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Microinjection of catalytic subunit of cyclic AMP-dependent protein kinase enhances calcium action potentials of bag cell neurons in cell culture

(*Aplysia*/protein phosphorylation)

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ABSTRACT **We have found that the calcium action potentials of bag cell neurons from the abdominal ganglion of *Aplysia* may be enhanced by intracellular microinjection of the catalytic subunit of cyclic AMP-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37). The catalytic subunit was purified from bovine heart and shown to be effective in stimulating the phosphorylation of bag cell proteins in homogenates at concentrations of 10–50 nM. Intracellular injection into isolated bag cell neurons maintained in primary culture was through pressure applied to microelectrodes filled at the tip with catalytic subunit (5–22 μM). In 11 of 16 injected cells, both the slope of the rising phase and the height of the action potentials evoked by a constant depolarizing current were markedly enhanced relative to the pre-injection control (mean increases, 73% and 35%, respectively). This effect could occur with no change in resting potential or in the latency of the action potential from the onset of the depolarizing pulse. The effect was observed with enzyme dissolved in three different salt solutions (Na phosphate, K phosphate, or KCl). In two experiments, tetrodotoxin (50 μM) added to the extracellular medium had no effect on the enhanced action potentials. Subsequent addition of the calcium antagonist Co^{2+}, however, diminished or abolished the spikes. In more than half of the experiments, the injection of catalytic subunit was accompanied by an increase in the input resistance of the cells as measured by applying small hyperpolarizing current pulses. In three experiments, subthreshold oscillations in membrane potential resulted from the injections. Control injections (24 cells), carried out either with carrier medium alone or with heat-inactivated enzyme preparations, did not produce spike enhancement, increased input resistance, or oscillations. Our data suggest that the stimulation of intracellular protein phosphorylation by the catalytic subunit of cyclic AMP-dependent protein kinase enhances the excitability of bag cell neurons by modifying calcium and potassium channels or currents.**

For many neurons, including the bag cell neurons in the abdominal ganglion of *Aplysia*, brief electrical stimulation or exposure to transmitter substances or to cyclic AMP analogues produces long-lasting changes in electrical excitability (1–4). These same agents can also produce changes in the phosphorylation state of neuronal proteins, supporting the hypothesis that an alteration in phosphorylation state may underlie certain changes in electrical activity (5, 6). In this report we describe the effects of a cyclic AMP-dependent protein kinase on the electrical properties of bag cell neurons. These neurons respond to brief electrical stimulation or to cyclic AMP analogues by generating a long-lasting afterdischarge, after which they become relatively refractory to further stimulation. Because the bag cells form an electrically coupled network in the intact abdominal ganglion (7, 8), intracellular injection experiments could be difficult to interpret. We therefore used isolated bag cell neurons in primary culture (7, 9) for this study. The action potentials in these neurons are predominantly due to activation of a calcium channel (10).

Cyclic AMP-dependent protein kinase (ATP:protein phosphotransferase, EC 2.7.1.37) is a tetrameric enzyme consisting of two cyclic AMP-binding subunits and two catalytic subunits (11). On binding to cyclic AMP, the tetramer dissociates, releasing the catalytic subunits which are then able to transfer the terminal phosphate of ATP to serine or threonine residues on substrate proteins. We present evidence that the catalytic subunit of cyclic AMP-dependent protein kinase (PKC) purified from bovine heart increases the phosphorylation of bag cell proteins and enhances the calcium action potentials.

In the companion paper, Castellucci *et al.* (12) describe effects of intracellularly injected PKC on the duration of calcium action potentials and on transmitter release in sensory cells of the abdominal ganglion incubated in the presence of tetraethylammonium ion.

MATERIALS AND METHODS

Culture of Bag Cell Neurons. *A. californica* was kept at 14°C, and all electrophysiological experiments were carried out at this temperature. Abdominal ganglia were dissected out and incubated at 22°C for 6 hr in filtered seawater containing 1.25% neutral protease (7, 9). The connective tissue capsules were removed and the bag cells were disaggregated by using a pasteur pipette. The cells were seeded into 35-mm Falcon tissue culture dishes containing L-15 medium (13) (GIBCO) made up in artificial seawater. The cells rapidly attached to the bottom of the culture dishes and within 2–3 days had developed elaborate neuritic branches. Electrophysiological studies were usually done within 1 week of seeding.

