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Intracellular injection of protein kinase inhibitor blocks the serotonin-induced increase in K^+ conductance in *Aplysia* neuron R15

(cAMP/protein phosphorylation/voltage clamp/ion channels)

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ABSTRACT **Previous work has shown that serotonin induces an increase in membrane K^+ conductance in *Aplysia* neuron R15 and that this response is mediated by cAMP. The present study examines the role of protein phosphorylation in the response to serotonin. A specific inhibitor of cAMP-dependent protein kinase was injected intracellularly into neuron R15. The injection blocked the serotonin-induced increase in K^+ conductance completely for at least 4 hours. The blockage was selective because the cell's response to dopamine was not inhibited. Furthermore, the blockage was specifically produced by protein kinase inhibitor because injection of other proteins (α-bungarotoxin and bovine serum albumin) did not affect the serotonin response. The serotonin response recovered fully 5–13 hours after the injection, presumably as a result of intracellular proteolysis of the protein kinase inhibitor. The results indicate that protein phosphorylation is a necessary step in the process that leads to activation of K^+ channels by serotonin in neuron R15.**

Several laboratories have implicated cAMP in the regulation of electrical activity in molluscan nerve cells (1–5). In some of these cells, cAMP causes an increase in membrane resistance and a broadening of action potentials, apparently resulting from a decrease in K^+ conductance (1–3). In *Aplysia* neuron R15, on the other hand, cAMP mediates a serotonin-induced increase in K^+ conductance (5, 6). In the latter case, the K^+ channel involved has been identified as the one responsible for anomalous rectification in this cell (unpublished data).

It has been proposed that cAMP acts exclusively by activation of cAMP-dependent protein kinases (7). It has been reported that injection of the active catalytic subunit of protein kinase into a sensory neuron (8) or bag cells (9) from *Aplysia* leads to spike broadening and an increase in membrane resistance. These effects are identical to those produced by cAMP in the same cells (1, 2), and it has been inferred that the catalytic subunit, like cAMP, is causing a decrease in K^+ conductance. In addition, voltage clamp experiments using internally perfused *Helix* neurons have demonstrated that intracellular application of physiological concentrations of the catalytic subunit can enhance Ca^{2+}-activated K^+ conductance (10). Thus, it is becoming evident that protein phosphorylation induced by exogenous catalytic subunit can alter the electrical properties of different nerve cells in a variety of ways. To examine the role of endogenous protein phosphorylation in a cAMP-mediated neuronal response, we have injected a specific protein kinase inhibitor (PKI) into neuron R15 to block endogenous protein kinase activity. We demonstrate here that the increase in K^+ conductance, normally elicited by serotonin in this cell, is blocked completely by PKI injection.

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MATERIALS AND METHODS

Aplysia californica, maintained as described (5), were used for these experiments. For measurement of protein kinase activity, ganglia were removed and rinsed in normal *Aplysia* medium (460 mM NaCl/55 mM $MgCl_2$/11 mM $CaCl_2$/10 mM KCl/1% glucose/10 mM Tris·HCl, pH 7.4) prior to homogenization as described (11). For electrophysiological experiments, the abdominal ganglion was pinned dorsal side up in a Sylgard dish and perfused with normal medium. PKI, purified to homogeneity from rabbit muscle (12) and provided by E. Fischer (University of Washington, Seattle) was dissolved in water to a concentration of 1 mg/ml. A 50-nl aliquot was back-loaded into the tip of a microelectrode pulled from either Omega-Dot or Microstar (Radnoti Glass) capillary tubing. An internal silver wire in contact with the wall of the electrode provided a sufficiently good electrical connection for determining that the tip of the electrode was in the cell. The diameter of the meniscus of the PKI solution was measured with an optical micrometer, and movement of the meniscus during application of pressure (1–10 atm; 1 atm = 1.013×10^5 Pa) was noted to estimate the volume of the injections, which ranged between 0.1% and 10% of the cell volume. In some experiments, neuron R15 was voltage clamped with two 3 M KCl-filled microelectrodes during the injection, whereas in others the injection was into an unclamped cell, and the injection electrode was removed prior to impalement with the KCl electrodes. The cell was voltage clamped by conventional techniques, and the membrane potential was swept between −120 and −40 mV at a rate of 4 mV/sec, and the resulting current was recorded. Current-voltage (*I–V*) curves were generated, first in normal medium after injection with PKI, then during perfusion with medium containing 5 μM serotonin, and finally after washing again in normal medium. The response of R15 to bath application of serotonin reaches its full extent in 10–15 min and continues undiminished for at least 90 min with continued perfusion of serotonin. The changes in *I–V* curves were monitored throughout the perfusion, but as a matter of routine, the curves presented in Figs. 2 and 3 were obtained 20 min after the beginning of perfusion with serotonin (Fig. 2) and dopamine (Fig. 3).

