MODULATION OF CALCIUM-MEDIATED INACTIVATION OF IONIC CURRENTS BY CA^{2+}/CALMODULIN-DEPENDENT PROTEIN KINASE II

MANABU SAKAKIBARA,* DANIEL L. ALKON,* ROBERT DELORENZO,‡ JAMES R. GOLDENRING,‡ JOSEPH T. NEARY,* AND ELIAHU HELDMAN§

*Section on Neural Systems, Laboratory of Biophysics, IRP National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health at the Marine Biological Laboratory, Woods Hole, Massachusetts 02543; ‡Department of Neurology, Yale University School of Medicine, New Haven, Connecticut 06510; and §Department of Pharmacology, Israel Institute for Biological Research, Ness-Ziona, Israel

ABSTRACT Iontophoretic injection of Ca^{2+} causes reduction of I_0A (an early rapidly activating and inactivating K^+ current) and I_0C (a late Ca^{2+}-dependent K^+ current) measured across the isolated type B soma membrane (Alkon et al., 1984, 1985; Alkon and Sakakibara, 1984, 1985). Similarly, voltage-clamp conditions which cause elevation of $[Ca^{2+}]_i$ are followed by reduction of I_0A and I_0C lasting 1–3 min. Iontophoretic injection of highly purified Ca^{2+}/CaM-dependent protein kinase II (CaM kinase II) isolated from brain tissue (Goldenring et al., 1983) enhanced and prolonged this Ca^{2+}-mediated reduction of I_0A and I_0C. $I_{Ca^{2+}}$, a voltage-dependent Ca^{2+} current, also showed some persistent reduction under these conditions. Iontophoretic injection of heat-inactivated enzyme had no effect. Agents that inhibit or block Ca^{2+}/CaM-dependent phosphorylation produced increased I_0A and I_0C amplitudes and prevented the effects of CaM kinase II injection. The results reported here and in other studies implicate Ca^{2+}-stimulated phosphorylation in the regulation of type B soma ionic currents.

Protein phosphorylation is one post-translational biochemical modification that can serve to express the reception of a signal by one cell from another. Synaptic transmitters or neurohormones released by one cell or cell population can cause an increase of intracellular cyclic-AMP and/or Ca^{2+} levels, thereby activating cyclic-AMP and/or Ca^{2+}-dependent phosphorylation. Similarly, a positive shift of membrane potential, i.e., depolarization (in response to external signals) can activate a voltage-dependent Ca^{2+}-current, elevate intracellular Ca^{2+}, $[Ca^{2+}]_i$, and thereby influence Ca^{2+}-dependent and/or cyclic-AMP dependent phosphorylation. Intercellular signalling, be it by release of neurochemical substances or by changes of membrane potential also initiates sequential cellular processes that somehow result in prolonged transformations such as those of hormonal regulation, learning, or development. Several different types of experiments suggest that protein phosphorylation may be crucially involved in such prolonged transformations which underlie biophysical encoding of associative memory.

Neary et al. (1981) observed a change of phosphorylation of a 20,000 mol wt protein in eyes isolated from the nudibranch *Hermissenda crassicornis* after training with an associative conditioning procedure but not with control regimens. Routtenberg and co-workers (1984, 1985) measured differences in phosphorylation within the hippocampus after inducing (by electrical stimulation) long-term potentiation in an intact preparation, and Gispen, Lynch, and others have shown changes in protein phosphorylation in hippocampal slices after tetanic stimulation (Browning et al., 1979; Bar et al., 1980). Differences in phosphorylation also occurred with "kindling" (an epileptic-like hyperexcitability of brain tissue; Wasterlain and Farber, 1984) and during perfusion with drugs such as serotonin (Saitoh and Schwartz, 1983). Ca^{2+}-calmodulin (CaM) dependent protein phosphorylation has also been implicated in mediating some of the effects of calcium on neuronal excitability (DeLorenzo, 1981, 1984; Goldenring et al., 1984).

Simulation of conditions that occur during *Hermissenda* classical conditioning have also produced specific changes of phosphorylation. Paired presentations of light and rotation cause progressive and prolonged depolarization of the type B membrane (Alkon, 1980). Such depolarization is also accompanied by prolonged elevation of intracellular Ca^{2+} (Connor and Alkon, 1984). Exposure of *Hermissenda* nervous systems to many minutes of ASW with 100 mM K^+_o (to depolarize membranes) causes increased phosphorylation of a 56,000 mol wt protein (Naito et al., 1985) and decreased phosphorylation of the 20,000 mol wt and a 25,000 mol wt protein (Neary and Alkon, 1983; Naito et al., 1985). Of particular interest is the persistence

of the effect of the 25,000 mol wt protein for 30 min or more after removal of the elevated K^+_o solution (Naito et al., 1985).

Iontophoretic injection of Ca^{2+} caused reduction of I_0A (an early rapidly activating and inactivating K^+ current) and I_0C (a late Ca^{2+}-dependent K^+ current) measured across the isolated type B soma membrane (Alkon et al., 1982*a*; Alkon and Sakakibara, 1984, 1985). Similarly, voltage clamp conditions that cause elevation of $[Ca^{2+}]_i$ are followed by reduction of I_0A and I_0C lasting 1–3 min. Iontophoretic injection of a Ca^{2+}/CaM-dependent protein kinase (phosphorylase kinase) enhanced and prolonged this Ca^{2+}-mediated reduction of I_0A and I_0C (Acosta-Urquidi et al., 1984). These same two currents were shown to remain reduced 1–2 d after associative conditioning (but not control regimens; Alkon et al., 1982*b*; 1985).