Intracellular Injection and Electrophysiological Recording. Glass microelectrodes were pulled on a Brown–Flaming electrode puller. The tips were then brushed against a ground glass plate to produce final tip diameters of ≈1.0 μm. When filled with enzyme or control solutions, these electrodes typically had resistances of 30–60 MΩ. Pressure injection (14) was used for moving material out of the electrodes. They were mounted in an electrode holder connected to a cylinder of N_2 gas so that pulses of pressure (4–40 psi; 10 sec–4 min) could be applied under the control of a solenoid-operated three-way valve (15). Cultured bag cell neurons were penetrated, under visual control, on a Leitz Diavert microscope. Recording of

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Abbreviations: $PhMeSO_2F$, phenylmethylsulfonyl fluoride; R-II, regulatory subunit of cyclic AMP-dependent protein kinase (type II); PKC, catalytic subunit of cyclic AMP-dependent protein kinase; PEM, phosphate/EDTA/2-mercaptoethanol buffer.

membrane potential was through an M701-WPI electrometer, and the intensity of current applied to depolarize or hyperpolarize the cell was monitored by using both the current monitor circuit of the electrometer (shown on figures) and an independent virtual ground circuit. When cells were impaled with a single electrode, the input resistance of the cells was estimated by measuring the maximal voltage displacements to a range of small hyperpolarizing currents after correction for bridge imbalance. In some experiments the cells were also penetrated with a second microelectrode filled with 2 M potassium citrate (resistance, 30–100 MΩ) and used for voltage recording only.

Preparation of the PKC (Type II) from Bovine Heart. The procedure used was an adaptation of the combined methods of Beavo *et al.* (16) and Corbin *et al.* (17). All steps were carried out at 4°C unless indicated otherwise. Bovine heart (4 kg) was homogenized in 3 vol (vol/wt) of PEM buffer (10 mM potassium phosphate/1 mM EDTA/15 mM 2-mercaptoethanol, pH 6.8) to which had been added 100 mM NaCl and 50 μM phenylmethylsulfonyl fluoride ($PhMeSO_2F$). The homogenate was centrifuged (11,000 × *g*, 30 min), and the supernatant was filtered through glass wool and stirred in 4 liters of packed DEAE-cellulose (Whatman DE-52) for 15 min. The resin was washed on a large Buchner funnel with 16 liters of homogenization buffer, resuspended in 4 liters of PEM to which 300 mM NaCl had been added, and stirred for 15 min. The filtrate was removed and the washing step repeated. The filtrate from both steps (8 liters) was combined, solid ammonium sulfate was added (314 g/liter), and the solution was stirred for 1 hr and centrifuged (11,000 × *g*, 30 min). The pellet was resuspended in 200 ml of PEM and dialyzed overnight against three 4-liter changes of the same buffer. The dialyzed sample was centrifuged (10,000 × *g*, 30 min) and the supernatant was adjusted to pH 6.1 with 1 M acetic acid.

This supernatant was then subjected to serial adsorption of unwanted protein to CM-Sephadex C-50 (three times, 50 ml of resin) equilibrated in PEM at pH 6.1. The filtrate was saved each time, and its pH was adjusted back to pH 6.8 with 1 M NaOH prior to subjecting it to four cycles of adsorption to CM-Sephadex C-50 equilibrated in PEM (pH 6.8). The filtrate was saved and applied, in series at 10–20 ml/hr, first to a cyclic AMP-Sepharose affinity column (0.5 × 4 cm) (17) equilibrated with PEM and then, by means of continuous tubing, to a column of CM-Sephadex C-50 (1 × 4 cm) equilibrated in the same buffer. At this stage the PKC was bound to the CM-Sephadex column and the regulatory subunit (R-II) was bound to the affinity column. The PKC was eluted by using a linear gradient (60 ml) of 10–300 mM potassium phosphate (pH 6.8). The position of elution was identified by assaying for histone kinase activity (18). The active fractions were pooled, dialyzed against PEM, and concentrated to about 0.3 mg/ml by reapplying the sample to a CM-Sephadex column (0.5 × 2 cm) equilibrated in PEM. The enzyme was eluted with a step of 300 mM potassium phosphate (pH 6.8). R-II was removed from the cyclic AMP-Sepharose affinity column at 24°C. The column was washed with: 5 ml of PEM at pH 6.8; 5 ml of 10 mM AMP in PEM; 5 ml of PEM; and 5 ml of 10 mM cyclic AMP (pH 6.8) seven times. The elution position of R-II was identified by using $NaDodSO_4$/polyacrylamide gel electrophoresis.

