Mechanism of Calcium-Dependent Inactivation of a Potassium Current in *Aplysia* Neuron R15: Interaction Between Calcium and Cyclic AMP

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In the preceding paper (Kramer and Levitan, 1988), we presented evidence that an inwardly rectifying K⁺ current (I_R) is inactivated by Ca²⁺ influx accompanying spontaneous bursting activity in the *Aplysia* neuron R15. In this paper we examine the mechanism that enables Ca²⁺ to inactivate I_R . Since I_R is enhanced by cyclic AMP in neuron R15 (Drummond et al., 1980; Benson and Levitan, 1983), we examined the Ca²⁺-dependent inactivation of I_R after application of either serotonin (5-HT), the adenylate cyclase activator forskolin, or a membrane-permeable cAMP analog, all agents that increase cAMP and hence the magnitude of I_R . Even though more active I_R channels are available under these conditions, less Ca²⁺-dependent inactivation is observed. This is contrasted with the Ca²⁺-dependent inactivation of the voltage-gated Ca²⁺ current (I_{Ca}). Elevating cAMP enhances I_{Ca} in R15 and also increases its Ca²⁺-dependent inactivation. Hence the mechanisms whereby Ca²⁺ inactivates I_R and I_{Ca} appear to differ from each other.

Elevating internal Ca²⁺ by repeatedly depolarizing the neuron suppresses the response of I_R to brief applications of 5-HT, and speeds the relaxation of the response, suggesting that Ca²⁺ can interfere with the cAMP-dependent activation of I_R . One biochemical site where Ca²⁺ can reduce cellular cAMP is by activating the Ca²⁺/calmodulin-sensitive form of phosphodiesterase. We have detected such enzyme activity in homogenates of *Aplysia* abdominal ganglia and extracts of single R15 somata. Inhibitors of the phosphodiesterase activity suppress the Ca²⁺-dependent inactivation of I_R . Finally, we have used a radioimmunoassay to measure cAMP in individual R15 somata, and have found that R15 neurons hyperpolarized for prolonged periods contain more cAMP than do R15 neurons allowed to burst, consistent with the hypothesis that Ca²⁺ influx reduces cAMP. Therefore we conclude that the Ca²⁺-dependent inactivation of I_R is due to an interaction between Ca²⁺ and cAMP metabolism, and that stimulation of the Ca²⁺/calmodulin-dependent phosphodiesterase contributes to the inactivation of I_R in neuron R15.

Ca²⁺ and cyclic AMP are well-established intracellular messengers in neurons. Studies of physiological interactions between these messengers, however, are still in their infancy. The *Aplysia* bursting pacemaker neuron R15 is ideal for studying such interactions because the individual roles that Ca²⁺ and cAMP play are well understood. Intracellular Ca²⁺, which increases during bursts of action potentials, regulates several ionic currents that participate in generating the bursting pattern of electrical activity (Gorman et al., 1982; Adams and Levitan, 1985; Kramer and Zucker, 1985a, b). cAMP mediates the effects of serotonin (5-HT; Drummond et al., 1980) and of the *Aplysia* neuropeptide egg-laying hormone (ELH; Levitan et al., 1987) on several ionic currents in neuron R15. The cAMP-mediated responses include an increase of an inwardly rectifying K⁺ current, I_R (Benson and Levitan, 1983), and an increase of a voltage-gated Ca²⁺ current, I_{Ca} (Lotshaw et al., 1986; Levitan and Levitan, 1988).

Studies in neuron R15 have shown that Ca²⁺ and cAMP do not modulate electrical activity by privately regulating different ionic currents. Rather, there are several examples where Ca²⁺ and cAMP converge to modulate the same ionic currents (see Kramer et al., 1988). In the preceding paper (Kramer and Levitan, 1988), we reported that I_R is modulated by intracellular Ca²⁺, in addition to the enhancement by cAMP reported earlier (Drummond et al., 1980; Benson and Levitan, 1983). Ca²⁺ influx leads to a large and prolonged inactivation of I_R . Hence, increasing Ca²⁺ or cAMP results in opposite changes in the magnitude of I_R in neuron R15. In this paper, we examine the biochemical mechanism that enables internal Ca²⁺ to inactivate I_R . In particular, we have explored the possibility that Ca²⁺ operates by interacting with cAMP metabolism.

The molecular mechanisms that enable Ca²⁺ and cAMP to modulate ionic currents are of great contemporary interest. All of the effects of cAMP in eukaryotic cells are thought to be due to activation of a specific cAMP-dependent protein kinase (Greengard, 1978; but see Nakamura and Gold, 1987). Indeed, this enzyme has been shown to mediate the effect of cAMP on a variety of ionic currents, including I_R in neuron R15 (for review, see Levitan, 1985). In contrast, intracellular Ca²⁺ may modulate ionic currents by at least 3 different mechanisms. First, Ca²⁺ directly regulates certain types of ion channels, such as the "maxi" Ca²⁺-activated K⁺ channel (Moczydlowski and Latorre, 1983), by binding to the channel protein and allosterically affecting its ability to open and close. Second, Ca²⁺ may regulate ionic currents through a more indirect biochemical route by changing the phosphorylation state of ion channels. For example, Ca²⁺/calmodulin-dependent protein kinases are thought

