primed synthesis of POMC cDNA in fixed pituitary sections was demonstrated by: (i) intermediate lobe localization of the autoradiographic signal, (ii) primer dependence and specificity of the signal, (iii) patterns of signal intensity in drug-treated animals consistent with the known regulation of POMC mRNA, and (iv) the hybridization to POMC cDNA of IST transcripts, which formed a discrete pattern of bands upon electrophoresis. The ability to generate cDNA in tissue sections, without the need for RNA extraction procedures, permits the rapid anatomical localization of mRNA. The sensitivity of the generated autoradiographic POMC IST signals to dopamine agonists and antagonists demonstrates that the method may be used in studies of the regulation of mRNA levels. In addition, cDNA may be obtained from small amounts of tissue, potentially permitting the cloning of specific cDNAs from tissues for which cDNA libraries do not currently exist.

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25. Supported by National Institute of Mental Health (NIMH) MH-23861, National Institute of Drug Abuse DA-05010, and Office of Naval Research N00014-86-K0251 grants, and by an NIMH Postdoctoral Research Service Award MH09099 to L.H.T. We thank C. J. Evans and K. L. Valentino for helpful discussions, S. Zalcman, S. Szara, and D. Woodward for their encouragement, J. C. Bulinski and A. Hoffman for critical reading of this manuscript, P. Erikson for help in the preparation of the manuscript, and A. L. R. Fritchle for help in the preparation of figures.

4 February 1988; accepted 15 April 1988

Intracellular Injection of cAMP Induces a Long-Term Reduction of Neuronal K^+ Currents

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Intracellular signals that trigger long-term (24-hour) changes in membrane currents in identified neurons of *Aplysia* have been examined in order to understand the cellular mechanisms underlying long-term sensitization. Adenosine 3′,5′-monophosphate (cAMP) was directly injected into individual sensory neurons to mimic the effects of sensitization training at the single cell level. Potassium currents of these cells were reduced 24 hours after injection of cAMP; these currents were similar to those reduced 24 hours after behavioral sensitization. These results suggest that cAMP is part of the intracellular signal that induces long-term sensitization in *Aplysia*.

THE LARGE AND IDENTIFIABLE NEURONS of the mollusk *Aplysia* have made it a useful model to study cellular changes in neurons that accompany alterations of behavior (*1*). For example, long-term sensitization of certain reflex responses can last for several days (*2, 3*). These behavioral changes are associated with changes in (i) morphology of sensory neuron synaptic contacts (*4*), (ii) amplitude of evoked excitatory postsynaptic potentials (EPSPs) from sensory neurons to motor neurons (*5, 6*), (iii) sensory neuron excitability (*7, 8*), and (iv) membrane currents of the sensory neurons (*3*). In addition, these changes require protein synthesis (*6, 9*). The intracellular signals that lead to the induction of long-term sensitization are unknown. Because cAMP is elevated in the sensory neurons during the application of the stimuli that lead to long-term sensitization (*10*), we have assessed the role of cAMP

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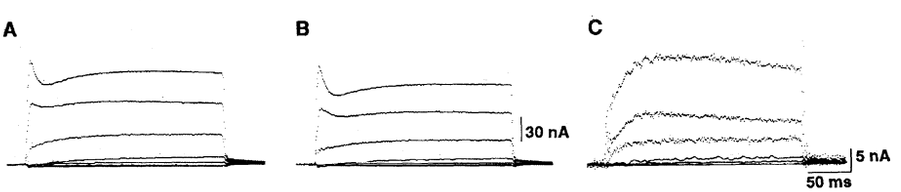

Fig. 1. Membrane currents in response to voltage-clamp pulses from a holding potential of −50 mV. Voltage-clamp pulses were elicited to membrane potentials between −80 and +25 mV in steps of 15 mV. (**A**) Overall average of current response families representing all cells injected with 5′-AMP 24 hours before voltage clamping. (**B**) Overall average of current response families representing all cells injected with cAMP 24 hours before voltage clamping. (**C**) The averaged cAMP difference families [(A) − (B)] from all ten clusters. The current response family in (C), therefore, represents the net outward currents that are reduced as a consequence of previous injection of cAMP (I_{cAMP}). Note different vertical calibration in (C).