The R-II and PKC were then recombined in a 55:40 weight ratio in the presence of 10 mM $MgCl_2$ and 0.1 μM ATP. After 30 min, the holoenzyme was dialyzed three times against 4 liters of PEM. The protein was then applied to a DEAE-cellulose column (1 × 3 cm) equilibrated in PEM. The column was washed with: 7 ml of 80 mM NaCl/PEM four times; 5 ml of 40 mM NaCl/PEM; and 5 ml of 40 mM NaCl/100 μM cyclic AMP/PEM three times. The pure PKC was eluted at this stage. The fractions were pooled, concentrated by vacuum dialysis to 0.22–1.13 mg/ml, and finally dialyzed against 300 mM sodium or potassium phosphate (pH 6.8) with 5–15 mM 2-mercaptoethanol. This purified enzyme preparation was used to fill the tips of the injection microelectrodes. For some experiments, 200 μl of the enzyme was further dialyzed against 250 ml of 0.3 M KCl/15 mM 2-mercaptoethanol for 24 hr prior to intracellular injection. In some preliminary experiments, 10% glycerol was also present in the injection solution. For control experiments with heat-inactivated enzyme, solution containing active enzyme was heated in a water bath at 80°C in a sealed vial for 20 min.

For the assay of PKC activity in bag cell homogenates, intact bag cell clusters and surrounding connective tissue were dissected away from the abdominal ganglion. Four clusters were homogenized in 100 μl of ice-cold 50 mM sodium acetate, pH 6.5/10 mM $MgCl_2$/1 mM Zn acetate/1 mM theophylline. The reaction was initiated by adding 50 μl of homogenate to plastic tubes containing 20 μl of 70 μM [γ-^{32}P]ATP (25 Ci/mmol; 1 Ci = 3.7 × 10^{10} becquerels; ICN) and PKC at varying concentrations at 24°C. After 2 min, the reaction was terminated by addition of 75 μl of 10% (wt/vol) glycerol/2% (vol/vol) $NaDodSO_4$/5% (vol/vol) 2-mercaptoethanol/0.001% bromophenol blue followed by heating at 90°C for 15 min. The solubilized proteins were separated by 0.5% $NaDodSO_4$/10% polyacrylamide slab gel electrophoresis with 0.05 M Tris/0.6 M glycine, pH 8.5, as buffer. The gels were stained and subjected to autoradiography. The stimulation of ^{32}P incorporation into individual phosphoprotein bands was quantitated by scanning of the autoradiograms on a densitometer.

RESULTS

Activity of the Catalytic Subunit. Addition of the PKC from bovine heart to homogenates of bag cells containing [γ-^{32}P]ATP enhanced the incorporation of ^{32}P into all observed phosphoprotein bands in a dose-dependent manner (Fig. 1). The range of PKC concentrations used was about 1/2000th to 1/400th the concentrations of the most concentrated solutions used to fill the tips of the microelectrodes (1.13 mg/ml).

Injection of PKC into Bag Cell Neurons. After penetration of a bag cell neuron with either one or two microelectrodes, a series of 10 suprathreshold depolarizing current pulses (250-msec pulses, 0.05–0.25 nA, 0.83 pulse/sec) was applied. The usual response to this type of stimulation was a progressive increase in the height and width of the action potentials elicited by each pulse (Fig. 2*B*, cells 1 and 2). In some cases, however, adaptation occurred such that later pulses in the train failed to

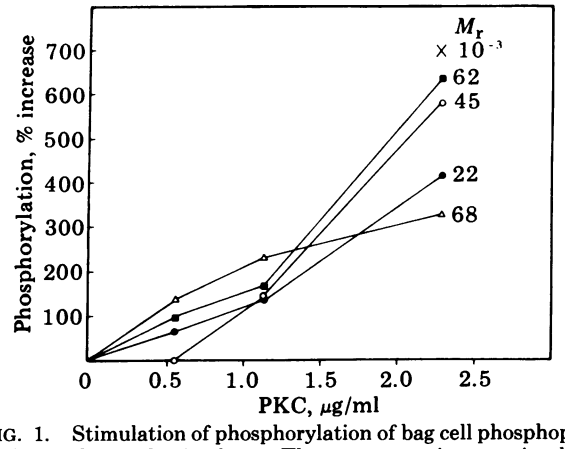

FIG. 1. Stimulation of phosphorylation of bag cell phosphoproteins by PKC from bovine heart. The percentage increase in phosphorylation was quantitated by densitometric scanning of autoradiograms and is shown for four representative bag cell phosphoproteins of M_r 22,000, 45,000, 62,000, and 68,000. The PKC concentrations shown are equivalent to ≈12 to 54 nM.