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to modulate ionic currents in *Hermisenda* photoreceptors (Sakakibara et al., 1986) and mammalian brain (DeLorenzo, 1984), while Ca^{2+} /calmodulin-dependent phosphatases may underlie Ca^{2+} -dependent inactivation of Ca^{2+} currents (Eckert and Chad, 1984; Armstrong and Eckert, 1987). Finally, Ca^{2+} may regulate ionic currents through an even more circuitous pathway involving regulation of cAMP metabolism. Ca^{2+} , via calmodulin, can regulate cAMP synthesis by modulating adenylate cyclase activity (Brostrom et al., 1978) and can regulate cAMP hydrolysis by activating a form of phosphodiesterase (PDE; Wolff and Brostrom, 1979). Such modulation of cAMP metabolism by internal Ca^{2+} is thought to be involved in the changes of electrical activity that accompany associative conditioning in *Aplysia* sensory neurons (Abrams et al., 1985; Ocorr et al., 1985).

In this study we have used a combined electrophysiological and biochemical approach to ask which of the above mechanisms is responsible for the Ca^{2+} -dependent inactivation of I_R . The results indicate that Ca^{2+} inactivates I_R by reducing the cAMP-dependent activation of I_R . A preliminary report of this work has appeared previously (Kramer and Levitan, 1987).

Materials and Methods

All experiments were performed on neuron R15 of the abdominal ganglion of *Aplysia californica*. Methods of dissection, 2-electrode voltage clamping, and the composition of artificial seawater (ASW) solutions are as described in Kramer and Levitan (1988) and Kramer and Zucker (1985a). The Ca^{2+} -dependent inactivation of I_R was elicited by using the voltage-clamp protocol introduced in Kramer and Levitan (1988), except where noted otherwise. Ganglia were superfused continuously with saline solutions (21–23°C) at 1–3 ml/min. Neurotransmitters and pharmacological agents were included in the perfusing medium except for in the experiments illustrated in Figure 7. In these experiments, 5-HT was applied with pressure from the tip of a micropipette, and 1 mM phenol red was used as a marker dye to aid in visualizing the local application of 5-HT on the soma of R15.

5-HT, 3-isobutyl-1-methylxanthine (IBMX), and 8-(4-chlorophenylthio) cAMP (8-PCPT-cAMP) were obtained from Sigma. Forskolin was obtained from Calbiochem. RO-20-1724 was kindly provided by W. Burkhardt, Hoffman-LaRoche. Saline solutions containing these substances were made up immediately prior to use. Forskolin and RO-20-1724 stock solutions were made in ethanol, and working solutions did not contain in excess of 0.2% ethanol. This concentration of ethanol had no effect on ionic currents in control experiments.

Phosphodiesterase assay. PDE assays were performed using the method of Walter and Kiger (1984) with either *Aplysia* abdominal ganglia or isolated R15 somata. Ganglia were homogenized in 100 μl of homogenization buffer (50 mM Tris-HCl, pH 8.0) with a ground-glass tissue homogenizer; single somata were disrupted by sonication and vortexing in 100 μl of homogenization buffer. Five microliters of the ganglion homogenate or 20 μl of the single-cell homogenate were added to 100 μl of assay buffer, consisting of 50 mM NaCl, 10 mM MgCl_2 , 10 mM EGTA, 1 mg/ml BSA, 4 mM β -mercaptoethanol, and 50 mM Tris-HCl (pH 8.0). CaCl_2 was added at appropriate concentrations (0.36–9.95 mM) to give calculated free Ca^{2+} concentrations of 10^{-9} – 10^{-4} M. Calmodulin (Sigma), 1 μM , was also included in the assay medium, although the homogenates exhibited considerable Ca^{2+} -dependent, trifluoperazine-sensitive PDE activity in the absence of exogenous calmodulin, implying a high level of endogenous calmodulin in the tissue. Reactions were initiated by the addition of 5 μl substrate containing 3 μCi ^3H -cAMP, 20 μM cAMP, and 20 mM AMP. Reaction mixtures were incubated for 15–20 min at 37°C, and the reaction was terminated by addition of 5 μl of carrier (10 mM cAMP and 10 mM AMP) and immersion of the assay tube in a boiling water bath. A volume of 5 μl was spotted onto a strip of PEI-cellulose thin-layer chromatography (TLC) sheet containing fluorescence indicator (Brinkmann Instruments) and chromatographed in an ascending system with 50 mM KCl. After about 45 min, cAMP and AMP were identified under a shortwave UV lamp, cut from the strips, eluted with 1 ml of 100 mM MgCl_2 in 20 mM Tris-HCl (pH 7.4), and then counted in a liquid-scintillation counter.

Single somata isolation. Single R15 somata were removed from frozen

Figure 1. Effect of 50 μM 5-HT on I_R , I_{Ca} , and their Ca^{2+} -dependent inactivation in R15. *A*, Membrane currents resulting from 1 sec hyperpolarizing voltage-clamp pulses from -80 to -110 mV. The membrane current is due primarily to I_R (see Kramer and Levitan, 1988). *B*, Membrane currents resulting from 1 sec depolarizing pulses from -75 to -45 mV. The inward current is due to I_{Ca} (Gorman et al., 1982; Kramer and Zucker, 1985a, b; Levitan and Levitan, 1988). In both *A* and *B*, the currents were recorded before (*left*) and 10 min after (*right*) bath application of 50 μM 5-HT. Each of the 4 traces shows 2 superimposed recordings: the larger current was obtained immediately before, and the smaller (partially inactivated) current (filled circles) 90 sec after a "simulated burst" of depolarizing pulses designed to induce Ca^{2+} influx. The simulated burst consisted of five 150 msec depolarizing pulses to 0 mV.