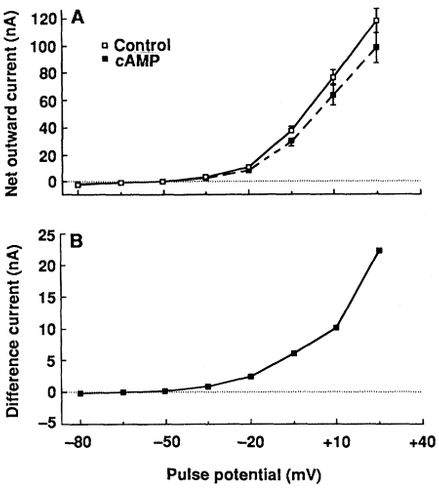

Fig. 2. The *I-V* relations of response families in Fig. 1. Values plotted are the amplitude of the net outward current at the end of a 300-ms pulse to the indicated potential. (**A**) The *I-V* relation of cells injected with 5′-AMP (open squares, 17 cells) or cAMP (closed squares, 19 cells). Error bars represent SEM. (**B**) The *I-V* relation of the cAMP difference current.

in triggering the cellular changes that accompany long-term sensitization.

Aplysia californica were anesthetized by injection of a volume of $MgCl_2$ equal to approximately one-half the animal's weight. The right pleural-pedal ganglia, which contain the cluster of sensory neurons that innervate the right side of the tail and posterior part of the animal (*11*), were removed and pinned to the floor of an experimental chamber. The ganglia were bathed in artificial seawater (ASW) at room temperature (20° to 22°C) buffered to *p*H 7.6 with 10 m*M* tris. The connective tissue sheath that covers the pleural ganglion was surgically removed to expose the cell bodies of the tail sensory neurons. Individual sensory neurons (*11*) were impaled with one microelectrode (18 to 35 megohms) that was filled with 30 m*M* fast green dye and either 200 m*M* cAMP or 200 m*M* 5′-adenosine monophosphate (5′-AMP) in distilled water (*12*) (5′-AMP was used as a control because it is the immediate breakdown product of cAMP).

After impalement of a cell, cAMP or 5′-AMP was injected into the cell by iontophoresis (*13*). Although cAMP characteristically induced a depolarization and decrease in membrane conductance, no consistent responses were observed in cells injected with 5′-AMP. After successful iontophoresis into five to eight cells per sensory neuron cluster, the electrode was removed, and the ganglia were placed in organ culture (*14*) for 24 hours.

Twenty-four hours after injection of cAMP and 5′-AMP into sensory neurons, the ganglia were removed from organ culture media and gradually returned to buffered ASW at room temperature. We were able to identify the sensory neurons that had been injected by observing the presence of fast green dye and by using a map of the sensory neuron cluster generated during the initial injection. Sensory neurons that had been injected were then reimpaled with two microelectrodes filled with 3*M* potassium acetate (resistance 4 to 10 megohms). All cells were voltage-clamped at a holding potential (V_h) of −50 mV (*15*). Voltage pulses to seven different membrane potentials between −80 and +25 mV (300-ms pulses at 40-s intervals) were delivered to each cell (*3*).

To analyze the long-term effects of cAMP on the membrane currents of the sensory neurons, current responses to the series of voltage-clamp commands were digitized and stored on computer for averaging and subtraction. For each cluster of sensory neurons, the membrane currents elicited at corresponding potentials from the cells injected with cAMP or 5′-AMP were averaged. Thus, two families of current responses were obtained from each cluster: a control (5′-AMP) family and a cAMP family, representing the current-voltage (*I-V*) relation of each of the two groups. An overall average response family for all of the experiments (ten clusters) was obtained for 5′-AMP cells by averaging all the response families of the individual clusters (Fig. 1A), a similar process was used for cAMP cells (Fig. 1B). The cAMP difference family was obtained by subtracting the cAMP family from the control family for each cluster; the resulting subtracted families from all the clusters were then averaged (Fig. 1C). Figure 1C, therefore, represents the net outward membrane currents elicited at the various test potentials that are reduced for at least 24 hours as a consequence of injection of cAMP (I_{cAMP}). At membrane potentials from −35 to +25 mV, the net outward current at the end of the pulse in cells that had been injected with cAMP was significantly less than the current in sensory neurons that had been injected with 5′-AMP ($t = 3.04$; $P < 0.01$; $n = 10$ clusters) (*16*). Although cAMP induced a slight reduction in steady-state membrane conductance, as measured by hyperpolarizing pulses to −65 and −80 mV, this was not a significant effect ($t = 0.51$; not significant).

The voltage dependence of the net membrane current that was reduced by cAMP was determined by plotting an *I-V* relation of the data in Fig. 1. The net membrane current at the end of the voltage-clamp pulse in sensory neurons that had been injected with 5′-AMP and with cAMP was plotted against the membrane potential of the voltage-clamp pulse (Fig. 2A). Figure 2B is the difference *I-V* relation; this curve represents the voltage sensitivity of the current that is reduced 24 hours after injection of cAMP. I_{cAMP} is present near the resting potential and the threshold potential for generation of an action potential.