produce full action potentials (Fig. 2*B*, cells 3 and 4). A series of small hyperpolarizing current pulses (0.01–0.15 nA, 1–4 sec) was also delivered to determine the input resistance of the cell. The series of depolarizing and hyperpolarizing current pulses was repeated at 5-min intervals. When the response to both types of stimulus had remained stable for at least 20 min, a pulse of pressure (2–10 psi, 5 sec) was applied to the enzyme-containing microelectrode. If no change in the response of the cell to the depolarizing and hyperpolarizing pulses was observed, the pressure pulse was repeated at higher pressure and for longer times (up to 45 psi, 4 min) until a change was seen.

In 11 of 16 cells injected with the PKC, the first detectable change in electrical response was an increase in the height and in the slope of the rising phase of the action potentials evoked by a constant depolarizing current pulse. This can be seen in Fig. 2*A* by examining the tracings of the first spikes evoked by the repetitive pulses. The mean (±SEM) increase in the amplitude of these first action potentials for the 11 cells was 35 ± 10.1% (measured from overshoot to undershoot) and the increase in the slope of the rising phase of the action potentials was 72.9 ± 17.3%. Fig. 2*B* shows that the subsequent action potentials evoked by the later pulses also were greater in amplitude and, in some cases, significantly broader than their control counterparts. The change in the shape of the spikes could occur with no detectable change in either the resting potential of the cell or the latency of the spike from the onset of the depolarizing pulse. The effect was observed with enzyme that was dissolved in each of the three salt solutions used as carrier [Na phosphate, three experiments (Fig. 2, cell 2); K phosphate, six experiments (Fig. 2, cells 1 and 3); KCl, two experiments (Fig. 2, cell 4)]. After injection of PKC, the effect of enhanced spike electrogenesis generally remained stable for the remainder of the experiment (up to 60 min).

To determine if the enhanced spikes that follow injection of PKC were due to an enhancement of sodium or calcium components, we added tetrodotoxin (50 μM) to the extracellular medium after intracellular injection in two experiments. In both cases, tetrodotoxin had no effect on the enhanced spikes, suggesting that they were carried by Ca^{2+}. Subsequent addition of the calcium antagonist $CoCl_2$ (12 mM) to the extracellular medium substantially diminished the evoked action potentials in one case and totally abolished them in the other.

The change in the configuration of evoked spikes was often accompanied by an increase in the input resistance of the cells (Fig. 3). Input resistance was monitored in 9 of the 11 positively responding cells. Five had input resistances that were significantly increased over the pre-injection control values (mean increase, 65%). The remaining cells' resistances remained unchanged or diminished slightly compared with control values. The mean increase for the nine cells was 32 ± 16% (SEM).

In three of the cells injected with PKC, subthreshold oscillations in membrane potential developed after injection and persisted for the remainder of the experiment. An example of such subthreshold oscillations is shown in Fig. 4. In one of the three cells, the oscillations reached spike threshold and resulted in irregular repetitive firing for 9 min after the injection (not shown). The subthreshold oscillations were membrane potential-dependent, being increased both in frequency and amplitude by subthreshold depolarizing current pulses and diminished or abolished by hyperpolarizing pulses (Fig. 4*B*).