RESULTS

cAMP-dependent PKI is a heat-stable 10,000-dalton protein that binds with high affinity to the catalytic subunit of cAMP-dependent protein kinase and inhibits its activity (12, 13). PKI has been used to inhibit cAMP-mediated responses in several nonneuronal cell types (14, 15). We have found that PKI derived

Abbreviation: PKI, protein kinase inhibitor.

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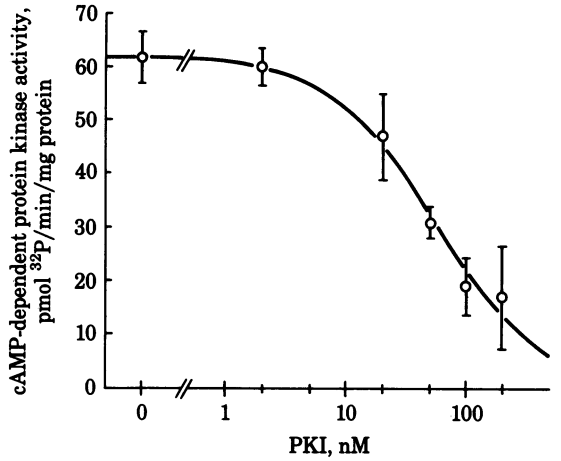

FIG. 1. Inhibition of cAMP-dependent protein kinase activity from *Aplysia* ganglia by PKI. Protein kinase activity was measured in a 12,000-g supernatant prepared from *Aplysia* ganglion homogenates. The tissue preparation and assay conditions were as described (11) except that the total assay volume was 50 μl. cAMP-dependent protein kinase activity was measured (20 μg of *Aplysia* protein per assay) as the difference in activity in the presence and absence of 1 μM cAMP. The basal activity measured in the absence of cAMP was 51 pmol of ^{32}P per min/mg of protein. The values on the abscissa are the final concentrations of PKI in the assay. Each point is the mean ± SEM for 3–6 samples. The solid line is a binding curve fit by least squares to the data.

from rabbit skeletal muscle inhibits cAMP-dependent protein phosphorylation in extracts from *Aplysia* ganglia in a dose-dependent manner (Fig. 1). The basal level of protein phosphorylation, measured in the absence of cAMP, was decreased no more than 5% by PKI. Gel electrophoretic analysis of the endogenous *Aplysia* proteins, whose phosphorylation is stimulated by cAMP, has demonstrated that PKI inhibits the phosphorylation of all of them to the same extent (not shown). Considering the phylogenetic distance between molluscs and mammals, these results suggest that the cAMP-dependent protein kinase system has been highly conserved during evolution.

Having found that PKI blocks *Aplysia* cAMP-dependent protein kinase activity *in vitro*, we injected it under pressure directly into neuron R15 to give intracellular concentrations in the 0.1–10 μM range and examined the cell's electrical properties and responses to neurotransmitters. For these measurements we used a voltage clamp to generate so-called steady-state *I–V* curves, the slopes of which are a measure of the total ionic conductance of the cell's membrane (Figs. 2 and 3). Except for transient changes in membrane voltage or current that accompanied the larger injections, PKI did not alter the resting steady-state *I–V* relationship of neuron R15. However, the increase in K^+ conductance normally elicited by serotonin (Fig. 2 *a* and *b*) was inhibited completely by PKI (Fig. 2 *c* and *d*).

Similar results were obtained in 8 out of 11 cases in which we observed a movement of the meniscus in the injection electrode, indicative of a successful injection of PKI. In the three experiments in which we injected what appeared to be large amounts of PKI without effect (16), we were using Omega-Dot capillary tubing to prepare injection microelectrodes. We have since found that with electrodes pulled from such tubing, movement of the meniscus can occur without ejection of material from the electrode tip, apparently by movement of the solution