The currents reduced 24 hours after injection of cAMP are similar to those that were found to be reduced 24 hours after sensitization training of the animal (*3*). In both instances, the current shows relatively slow kinetics of activation (Fig. 1C), mild voltage

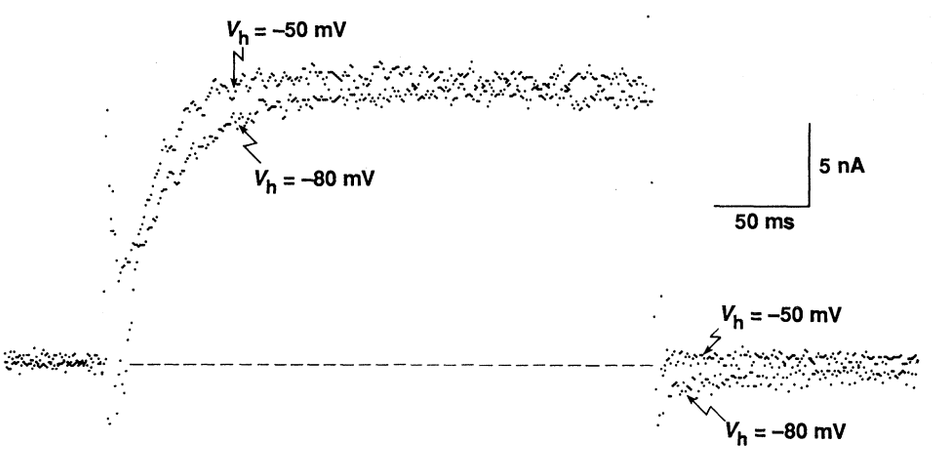

Fig. 3. Mean cAMP difference currents (representing all cells) elicited by pulses to a membrane potential of +10 mV from a V_h of −50 mV and of −80 mV. Arrows at the end of the pulse indicate tail currents after return of the membrane potential to the holding potential. The baselines (holding currents) have been aligned to aid viewing of tail currents.

dependence (Fig. 2B), and very little inactivation during the pulse (Fig. 1C).

Although both sensitization training and injection of cAMP induce a long-term reduction of net outward current in the sensory neurons, this change could represent an increase of an inward current rather than a true reduction of an outward current. One way to distinguish between these two possibilities is to determine the reversal potential of I_{cAMP}. We used tail currents, which were present upon return of the membrane potential to the holding potential, to examine the reversal potential of I_{cAMP}. These tail currents represent the flow of current through membrane channels before the channels have had time to close after repolarization. If I_{cAMP} is a K^+ current, then the tail currents of I_{cAMP} should reverse near the K^+ equilibrium potential.

After the generation of an *I-V* relation at a holding potential of −50 mV in each cell, the V_h of each sensory neuron was shifted to −80 mV. A 300-ms pulse from −80 mV to a single test potential of +10 mV was used to activate voltage-dependent membrane currents. Upon return of the membrane potential to −80 mV, the tail current was recorded. This allowed for the comparison of I_{cAMP} obtained from two different V_h values and for the comparison of tail currents at two different repolarization potentials. Because the reversal potential for K^+ currents is presumably between the two V_h values, the tail current of I_{cAMP} would be expected to reverse direction between −50 and −80 mV if I_{cAMP} is a K^+ current. Current traces were averaged and subtracted as described above to obtain I_{cAMP} at two different holding potentials. The subtracted current traces that represent I_{cAMP} obtained with pulses from −50 to +10 mV (V_h = −50 mV) and with pulses from −80 to +10 mV (V_h = −80 mV) are shown in Fig. 3. The tail current after the pulse reverses between −50 and −80 mV. Although a possible contribution from other membrane currents cannot be excluded, these results indicate that a K^+ current is at least partially responsible for the net outward current that is reduced 24 hours after injection of cAMP.

The comparison of I_{cAMP} obtained from two different holding potentials (−50 and −80 mV) gives further insight into the nature of the K^+ current that is reduced by cAMP. Two K^+ currents, which are present in sensory neurons (*17, 18*), demonstrate voltage-dependent steady-state inactivation. These are the transient or "A" current (I_A) and the delayed rectifier current ($I_{K,V}$). A voltage-clamp pulse from −80 to +10 mV would cause greater activation of these currents than a pulse from −50 to +10 mV. However, I_{cAMP} obtained during pulses from −80 to +10 mV is not greater than I_{cAMP} obtained during pulses from −50 to +10 mV (Fig. 3). This suggests that neither I_A nor $I_{K,V}$ is a major component of I_{cAMP}. Thus, these currents do not appear to be regulated significantly by cAMP over a long period of time in the sensory neurons. The two remaining membrane K^+ currents that are present in the sensory neurons are a Ca^{2+}-activated K^+ current and the "S" current (*17, 18*). The presence of a cAMP difference current near the V_h (−50 mV) suggests that S current may be a component of I_{cAMP}. However, we cannot exclude the possibility that part of I_{cAMP} represents other K^+ currents.