Control Injections. Control data for the effects of injected PKC were obtained by injecting either heat-inactivated enzyme preparations [six experiments (Fig. 5, cell 1)] or carrier alone [0.3 M Na phosphate/10 mM 2-mercaptoethanol, 3 experiments (Fig. 5, cell 2); 0.3 M K phosphate/15 mM 2-mercaptoethanol, 11 experiments (Fig. 5, cell 3); 0.3 M KCl/15 mM 2-mercaptoethanol, 4 experiments (Fig. 5, cell 4)]. Of these 24 injected cells, only 1 gave a small increase in spike height after intracellular injection [7.5% when injected with K phosphate-based carrier medium (Fig. 5, cell 3)]. The other cells showed no change in their electrical properties until, after repeated injections, there was some loss of resting potential and spike height associated with a decrease in input resistance. The five cells that were injected with active PKC but failed to respond positively also showed these changes which are a nonspecific effect of excessive intracellular pressure. After some of these control injections the width of the spikes observed in response to a train of depolarizing current pulses was increased, compared with

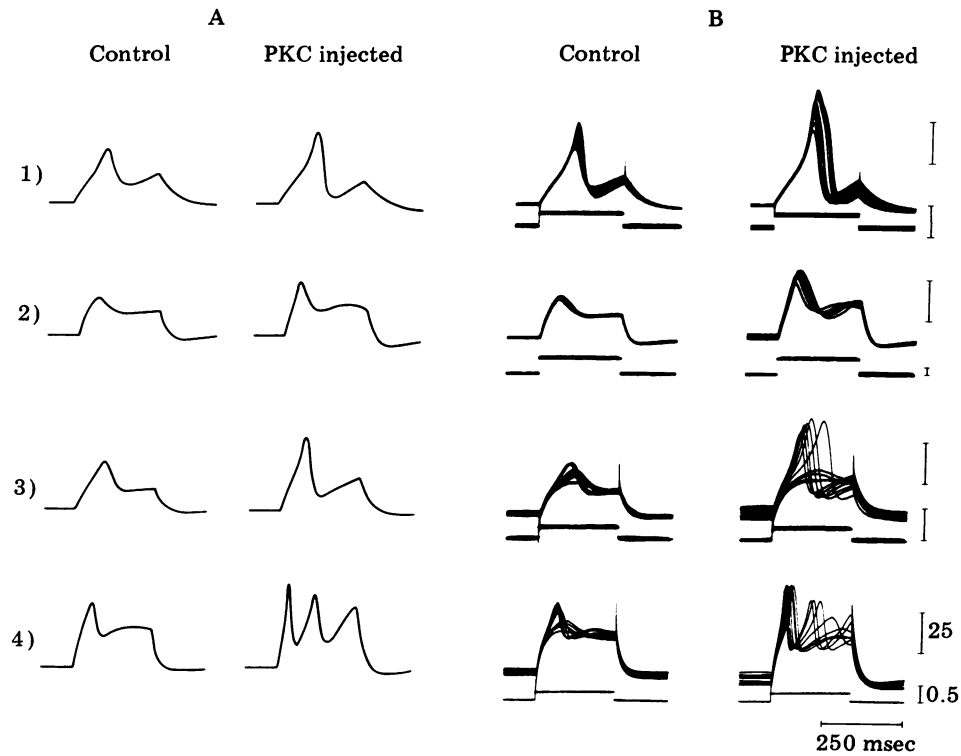

FIG. 2. Effect of intracellularly injected PKC on action potentials of bag cell neurons. (*A*) Control shows tracings of first action potential evoked by a train of depolarizing current pulses prior to injection of PKC. The second column shows the response of the cells to the same current pulses after injection. (*B*) Superimposed oscilloscope tracings of the response to multiple depolarizing current stimuli at a frequency of 0.83/sec before and after injection of PKC, for the same four cells as in *A*. Lower traces show the applied transmembrane current and upper traces show the action potential responses. The solutions at the electrode tips contained PKC (0.5–1.13 mg/ml), 2-mercaptoethanol (10–15 mM), and 0.3 M K phosphate (cells 1 and 3), 0.3 M Na phosphate (cell 2), or 0.3 M KCl (cell 4). The injection variables for cells 1–4 were: 7 psi, 40 sec; 2 psi, 12 sec; 43 psi, 240 sec; and 11 psi, 60 sec.