ganglia using a modification of the method of Giller and Schwartz (1971). After lowering the bath volume so that the desheathed ganglion was barely covered by saline, 2 ml of a solution of 70% propylene glycol/30% normal saline (vol/vol) cooled to below -20°C was dropped onto the surface. After about 5 sec, the ganglion was covered with powdered dry ice, and the microelectrodes were removed from R15. The frozen soma of R15 was located under a dissecting microscope, broken away from the ganglion with fine forceps, and transferred to an assay tube using a needle that had been precooled by immersion in dry ice. The assay tube contained either 10 μl of assay buffer below -20°C for PDE experiments, or 100 μl of 98% ethanol/2% HCl (vol/vol) for determination of cAMP content by radioimmunoassay (DuPont Rianen cAMP RIA kit). Individual cells, or pools of 5 cells, were stored at -70°C for PDE assays or cAMP radioimmunoassay, respectively.

Results

Effect of 5-HT on the Ca^{2+} -dependent inactivation of I_R

Experiments using 5-HT provided the first evidence suggesting that Ca^{2+} does not interact directly with I_R channels to cause their inactivation. If the inactivation of I_R were a result of direct Ca^{2+} interaction with the channels, then when the magnitude of I_R is increased, the Ca^{2+} -dependent inactivation of I_R should increase in parallel. One way to increase the magnitude of I_R is to treat R15 neurons with 5-HT. As determined by noise analysis, the increase in I_R caused by 5-HT is due to an increase in the number of functional channels, rather than to an increase in the probability that individual channels are open (Gunning, 1987). Hence, in the presence of increasing concentrations of 5-HT, one might expect an increasing number of I_R channels to be inactivated by a constant influx of Ca^{2+} . In fact, just the opposite was observed.

Figure 1 shows the effect of 50 μM 5-HT on I_R and I_{Ca} , and on the Ca^{2+} -dependent inactivation of these currents. I_R was

Figure 2. Effect of $50\ \mu\text{M}$ 5-HT on the steady-state I-V curve of neuron R15. *A*, I-V curves obtained in normal saline. *B*, I-V curves obtained 15 min after application of $50\ \mu\text{M}$ 5-HT. In both *A* and *B*, I-V curves were generated before and 90 sec after a simulated burst consisting of five 150 msec depolarizing pulses to 0 mV. Ionic currents used in constructing the I-V curves were elicited by applying 400 msec pulses from a holding potential of -70 mV.

elicited by applying 1 sec hyperpolarizing voltage-clamp pulses from -80 to -110 mV (Fig. 1*A*), while I_{Ca} was elicited by applying 1 sec depolarizing pulses from -75 to -45 mV (Fig. 1*B*). The Ca^{2+} -dependent inactivation of these currents was brought about by applying a series of voltage-clamp depolarizations that was designed to simulate the Ca^{2+} influx that occurs during a burst of action potentials (Kramer and Levitan, 1988). The inactivation of I_{R} and I_{Ca} was measured 90 sec after the end of the simulated burst.

Addition of $50\ \mu\text{M}$ 5-HT causes a cAMP-dependent increase in the magnitude of both I_{R} and I_{Ca} , as described previously (Benson and Levitan, 1983; Levitan and Levitan, 1988). However, $50\ \mu\text{M}$ 5-HT has different effects on the Ca^{2+} -dependent inactivation of these 2 currents. 5-HT reduces the Ca^{2+} -dependent inactivation of I_{R} caused by the simulated burst (Fig. 1*A*), while it enhances the Ca^{2+} -dependent inactivation of I_{Ca} (Fig. 1*B*). The effect of 5-HT on the inactivation of I_{R} and I_{Ca} can

best be illustrated by constructing steady-state current-voltage (I-V) curves from voltage-clamp pulse data. The inactivation of I_{R} following a simulated burst is apparent at membrane potentials more negative than -70 mV, while the inactivation of I_{Ca} is apparent positive to -60 mV (Fig. 2*A*). The different effects of $50\ \mu\text{M}$ 5-HT on the Ca^{2+} -dependent inactivations of the 2 currents can be seen clearly in Figure 2*B*. The effect of 10 – $100\ \mu\text{M}$ 5-HT on the 2 currents is consistent: it reduces the inactivation of I_{R} by 82 – 100% , while the Ca^{2+} -dependent inactivation of I_{Ca} is increased ($n = 7$).

It is possible that the apparent suppression of I_{R} inactivation by 5-HT is due to a change in the kinetics of recovery from inactivation rather than to a decrease in the amount of inactivation. Figure 3 shows the recovery of I_{R} and I_{Ca} from inactivation after a simulated burst. The peak inactivation of I_{R} following a burst in normal saline is normally delayed by 60 – 90 sec. In the example shown in Figure 3*A*, the small degree of I_{R}

Figure 3. Kinetics of recovery from Ca^{2+} -dependent inactivation in normal (squares) and $50\ \mu\text{M}$ 5-HT (circles) saline. *A*, Recovery of I_{R} from inactivation induced by a simulated burst. I_{R} was elicited by applying 400 msec pulses from -75 to -115 mV. *B*, Recovery of I_{Ca} from inactivation induced by a simulated burst. I_{Ca} was elicited by applying 400 msec pulse from -75 to -45 mV. The simulated bursts in *A* and *B* were applied at time 0, and consisted of five 150 msec pulses to 0 mV.