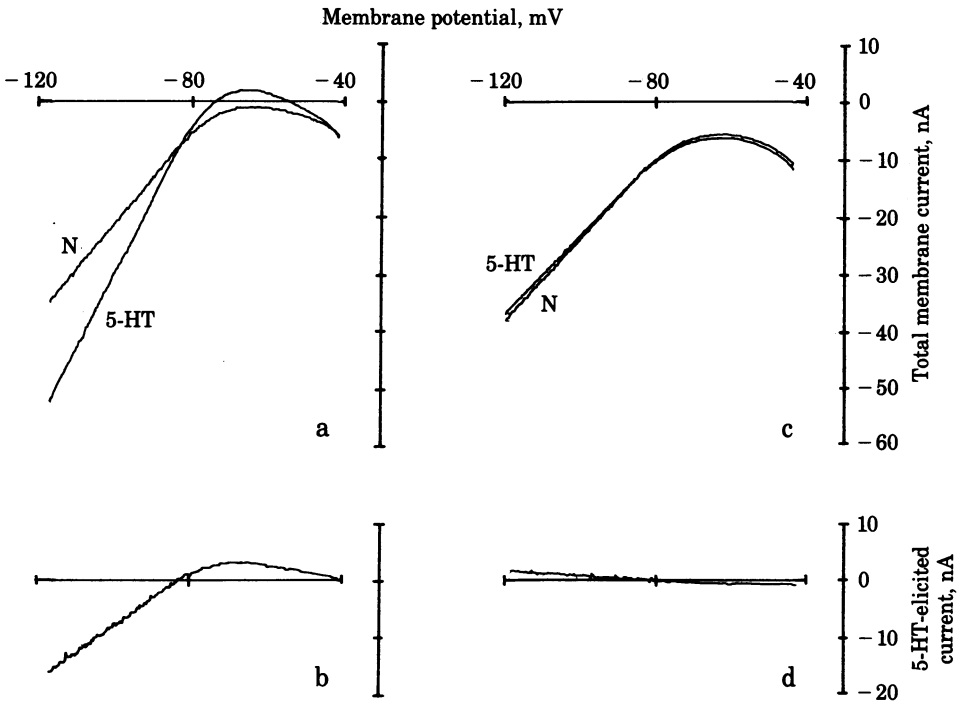

FIG. 2. Intracellular injection of PKI blocks the serotonin (5-HT) response in neuron R15. An estimated 2 nl of PKI solution was injected into a cell with a somal volume of ≈23 nl. The serotonin response was measured 2 hr (*Right*) and 10.5 hr (*Left*) after PKI injection. The curves show the total membrane current in the presence (5-HT) and absence (N) of serotonin (*a* and *c*) and the net current elicited by serotonin (*b* and *d*). The curves measured before serotonin application and after washing again with normal medium were averaged together with a Nicolet digital oscilloscope, and the averaged curve is shown as trace N in *c*, together with the curve (trace 5-HT) measured in the presence of serotonin. Note that serotonin produced virtually no change in the *I–V* curve 2 hours after injection of PKI (*c*). This is emphasized by an examination of the serotonin-elicited current (*d*), obtained by subtracting curve N from curve 5-HT using the digital oscilloscope. For comparison, the *I–V* curves and serotonin-elicited current recorded 10.5 hours after PKI injection are shown in *a* and *b*, respectively. The *I–V* curve in normal medium (N) had drifted up slightly at this time, but its shape remained unchanged. The response to serotonin, as measured by the increase in slope of the *I–V* curve (*a*) and by the magnitude of the serotonin-elicited current (*b*), had recovered by this time and was equivalent to that in noninjected cells (5).

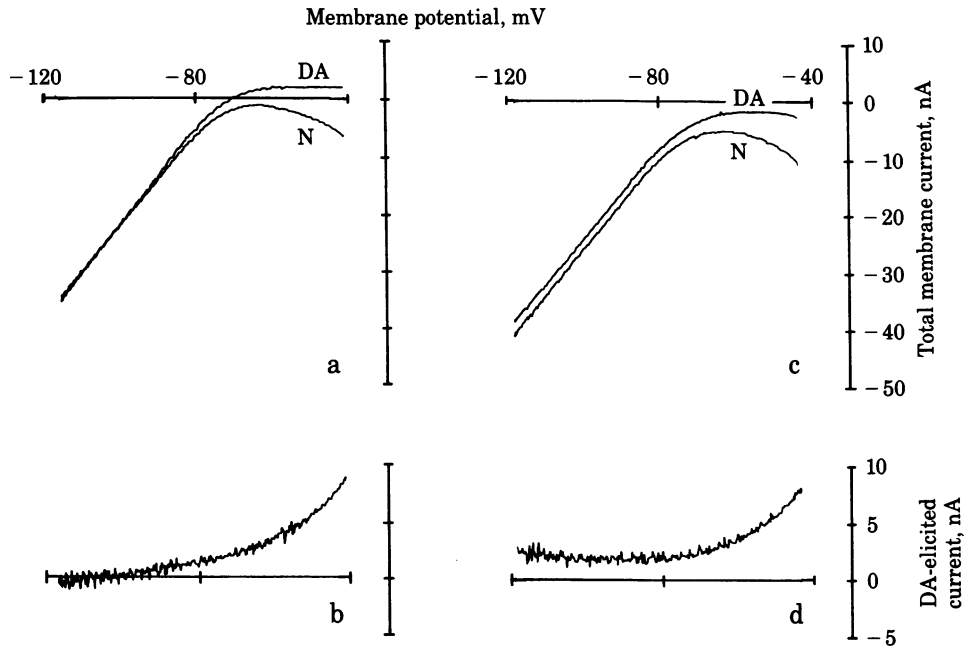

FIG. 3. Lack of effect of PKI injection on the dopamine response in neuron R15. Same cell and conditions as for Fig. 2 except that dopamine (DA; 100 μM) was used instead of serotonin and the response was measured 3 hr (*Right*) and 12 hr (*Left*) after PKI injection.

back up the fiber inside of the electrode.