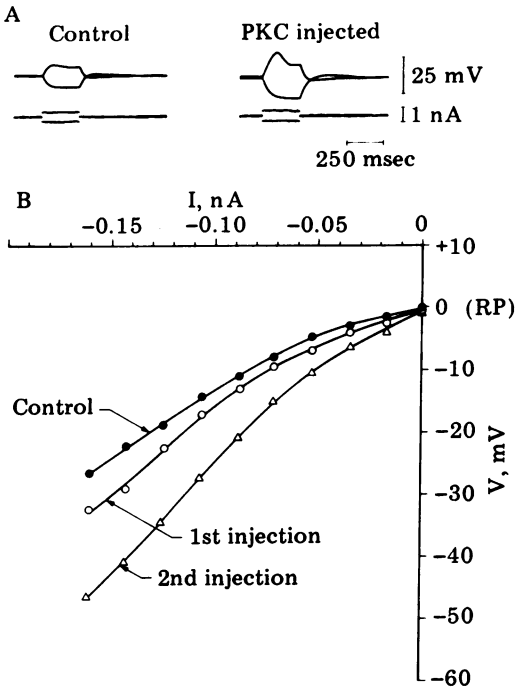

FIG. 3. Increased input resistance in a bag cell neuron after injection of PKC. (*A*) Voltage responses (upper traces) to constant depolarizing and hyperpolarizing current pulses (lower traces) before and after injection. The injection electrode contained PKC (0.54 mg/ml) in 0.3 M Na phosphate/10 mM 2-mercaptoethanol; injection was at 2 psi for 12 sec. (*B*) Current–voltage relationships for another bag cell neuron. The voltage axis is relative to the resting potential (RP) of the neuron. The electrode tip contained PKC (1.13 mg/ml) in 0.3 M K phosphate/15 mM 2-mercaptoethanol. The first PKC injection (18 psi, 10 sec) produced an increase in resistance with no change in resting potential. A second, larger, injection (43 psi, 240 sec) further increased input resistance. This second injection was associated with a small depolarization (4 mV) from the preinjection resting potential of 37 mV.

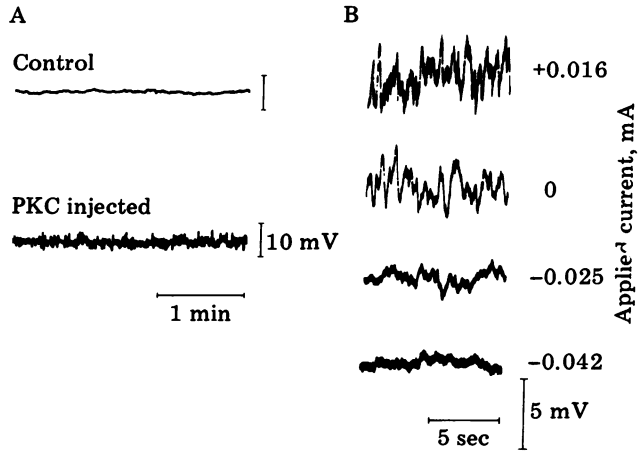

FIG. 4. Emergence of subthreshold oscillations in membrane potential of a bag cell neuron after injection of PKC. (*A*) Slow-speed chart tracings of membrane potential before and after injection (11 psi, 60 sec). The electrode tip contained PKC at 1.13 mg/ml in 0.3 M KCl/15 mM 2-mercaptoethanol. (*B*) Oscilloscope tracings of the induced subthreshold oscillations in the same cell as in *A*. Depolarizing current (top trace) increased frequency and amplitude; hyperpolarizing current (bottom two traces) diminished oscillations.

controls, even though their amplitude was not enhanced. This change was always accompanied by a large depolarization (≥10 mV) and shortening of the latency from the onset of the current pulse to the onset of the action potential. In Fig. 5, cell 2 shows the most extreme example of this effect which was most likely due to inactivation of delayed outward currents by the injection-induced depolarization (19). In no control experiments was a sustained increase in input resistance observed after injection. The mean (±SEM) pre-injection resting potential of the control group of injected cells was 34.7 ± 1.9 mV compared with 35.3 ± 2.3 mV for the experimental group. These resting potentials are lower than those of bag cells in the intact abdominal ganglion or of isolated bag cells used in other studies (9, 20) and may result from penetration by the larger tips of pressure-injection microelectrodes.