Figure 4. Effect of forskolin and 8-PCPT-cAMP on the inactivation of I_R . *A*, Time course of I_R inactivation in normal saline (control) and 30 min after application of 100 μ M forskolin. *B*, Time course of I_R inactivation in normal saline (control) and 90 min after application of 1 mM 8-PCPT-cAMP. I_R was measured as in Figure 3. The simulated burst given at time 0 consisted of five 150 msec pulses to 0 mV.

inactivation measured in 50 μ M 5-HT does not exhibit a delay, but is maximal immediately after the end of the burst. This result, however, was quite variable. In some experiments, the residual I_R inactivation observed in 50 μ M 5-HT exhibited no delay ($n = 3$), while in other experiments 50 μ M 5-HT seemed to scale down the inactivation of I_R without changing the recovery kinetics ($n = 4$). In contrast, 5-HT increases the Ca^{2+} -dependent inactivation of I_{Ca} without changing the kinetics of its recovery from inactivation (Fig. 3*B*).

Effect of forskolin and 8-PCPT-cAMP on the inactivation of I_R

The above results demonstrate that 5-HT can reduce the Ca^{2+} -dependent inactivation of I_R , even though 5-HT increases the number of I_R channels available to be inactivated, and even though the Ca^{2+} current (and hence Ca^{2+} influx during the burst) is increased by 5-HT. Since 5-HT stimulates an adenylate cyclase in R15 (Levitan, 1978), leading to an increase in the level of cAMP (Cedar and Schwartz, 1972; Levitan and Drummond, 1980), we postulated that the reduction in Ca^{2+} -dependent inactivation of I_R elicited by 5-HT was mediated by cAMP. To test this hypothesis, the Ca^{2+} -dependent inactivation of I_R was measured after application of forskolin (Fig. 4*A*), which directly activates adenylate cyclase (Seamon et al., 1981). Addition of 100 μ M forskolin reduces the Ca^{2+} -dependent inactivation of I_R by 75–94% ($n = 3$). In order to test whether an increase in cAMP per se can reduce the Ca^{2+} -dependent inactivation of I_R , the inactivation of I_R was measured after application of the membrane-permeable analog 8-PCPT-cAMP (Fig. 4*B*). Addition of 1 mM 8-PCPT-cAMP reduces the Ca^{2+} -dependent inactivation of I_R by 55–83% ($n = 3$). Hence increasing cAMP in R15 can suppress the Ca^{2+} -dependent inactivation of I_R .

Sensitivity of the Ca^{2+} -dependent inactivation of I_R to 5-HT

Only high concentrations of 5-HT reduce the Ca^{2+} -dependent inactivation of I_R in neuron R15. Figure 5*A* shows dose-response curves of the effect of 5-HT on I_R both before and 90 sec after a simulated burst. Increasing concentrations of 5-HT result in an increase in the amplitude of I_R , up to a saturating concentration of 1–10 μ M. Higher 5-HT concentrations produce no further increase of I_R . The curve of Figure 5*B* shows the

decrease in I_R resulting from the simulated burst as a function of 5-HT concentration, taken from the difference between the 2 curves of Figure 5*A*. In contrast to the cAMP-dependent activation of I_R by 5-HT, the Ca^{2+} -dependent inactivation of I_R increases only up to a 5-HT concentration of between 1 and 10 μ M, and drops dramatically at higher concentrations. Thus, it is only at saturating concentrations of 5-HT that the inhibition of Ca^{2+} -dependent inactivation begins to occur. At less than saturating 5-HT concentrations, a nearly constant fraction of the total magnitude of I_R is inactivated by depolarizing pulses (Fig. 5*C*). Above 1 μ M 5-HT, there is an abrupt decrease in the fraction of I_R inactivated by Ca^{2+} influx.

These results demonstrate that concentrations of 5-HT that result in a maximal activation of I_R reduce the ability of intracellular Ca^{2+} to inactivate I_R . This observation seems to be inconsistent with a direct action of Ca^{2+} on I_R channels. One scenario that would better explain the results is that Ca^{2+} counteracts the cAMP-dependent activation of I_R , and, at high levels of cAMP, the effect of Ca^{2+} is overwhelmed. We have considered an alternative explanation, however.

It is known that the cAMP-dependent modulation of I_R is due to phosphorylation of some protein substrate, possibly the I_R channel itself (Lemos et al., 1986). Perhaps Ca^{2+} does interact directly with I_R channels, but phosphorylated I_R channels are insensitive to Ca^{2+} -dependent inactivation. Two observations argue against this hypothesis. First, the Ca^{2+} -dependent inactivation of I_R is actually enhanced by addition of up to 1 μ M 5-HT, suggesting that modulated channels recruited by 5-HT can be inactivated. Second, the suppressive effect of high concentrations of 5-HT on inactivation can be overcome with a very large influx of Ca^{2+} , as shown in Figure 6. In this experiment, the amplitude of the inward current elicited by hyperpolarizing pulses increased from 18 to 48 nA following addition of 50 μ M 5-HT, indicating an enhancement of I_R . Under these conditions, a single burst of depolarizing pulses would produce little inactivation of I_R (e.g., see Figs. 1–3, 5). At the arrow (Fig. 6), the neuron was released from voltage-clamp, and spontaneously fired bursts of action potentials. After 5 min of continuous bursting activity, the neuron was voltage-clamped once again. The hyperpolarizing pulses now elicited only 28 nA of

Figure 5. Dose-response curves of 5-HT action on I_R . *A*, Activation of I_R as a function of 5-HT concentration. Currents were elicited by applying 400 msec pulses from -75 to -115 mV before and 90 sec after simulated bursts in saline containing 0–100 μM 5-HT. *B*, Inactivation of I_R as a function of 5-HT concentration. The decrease in current resulting from the simulated burst is taken as the difference between the 2 curves shown in *A*. *C*, Percentage of I_R inactivated as a function of 5-HT concentration. Data points show the decrease in current (as in *B*) divided by the current elicited before the simulated burst.