We examined the kinetics of this PKI inhibition by applying serotonin at different times after injection and found no serotonin response between 1 and 4 hours postinjection (the experimental design precluded testing earlier than 1 hour). Typically, we observed a recovered response 7–13 hours after PKI injection, although in one experiment recovery was apparent as early as 5 hours. The recovered response was identical in all respects to the serotonin response observed in noninjected cells (5). In one experiment we elicited a normal serotonin response prior to blockage with PKI. In this case a second injection of PKI was made 26 hours after the first one and 21 hours after the cell had shown a normal response to serotonin. This second injection completely blocked the effect of serotonin applied 1.5 hours later.

To test whether the inhibition observed was due specifically to PKI, we injected two other proteins into R15 and examined the response to serotonin. The very small quantities of PKI available and the high stability of this low molecular weight protein (12) prevented us from using inactivated PKI in these control experiments. We chose instead α-bungarotoxin (Amersham) which has a molecular weight (8,000) similar to that of PKI and which, in addition, was available in tritiated form, allowing us to verify the size of the injections after the experiments. α-Bungarotoxin was dissolved at the same concentration (1 mg/ml) as was used for PKI, along with bovine serum albumin (2 mg/ml) as a stabilizer. The volumes injected were at least as large as the largest PKI injections (5–10% of the cell volume), resulting in intracellular protein concentrations at least 3 times as high as in the PKI experiments. Although these large injections of protein often led to depolarization (in unclamped cells) or an increase in voltage clamp holding current, the serotonin response was normal in all respects in the three cells that we tested within 2 hours after the injection. These results indicate that injection of protein *per se* is not sufficient to block the increase in K^+ conductance elicited by serotonin.

The selectivity of the PKI inhibition was investigated by examining the response of R15 to dopamine (Fig. 3). Dopamine elicits a decrease in inward current, which is most prominent in the depolarized range of membrane potentials, where the *I–V* curve displays a negative slope resistance (ref. 17 and Fig. 3 *a* and *c*). This response, which does not appear to involve cAMP (6, 18), is thought to be due to a decrease in Na^+ conductance (17, 19). Comparison of Figs. 3 *b* and *d* (which illustrate data from the same cell as in Fig. 2) shows that the dopamine response was normal after PKI injection. A dopamine response was observed in every PKI-injected cell immediately after it had been ascertained that the serotonin response of the cell had been blocked. Furthermore, the spontaneous excitatory synaptic currents often observed in R15, which probably result from an acetylcholine-induced increase in Na^+ conductance (20), also remained unchanged after PKI injection (not shown). Thus, the inhibitory effects of PKI in R15 appear to be selective for the serotonin-induced increase in K^+ conductance.

DISCUSSION

Work from several laboratories has shown that introduction of an exogenous catalytic subunit of cAMP-dependent protein kinase into certain molluscan nerve cells can produce decreases (8, 9) or increases (10) in K^+ conductance. In two of these cases (8, 9), the changes in K^+ conductance resemble those produced by increases in intracellular cAMP, suggesting that the same or similar targets are being phosphorylated by endogenous and exogenous protein kinase. The present study examines directly the role of endogenous cAMP-dependent protein kinase in controlling K^+ conductance in neuron R15. Our results demonstrate that *in vivo* inhibition of endogenous cAMP-dependent protein kinase blocks the increase in K^+ conductance normally elicited by serotonin in R15, thereby implicating protein phosphorylation as a necessary step in the process that leads to activation of a K^+ channel by a neurotransmitter.

These results, together with those from other laboratories, give rise to at least two interesting questions. The first concerns the identity of the protein substrates phosphorylated by cAMP-dependent protein kinase. In particular, one would like to know whether the proteins that form K^+ channels are phosphorylated

directly by protein kinase or whether they are activated or inactivated as the end result of some longer chain of events. A second, more general question is how cAMP-dependent protein phosphorylation gives rise to the variety of electrical responses observed in different nerve cells. In each case thus far described, the end-target is a K^+ channel, but in two instances K^+ channels are activated (5, 10), and in two others they are inhibited (8, 9). Furthermore, at least three pharmacologically distinct K^+ channels appear to be affected—the anomalous rectifier (unpublished data), the Ca^{2+}-activated K^+ channel (10, 21), and possibly a novel K^+ channel (22). And yet, probably all of these K^+ conductance systems are present in each of the cell types studied. It will be of interest to determine the molecular mechanisms by which specificity is conferred in the regulation of neuronal K^+ conductance.

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