DISCUSSION

We have shown that, in bag cell neurons, presumed calcium spikes may be enhanced by intracellular injection of PKC. Although we have shown that bovine PKC enhances phosphorylation of *Aplysia* bag cell proteins *in vitro*, we can make no statement about the degree of intracellular phosphorylation

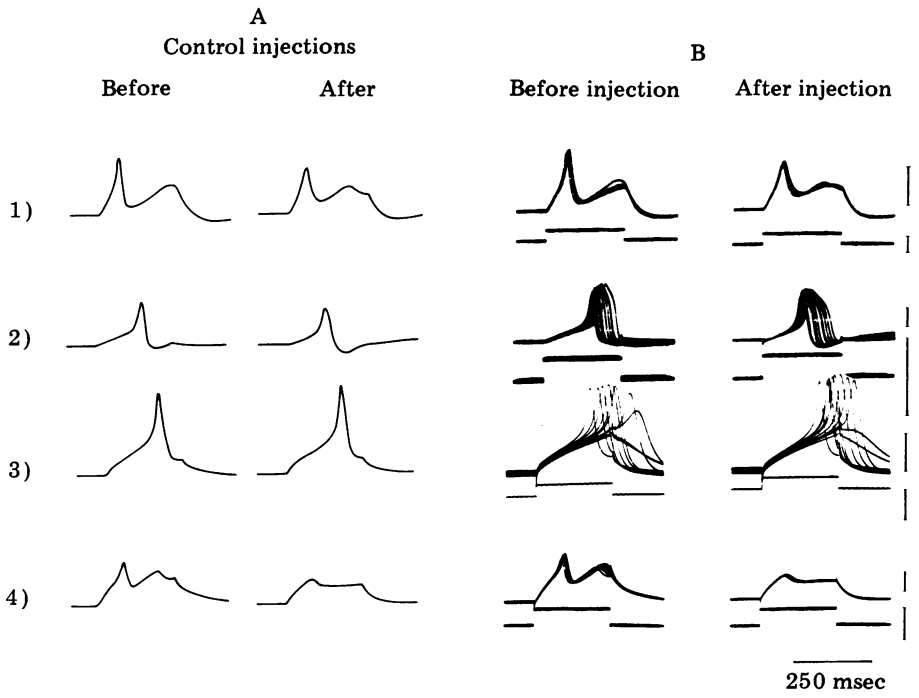

FIG. 5. The effects of control injections of heat-inactivated enzyme solutions and carrier solutions on action potentials of bag cell neurons. (*A*) Tracings of first action potential evoked by a train of depolarizing current pulses before and after injection. (*B*) Superimposed oscilloscope tracings of the response to multiple depolarizing current stimuli at a frequency of 0.83/sec before and after injection for the same four cells as in *A*. Lower traces give the applied transmembrane current; upper traces show the action potential responses. Cell 1 was injected (6 psi, 3 min) with heat-inactivated PKC (0.54 mg/ml) in 0.3 M Na phosphate/10 mM 2-mercaptoethanol. Cells 2, 3, and 4 were injected with 0.3 M Na phosphate (5 psi, 4.5 min), 0.3 M K phosphate (40 psi, 12 min), and 0.3 M KCl (6 psi, 8 min), respectively, with 10–15 mM 2-mercaptoethanol present in each case.

that was induced in the injection experiments or about its spatial extent. The effect of PKC could be a direct effect on components of the calcium or potassium channels or could be secondary to some less-specific metabolic effect of kinase injection—for example, calcium currents could be enhanced by a kinase-induced decrease in intracellular pH (21). In addition, the state of excitability of neurons in primary culture may be subject to metabolic influences that do not play a role in naturally occurring excitability changes of the same neurons *in vivo*. Nevertheless, the fact that changes similar to those that we have described for kinase injection do occur in the natural activity of these cells (see below) suggests that changes in phosphorylation state do control electrical excitability.

The bag cell neurons in the abdominal ganglion of *Aplysia* generate a long-lasting afterdischarge (≈30 min) in response to brief synaptic stimulation or to cyclic AMP analogs (1, 3, 22). The shape and amplitude of the action potentials change dramatically during the course of the afterdischarge, with the maximal height and width of the intracellularly recorded action potentials being observed 2 min after the onset of the afterdischarge (10). This maximum is also correlated with a peak in cyclic AMP levels within the bag cell cluster (3). Although bag cells show frequency-dependent broadening and augmentation of spike height with repetitive depolarizing current pulses (≈1/sec), this effect occurs too quickly to account for the slow increase in spike height and width over the first 2 min of afterdischarge, during which the cells discharge at a relatively fast rate (2–4 spikes/sec). The data of this paper suggest that the enhanced spikes during afterdischarge may result from the increased phosphorylation of certain bag cell proteins.