Figure 6. Suppression of I_R inactivation by 5-HT can be overcome with a large influx of Ca^{2+} . Current in normal saline, after addition of 50 μM 5-HT, and after addition of 1 mM Ba^{2+} . At the bold arrow, the cell was released from voltage-clamp and fired bursts of spikes for 5 min; then the voltage-clamp was turned on once again. Note the inactivation of I_R induced by the prolonged bursting activity. Currents were elicited by applying 400 msec pulses from -75 to -110 mV. The dashed line indicates the leak current present after addition of Ba^{2+} .

current, indicating that 20 nA of I_R was inactivated by the bursting activity. After recovery from the bursts, 1 mM Ba^{2+} was added to block all of the I_R (Benson and Levitan, 1983; Kramer and Levitan, 1988), revealing a residual leak current of 9 nA. Hence, only about 9 nA of the initial 18 nA of current present before 5-HT application was I_R , but the prolonged bursting activity inactivated 20 nA of current. Therefore, at least a portion of the I_R inactivated by the bursts must have been recruited by 5-HT, implying that the modulated channels are indeed susceptible to Ca^{2+} -dependent inactivation.

Internal Ca^{2+} antagonizes the response of R15 to 5-HT

There is an additional consequence of the apparent interaction between Ca^{2+} and cAMP in R15, as illustrated in Figure 7. In the experiments discussed above, we examined the effect of Ca^{2+} on I_R in the presence of a given concentration of 5-HT, after the cAMP-dependent activation of I_R had reached steady state. In the experiment illustrated in Figure 7, we examined the effect of internal Ca^{2+} on the activation of I_R brought about by a transient application of 5-HT. 5-HT was applied transiently as follows: A micropipette (tip diameter, about 10 μm) filled with 100 μM 5-HT was positioned 300 μm from R15 during each puff, and was removed from the recording chamber after each

Figure 7. Ca^{2+} influx reduces the activation of I_R by 5-HT. *A*, Example of modulation of I_R by local application of a 10 sec "puff" of $100 \mu\text{M}$ 5-HT at the soma of R15. Currents were elicited by applying 800 msec pulses from -80 to -120 mV. *B*, 5-HT responses in an R15 continuously hyperpolarized negative to -80 mV or " Ca^{2+} -loaded" by applying 150 msec pulses to -5 mV every 5 sec. These 2 treatments were alternated, and the I_R was allowed to reach steady state in each of the 2 conditions before the 5-HT puff was applied. Data represent mean $\pm$ SEM of the increase in I_R elicited by three 10 sec puffs of $100 \mu\text{M}$ 5-HT delivered to an R15 in each of the 2 conditions. I_R was measured by applying 400 msec pulses from -80 to -120 mV.

puff was complete. The 5-HT puff was applied to an R15 neuron that was either continuously voltage-clamped to a hyperpolarized membrane potential (-80 mV) to prevent Ca^{2+} influx through voltage-gated Ca^{2+} channels, or periodically depolarized to 0 mV to induce Ca^{2+} influx. I_R was measured at 5 sec intervals by hyperpolarizing the cell from -80 to -115 mV (Fig. 7*A*). The 5-HT puff results in a prolonged increase in I_R when the cell is kept hyperpolarized to prevent Ca^{2+} influx. In contrast, the response of I_R to the 5-HT puff is smaller, and relaxes more rapidly, when the neuron is "loaded" with Ca^{2+} by repeated depolarizations. Figure 7*B* shows a summary of the results from 6 puffs of 5-HT, alternately delivered to R15 in the hyperpolarized or the Ca^{2+} -loaded state. The peak increase of I_R induced by the 5-HT puff in the Ca^{2+} -loaded state is only 66% as large as that in the hyperpolarized state. In addition, I_R recovers more quickly to its initial amplitude when 5-HT is applied in the Ca^{2+} -loaded state. Similar results are obtained when $100 \mu\text{M}$ egg-laying hormone (ELH), instead of 5-HT, is puffed onto the neuron. Hence, Ca^{2+} -loading antagonizes the response of R15 to neurotransmitters whose effects are mediated by cAMP, suggesting once again that Ca^{2+} interacts with cAMP metabolism.

Figure 8. Phosphodiesterase activity in abdominal ganglion homogenates. PDE activity measured as the degradation of ^3H -cAMP in buffer containing 10^{-8} – 10^{-4} M Ca^{2+} . Assay was performed in control assay buffer, assay buffer containing $100 \mu\text{M}$ RO-20-1724, or assay buffer containing $100 \mu\text{M}$ IBMX. Data are normalized with respect to maximal PDE activity (control assay at 10^{-6} M Ca^{2+}). Error bars represent range of results from 2 separate assays.