Although we have not tested the role of protein synthesis during long-term sensitization or cAMP injection, Schacher *et al.* (*19*) have demonstrated that protein synthesis is required for the long-term effects of membrane-permeable cAMP analogs in cultured sensory neurons. In addition, at least some of the long-term effects of serotonin in cultured sensory neurons (*6, 8*) are mimicked by cAMP (*19*). These results indicate that many of the cellular changes that accompany long-term sensitization are induced by transient elevation in the levels of cAMP. Also, many of these processes involve protein synthesis or the regulation of protein synthesis. In addition, a change in the ratio of subunits of the cAMP-dependent protein kinase (*20*) may contribute to long-term effects on K^+ currents observed in the sensory neurons after injection of cAMP.

The current that is reduced over a long period of time as a consequence of cAMP injection has relatively slow kinetics and mild voltage dependence. However, it appears to be active close to the resting potential and the threshold for action potential generation. This indicates that long-term reduction of I_{cAMP} may be involved in enhancing the excitability of the sensory neurons and in the control of repetitive firing (*7, 8, 17, 21*). Thus, at least one of the substrates for the long-term cellular ramifications of sensitization is a K^+ current that appears to control the excitability and repetitive firing of sensory neurons.

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12. The experiment was "blind" as to which electrodes contained cAMP and which contained 5′-AMP. Sensory neurons were chosen so that each cluster had a random spatial distribution of 5′-AMP and cAMP cells. An approximately equal number of cells in each cluster were injected with cAMP and with 5′-AMP. Thus, each cluster served as its own control.
13. The iontophoresis protocol was as follows: three trains of pulses (each train having a duration of 20 s) were delivered to the intracellular electrode to inject either cAMP or 5′-AMP. The trains consisted of 1-nA hyperpolarizing pulses (50 ms on, 50 ms off). Trains were separated by about 1 min. After the three trains, one long hyperpolarizing pulse (1 nA, 420 s) was delivered. Finally, two more trains of pulses were delivered as previously described. The total time of iontophoresis was 520 s, where 100 s was on a 50% duty cycle. This mixed protocol was developed during pilot studies and appeared to give the most successful iontophoresis. Only cells that could be visibly identified by the presence of the dye after iontophoresis were used in the study.
14. For organ culture, the entire recording dish was submerged in 500 ml of culture medium. The culture medium used was half-strength (50% v/v) McCoy's 5A Medium (Hazelton) that was made up with additional salts added to bring the medium to marine seawater conditions; the other 50% was ASW. The medium was brought to *p*H 7.6 with NaOH and buffered with 10 m*M* tris. This medium was supplemented with 5% sterile filtered *Aplysia* hemolymph and was sterile filtered before use. The incubation was carried out overnight at 15°C (the approximate temperature of the natural environment of *A. californica*).
15. Only cells that could initiate action potentials, had resting potentials more negative than −35 mV, and had input resistances greater than 7 megohms after impalement with both microelectrodes were voltage-clamped. A total of 36 of these cells in ten different clusters of sensory neurons were successfully voltage-clamped (17 5′-AMP cells and 19 cAMP cells). There was no detectable difference in the survival rate between cells injected with cAMP and those injected with 5′-AMP.
16. For statistical analysis, the data were reduced so that each cluster yielded two scores for the cAMP cells and two scores for the 5′-AMP cells. For example, the net outward current at the end of the five depolarizing pulses of the control response family were summed to generate one of the scores for the control cells. The net currents at the end of the two hyperpolarizing pulses of the response family were summed to generate the second score for the control cells. The same procedure was used to generate two scores for the cAMP cells. Differences (cAMP versus 5′-AMP) in corresponding depolarized scores from all the clusters were tested by a paired *t* test. The hyperpolarized scores were treated as independent from the depolarized scores and were also tested by a paired *t* test. A one-tailed analysis was used based on pilot experiments.
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22. We thank W. Scholz for assistance with organ culture and D. Baxter, L. Cleary, E. Kandel, and S. Schacher for comments on the manuscript. Supported by NIMH award K02 MH00649 and NIH grant NS 19895.

21 January 1988; accepted 13 April 1988