drummond, A. H., Bucher, F. & Levitan, I. B. (1980) *J. Biol. Chem.*, in press.
21. Gerschenfeld, H. & Paupardin-Tritsch, D. (1974) *J. Physiol. (London)* **243**, 427–456.
22. Pellmar, T. C. & Carpenter, D. O. (1979) *Nature (London)* **277**, 483–484.
23. Treistman, S. N., *Science*, in press.
24. Cedar, H. & Schwartz, J. H. (1972) *J. Gen. Physiol.* **60**, 570–587.
25. Levitan, I. B. & Drummond, A. H. (1980) in *Drug Receptors in the Nervous System* (Int. Science Services, Jerusalem, Israel), in press.
26. Levitan, I. B. (1978) *Brain Res.* **154**, 404–408.