Ca^{2+} -sensitive phosphodiesterase activity

We have focused our biochemical studies on one locus at which Ca^{2+} could regulate cAMP levels and potentially lead to a decrease in the magnitude of I_R : the Ca^{2+} /calmodulin-activated form of PDE. We have detected both Ca^{2+} -sensitive and Ca^{2+} -independent PDE activity in homogenates of *Aplysia* abdominal ganglia (Fig. 8). In control assay buffer, about 70% of the total PDE activity in the homogenate is apparent below 10^{-7} M free Ca^{2+} (Ca^{2+} -independent PDE), while the remaining 30% is stimulated by increasing Ca^{2+} to 5×10^{-7} M or higher concentrations (Ca^{2+} -sensitive PDE). Almost half of the Ca^{2+} -independent PDE activity is blocked by addition of $100 \mu\text{M}$ RO-20-1724, a selective inhibitor of a form of Ca^{2+} -independent PDE in mammalian tissue (Tanner et al., 1986), while the Ca^{2+} -stimulated PDE is unaffected (Fig. 8). In contrast, $100 \mu\text{M}$ IBMX blocks most of the PDE activity below 10^{-7} M Ca^{2+} , and completely eliminates PDE activity stimulated by higher Ca^{2+} concentrations. Addition of $150 \mu\text{M}$ trifluoperazine, a calmodulin inhibitor (Weiss and Levin, 1978), blocks all of the Ca^{2+} -stimulated, and about half of the Ca^{2+} -independent, PDE activity in the ganglion homogenate (data not shown).

The 2 forms of PDE activity can also be detected in extracts from single, isolated R15 somata (Fig. 9). In these experiments, the activity of Ca^{2+} -independent PDE was taken as the rate of cAMP degradation in 10^{-9} M Ca^{2+} (Fig. 9*A*). The activity of Ca^{2+} -sensitive PDE was taken as the difference in the rates of cAMP degradation in the presence of 10^{-4} M Ca^{2+} and 10^{-9} M Ca^{2+} , with $100 \mu\text{M}$ RO-20-1724 added to reduce the background Ca^{2+} -independent activity (Fig. 9*B*). In assays from 4 R15 somata, the Ca^{2+} -sensitive PDE accounted for $31 \pm 7\%$ of the total measured PDE activity, while the Ca^{2+} -independent PDE accounted for the remaining $69 \pm 7\%$. Hence, R15 exhibits Ca^{2+} -sensitive and Ca^{2+} -independent PDE activities in about the same proportions measured in whole abdominal ganglia.

Effects of PDE inhibitors on the Ca^{2+} -dependent inactivation of I_R

If Ca^{2+} -dependent stimulation of PDE is responsible for the inactivation of I_R , then the inactivation should be blocked by addition of PDE inhibitors. Figure 10 shows the effect of IBMX

Figure 9. PDE activity in extracts of an R15 soma. *A*, Ca^{2+} -independent PDE activity measured in control buffer containing 10^{-9} M Ca^{2+} . *B*, Ca^{2+} -sensitive PDE activity in assay buffer containing 10^{-4} M Ca^{2+} and 10^{-9} M Ca^{2+} . RO-20-1724, $100 \mu\text{M}$, was included to suppress background Ca^{2+} -independent PDE activity. Linear regression lines fitted to the data in both *A* and *B* were used to determine reaction rates.

on the Ca^{2+} -dependent inactivation of I_R . The standard voltage-clamp paradigm, consisting of bursts of depolarizing pulses, was used to induce the Ca^{2+} -dependent inactivation of I_R at 20 min intervals. Current-voltage curves were obtained both before and 90 sec after each burst, providing a measure of I_R and of the Ca^{2+} -dependent inactivation of I_R (see Kramer and Levitan, 1988). IBMX induces a small increase in I_R , presumably by causing a small increase in cAMP (Fig. 10, top). Furthermore, addition of IBMX results in a reversible decrease in the inac-

tivation of I_R (Fig. 10, bottom). At the end of the experiment, $50 \mu\text{M}$ 5-HT was added to the bathing medium, inducing a large increase in I_R . This demonstrates that the cAMP-dependent activation of I_R was not saturated by the IBMX alone. Thus, in contrast to the effects of 5-HT, forskolin, and 8-PCPT-cAMP, the inhibition of inactivation by IBMX does not seem to be caused by greatly increasing the level of cAMP in the neuron. We propose that the inhibition of inactivation by IBMX is due to direct inhibition of the Ca^{2+} -sensitive PDE per se.

We also tested the effect of RO-20-1724 on the Ca^{2+} -dependent inactivation of I_R . Addition of $100 \mu\text{M}$ RO-20-1724 results in an increase in I_R (Fig. 11, top), but only at higher concentrations ($300 \mu\text{M}$ RO-20-1724) does it begin to reduce the inactivation of I_R (Fig. 11, bottom). The large increase of I_R and corresponding decrease of inactivation at $300 \mu\text{M}$ RO-20-1724 suggest that the 2 phenomena may be caused by the same factor, namely, an increase in cAMP. Hence, it seems likely that high RO-20-1724, like high 5-HT, inhibits the inactivation of I_R by greatly increasing the level of cAMP in the neuron. Thus, we propose that RO-20-1724 leads indirectly to an inhibition of Ca^{2+} inactivation, in accord with the finding that RO-20-1724 inhibits the Ca^{2+} -independent, but not the Ca^{2+} -sensitive, PDE.