An increase in input resistance, the onset of subthreshold oscillations in membrane potential, and a long-lasting repetitive discharge are readily observed in isolated cultured bag cell neurons after the extracellular addition of a membrane-permeant cyclic AMP analogue (20, 22, 23). Subthreshold oscillations and repetitive discharge were observed in only some of the present experiments with injected kinase. Whether this is due to insufficient phosphorylation of specific substrate proteins or to factors unrelated to phosphorylation remains to be determined. An alternative approach to resolve this would be to attempt to block the response to cyclic AMP with protein kinase inhibitor (24) as is being done for other *Aplysia* neurons (25). The increase in membrane resistance in response to cyclic AMP analogues or intracellular protein kinase injection could be due to modification of the potassium channels by protein phosphorylation. We suggest (10) that phosphorylation increases the open time of the voltage-dependent calcium channel and decreases that of a potassium channel.

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1. Kupfermann, I. & Kandel, E. R. (1970) *J. Neurophysiol.* **33,** 865–876.
2. Treistman, S. N. & Levitan, I. B. (1976) *Nature (London)* **261,** 62–64.
3. Kaczmarek, L. K., Jennings, K. & Strumwasser, F. (1978) *Proc. Natl. Acad. Sci. USA* **75,** 5200–5204.
4. Dudek, F. E. & Blankenship, J. E. (1977) *J. Neurophysiol.* **40,** 1301–1311.
5. Greengard, P. (1978) *Cyclic Nucleotides, Phosphorylated Proteins, and Neuronal Function* (Raven, New York).
6. Jennings, K. R., Kaczmarek, L. K. & Strumwasser, F. (1979) *Soc. Neurosci. Abstr.* **5,** 249.
7. Kaczmarek, L. K., Finbow, M., Revel, J. P. & Strumwasser, F. (1979) *J. Neurobiol.* **10,** 535–550.
8. Blankenship, J. E. & Haskins, J. T. (1979) *J. Neurophysiol.* **42,** 347–355.
9. Strumwasser, F., Kaczmarek, L. K. & Viele, D. (1978) *Soc. Neurosci. Abstr.* **4,** 207.
10. Strumwasser, F., Kaczmarek, L. K., Jennings, K. R. & Chiu, A. Y. (1981) in *Eighth International Symposium on Neurosecretion,* eds. Lederis, K. & Farner, D. S. (Plenum, New York), in press.
11. Rubin, C. S. & Rosen, O. M. (1975) *Annu. Rev. Biochem.* **44,** 831–887.
12. Castellucci, V. F., Kandel, E. R., Schwartz, J. H., Wilson, F., Nairn, A. C. & Greengard, P. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 7492–7496.
13. Leibovitz, A. (1963) *Am. J. Hyg.* **78,** 173–180.
14. Strumwasser, F. (1965) in *Circadian Clocks* (North Holland, Amsterdam), pp. 442–462.
15. McCaman, R. E., McKenna, D. G. & Ono, J. K. (1977) *Brain Res.* **136,** 141–147.
16. Beavo, J. A., Bechtel, P. J. & Krebs, E. G. (1974) *Methods Enzymol.* **38C,** 299–308.
17. Corbin, J. D., Sugden, P. H., West, L., Flockhart, D. A., Lincoln, T. M. & McCarthy, D. (1978) *J. Biol. Chem.* **253,** 3997–4003.
18. Witt, J. J. & Roskoski, R. (1975) *Anal. Biochem.* **66,** 253–258.
19. Aldrich, R. W., Jr., Getting, P. A. & Thompson, S. H. (1979) *J. Physiol. (London)* **291,** 507–530.
20. Kaczmarek, L. K. & Strumwasser, F. (1980) *Soc. Neurosci. Abstr.* **6,** 214.
21. Moody, W. (1980) *J. Physiol.* **302,** 335–346.
22. Kaczmarek, L. K., Jennings, K. R. & Strumwasser, F. (1979) *Soc. Neurosci. Abstr.* **5,** 249.
23. Strumwasser, F., Kaczmarek, L. K., Chiu, A. Y., Heller, E., Jennings, K. R. & Viele, D. P. (1980) in *Peptides: Integrators of Cell and Tissue Functions,* ed. Bloom, F. E. (Raven, New York).
24. Ashby, C. D. & Walsh, D. A. (1972) *J. Biol. Chem.* **247,** 6637–6642.
25. Adams, W. B. & Levitan, I. B. (1980) *Soc. Neurosci. Abstr.* **6,** 704.