Finally, we tested directly whether Ca^{2+} influx during bursting activity regulates the level of cAMP in neuron R15. Figure 12 shows the results of a series of radioimmunoassays that were performed on R15 somata that were rapidly frozen and isolated from abdominal ganglia. The R15 neurons were either allowed to burst continuously for 20 min, or hyperpolarized to a potential negative to -75 mV under voltage-clamp for 20 min prior to rapid freezing. Five cells in each of these conditions were pooled, and the experiment was performed 3 times. The R15 neurons that were hyperpolarized have $41 \pm 7.3\%$ more cAMP than those that were allowed to burst spontaneously. Presumably, the spontaneously bursting neurons are "loaded" with Ca^{2+} , while the hyperpolarized neurons are relatively depleted of Ca^{2+} . Therefore, measurements of cAMP in R15 are consistent with the notion that Ca^{2+} activates PDE in the spontaneously bursting cell.

Discussion

We have considered 3 possible mechanisms whereby Ca^{2+} could inactivate I_R in neuron R15:

Figure 10. Effect of IBMX on Ca^{2+} -dependent inactivation of I_R . I_R (top) and the inactivation of I_R elicited by simulated bursts (bottom) were measured at 20 min intervals. Addition of 1 mM IBMX reversibly suppresses the inactivation of I_R . 5-HT, $50 \mu\text{M}$, was added at the end of the experiment to determine whether IBMX had saturated the activation of I_R . I_R was measured by applying 400 msec pulses from -75 to -115 mV.

Figure 11. Effect of RO-20-1724 on the Ca^{2+} -dependent inactivation of I_R . I_R (top) and the inactivation of I_R elicited by simulated bursts (bottom) were measured at 15 min intervals. RO-20-1724, 100 μM , increases I_R somewhat without reducing inactivation. A volume of 300 μM greatly increases I_R and does reduce inactivation. I_R was measured by applying 400 msec pulses from -75 to -115 mV.

1. Direct modulation of I_R channels

The evidence presented in this paper suggests that Ca^{2+} does not inactivate I_R directly by interacting with I_R channels. Increasing the magnitude of I_R by adding agents that elevate cAMP (5-HT, forskolin, 8-PCPT-cAMP) leads to a paradoxical inhibition of the Ca^{2+} -dependent inactivation of I_R . This occurs even though each of these agents enhances the voltage-gated Ca^{2+} current. We did not attempt to measure the level of Ca^{2+} in neuron R15; hence it is possible that cAMP changes either the background concentration of Ca^{2+} or the Ca^{2+} buffering capacity of the cytoplasm, and it is possible that changes in these parameters contribute to the suppression of I_R inactivation. The inhibition of inactivation does not appear to be caused by an acceleration of the recovery of I_R following Ca^{2+} influx (Figs. 3, 4), and is not due to an insensitivity of modulated I_R channels to Ca^{2+} (Fig. 6). In contrast, substances that increase cAMP do enhance the Ca^{2+} -dependent inactivation of I_{Ca} in R15 (Figs. 1–3; Levitan and Levitan, 1988), presumably by increasing both Ca^{2+} influx and the magnitude of I_{Ca} available for inactivation. Hence, there appears to be an important difference between the way Ca^{2+} inactivates I_R and I_{Ca} . Our results do not exclude a direct interaction of Ca^{2+} with Ca^{2+} channels as the critical step underlying Ca^{2+} -dependent inactivation of Ca^{2+} currents. However, electrophysiological (Chad and Eckert, 1986; Armstrong and Eckert, 1987) and biochemical (Hosey et al., 1986) studies suggest that the Ca^{2+} -dependent inactivation of at least some Ca^{2+} channels is not due to the direct binding of Ca^{2+} , but rather to stimulation of a phosphatase that reverses the cAMP-dependent phosphorylation of these channels. It is of course possible

Figure 12. CAMP content of spontaneous bursting and hyperpolarized R15 neurons. Three experiments were performed, each using 5 R15 neurons in each of the 2 conditions. The isolated somata were pooled and 2 separate determinations were made with each group of 5 cells. CAMP content is normalized with respect to the spontaneously bursting neurons in each experiment.

that the Ca^{2+} -dependent inactivation of Ca^{2+} currents in *Aplysia* neurons involves both direct and indirect mechanisms.

2. Modulation of I_R channels via activation of kinases or phosphatases

We have no direct evidence bearing on the hypothesis that Ca^{2+} causes I_R inactivation by activation of a Ca^{2+} -sensitive protein kinase or phosphatase. Despite the evidence for an interaction between Ca^{2+} and cAMP (see below), Ca^{2+} could still exert its effect on I_R by causing the phosphorylation of the same substrate that is phosphorylated by the cAMP-dependent protein kinase. Ca^{2+} - and cAMP-dependent protein kinases have been shown to cause dual phosphorylation of one substrate protein (synapsin I), both at identical and at different sites on the protein, allowing for possible interactions between the 2 intracellular messengers at their target effector molecules (for review, see Nestler and Greengard, 1983). It is also possible that Ca^{2+} activates a phosphatase that dephosphorylates the substrate responsible for I_R -channel activation. This seems less likely, as is discussed below.

3. Modulation of I_R by inhibition of cAMP-dependent activation

Our results support the hypothesis that Ca^{2+} causes the inactivation of I_R by decreasing the cAMP-dependent activation of the current. Ca^{2+} influx not only inactivates the basal I_R , but also decreases I_R that can be recruited by brief application of neuromodulators such as 5-HT (Fig. 7) and ELH, implying that Ca^{2+} inhibits the cAMP-dependent activation of I_R . Indeed, recent studies using a range of 5-HT concentrations have indicated that "loading" R15 with Ca^{2+} shifts the dose-response curve of the I_R response to higher 5-HT concentrations without reducing the maximal response (R. H. Kramer, unpublished observations). In addition Ca^{2+} -loading accelerates the recovery of I_R to its basal level after the neuron has been briefly exposed to these neuromodulators, suggesting that Ca^{2+} can affect a step in the cAMP cascade that persists after the 5-HT has been washed away from the neuron. The component of the cAMP cascade that remains active after the removal of agonist seems to be located somewhere before the phosphorylated substrate (e.g., ion channel protein), as is suggested by studies using a specific inhibitor of cAMP-dependent protein kinase (PKI). Injection of *Aplysia* sensory neurons (Castellucci et al., 1982) and

neuron R15 (J. Lemos and W. Adams, personal communication) with PKI rapidly reverses the effect of 5-HT on the modulated K^+ current, suggesting that the phosphorylation of the protein that regulates the current is labile, and that the continuous activity of the kinase is necessary to maintain the K^+ channels in their modulated state (Castellucci et al., 1982). If the phosphorylated substrate that activates I_R is short-lived in R15, then it is unlikely that Ca^{2+} acts at a step subsequent to the phosphorylation of this substrate. Hence, we consider it unlikely that a Ca^{2+} -stimulated phosphatase is the primary locus at which Ca^{2+} acts to cause the inactivation of I_R , although we have no direct evidence that would rule out a contribution from such a mechanism.

There are 2 steps preceding phosphorylation at which Ca^{2+} has been shown to regulate cAMP metabolism. First, Ca^{2+} , via calmodulin, regulates adenylate cyclase activity. The effect of Ca^{2+} on adenylate cyclase activity in both mammalian (Gnegy and Treisman, 1981) and *Aplysia* (Weiss and Drummond, 1985) tissues is biphasic: up to 10 μM Ca^{2+} activates the cyclase, while higher concentrations inhibit it. Conjoint activation of adenylate cyclase activity by agonists and Ca^{2+} (below 10 μM) has been proposed as a mechanism that enhances cAMP-dependent responses during associative conditioning (Abrams et al., 1985; Ocorr et al., 1985). The inhibition of the cyclase by Ca^{2+} concentrations exceeding 10 μM could, in theory, contribute to the Ca^{2+} -dependent inactivation of I_R in R15, but whether Ca^{2+} ever exceeds this concentration in R15 is unknown.

The second step at which Ca^{2+} regulates cAMP metabolism is at the activation of PDE via calmodulin. Our results show that the *Aplysia* abdominal ganglion, and R15 in particular, contains a Ca^{2+} /calmodulin-sensitive PDE, as well as a Ca^{2+} -independent PDE. Multiple forms of PDE with different substrate specificities, kinetics, sensitivity to pharmacological agents, and regulation by Ca^{2+} have been identified previously in mammals (Wells and Hardman, 1977; Tanner et al., 1986; Weishaar et al., 1986) and invertebrates (Davis and Kauvar, 1984). The Ca^{2+} /calmodulin-activated PDE has been immunocytochemically localized to dendrites and somata of brain neurons, suggesting a postsynaptic function (Kincaid et al., 1987). In a variety of tissues, this enzyme is thought to be regulated by physiological changes in Ca^{2+} , and now there is direct evidence that PDE is activated by physiological changes of Ca^{2+} in intact smooth muscle cells (Saitoh et al., 1985).

We propose that activation of Ca^{2+} /calmodulin-sensitive PDE is at least partly responsible for the inactivation of I_R in neuron R15. The Ca^{2+} -dependent inactivation of I_R is inhibited by the addition of the PDE inhibitor IBMX, and apparently this inhibition does not require a large increase in cAMP (Fig. 10). The observation that bursting pacemaker activity reduces cAMP content is consistent with the hypothesis that Ca^{2+} stimulates PDE activity. The inactivation of I_R is also inhibited by adding RO-20-1724 (Fig. 11), a selective inhibitor of the Ca^{2+} -independent PDE, but we suggest that RO-20-1724 only inhibits the inactivation of I_R when an overwhelming concentration of cAMP builds up in the neuron. The finding that RO-20-1724 increases I_R without inhibiting the Ca^{2+} /calmodulin-sensitive PDE suggests that the Ca^{2+} -independent PDE is important for maintaining the basal level of cAMP. Under the conditions used in our assays, there seems to be more Ca^{2+} -independent than Ca^{2+} /calmodulin-sensitive PDE activity in R15. The relative importance of these 2 enzymes in regulating cAMP levels in the intact cell remains to be determined.

Thus, the most likely mechanism by which intracellular Ca^{2+} leads to an inactivation of I_R is via an interaction with cAMP metabolism. The Ca^{2+} -dependent inactivation of I_R could be just one of the effects of the interplay between Ca^{2+} and cAMP in R15. Further studies of interactions between intracellular messenger systems are likely to reveal additional consequences important for the short- and long-term function of neurons.

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