In a similar vein we undertook in the present study to analyze the effects of iontophoretic injection of highly purified Ca^{2+}/CaM-dependent protein kinase II (CaM kinase II) isolated from brain tissue (Goldenring et al., 1983) that is part of a group of protein kinases activated by calcium and CaM. Of particular interest was the relationship of the enzyme's regulatory activity on membrane currents to the level of $[Ca^{2+}]_i$. We report here that the enzyme caused reduction (for 1–3 h) of I_0A and I_0C, and to a lesser extent of a voltage-dependent Ca^{2+} current, particularly after voltage clamp conditions that are known to cause a substantial and prolonged rise of $[Ca^{2+}]_i$. Agents that inhibit or block Ca^{2+}/CaM-dependent phosphorylation produced increased I_0A and I_0C amplitudes and prevented the effects of CaM kinase II injection.

METHODS

Preparation of Enzyme and Assay of Activity

CaM kinase II was prepared as described previously (Goldenring et al., 1983). The kinase preparation contained two major autophosphorylating enzyme subunits, with molecular weights of 50,000 and 60,000 D. Enzyme activity was determined by quantitating autophosphorylation of the enzyme subunits and of specific protein substrates (synapsin I, tubulin, and microtubule associated protein-2, MAP-2) satisfying standard incubation conditions and methods of quantitation (Goldenring et al., 1983).

Preparation of Enzyme Solutions (Control and Test Solutions)

Purified brain CaM kinase II was diluted into iontophoresis carrier solution (0.95 M KAc, 0.05 M Tris, pH 9.3) to a protein concentration of ~5 to 10 μg/ml. Iontophoresis of this carrier solution does not cause significant changes in the currents studied here (Alkon et al., 1983; Acosta-Urquidi et al., 1984). Inactivated calmodulin kinase II was prepared by heating the enzyme at 60°C for 30 min prior to dilution in the carrier solution. Heat inactivation of the enzyme at 65°C for 30 min produced >95% reduction in enzyme activity for Ca^{2+}/CaM-dependent autophosphorylation and substrate phosphorylation. Partially inactivated CaM kinase II was obtained by (*a*) storing the enzyme in the carrier solution at room temperature for 3–4 d (85–90% inactivated) or (*b*) subjecting the enzyme to several freeze-thaw cycles (50–60% inactivated).

Isolation and Preparation of Type B Photoreceptor Soma

Immediately after removing an animal from maintenance conditions the circumesophageal nervous system was dissected out and placed on a glass slide. Photoreceptor axons were cut with a single-edge razor 10–20 μm from their exit point from the eye (cf. Alkon, 1979). The eye was then rotated 90° laterally and 90° caudally to make the medial type B and the medial type A (as well as the lateral type B) photoreceptors clearly visible (cf. Alkon et al., 1985). Small pins with ends embedded in vaseline strips above and below the nervous system (see Alkon, 1975) placed on either side of the eye maintained the desired orientation. Other conditions of preparation, particularly enzymatic digestion of ensheathing connective tissue have been described elsewhere (Alkon et al., 1984).

Voltage-Clamp

Cells maintained at 20–22°C were bathed in ASW with the following composition: Na^+: 430 mM; K^+: 10 mM; Mg^{2+}: 50 mM; Ca^{2+}: 10 mM; Tris buffer (pH 7.4): 10 mM. Voltage clamp was effected by the insertion of two microelectrodes filled with 3 M KCl, made from A-M Systems thick-walled capillary glass, #6020. The microelectrode used for injection of current had a resistance of 10–15 MΩ. The microelectrode used for measuring intracellular voltage had a resistance of 20–25 MΩ. A current to voltage converter was used to ground (via a silver/silver chloride wire) the perfusion chamber as well as to measure membrane current. The capacitative transient of the voltage clamp current records settled within 5–12 ms. Command voltage steps occurred with a rise time of −0.25 ms.

Other details of the voltage clamp technique, protocol, etc., were as previously described (Alkon et al., 1982*a*; 1984; 1985).

Separation of Ionic Currents

In darkness there are four major voltage-dependent ionic currents that flow across the type B soma membrane (Alkon et al., 1982*a*, 1984; Alkon and Sakakibara, 1984); (*a*) I_0A, an early outward voltage-dependent K^+ current, eliminated by 1–2 mM external 4-aminopyridine (4-AP); (*b*) I_K, a delayed rectifying current, not significantly activated at voltages < 0 mV (absolute), and blocked by 100 mM tetraethylammonium; (*c*) $I_{Ca^{2+}}$, a sustained inward voltage-dependent current, blocked by 10 mM Cd^{2+} or Co^{2+} in the perfusing medium, and (*d*) I_0C, a delayed voltage-dependent outward current eliminated by substitution of Ba^{2+} for Ca^{2+} in the perfusing medium. In addition, illumination activates two major currents: (*a*) I_{Na^+}, a voltage-independent inward current, eliminated by removal of external Na^+ from the perfusion, medium, and (*b*) I_0C, an outward voltage-dependent current with the same properties as I_0C described above.

In this study, I_0A was measured as the peak outward current ~20 ms from the onset of the command depolarization. Because of its rapid activation, I_0A is maximally activated in the almost complete absence of other voltage-dependent outward current. At 20 ms after command onset, inward calcium current offers little contamination since it is much slower to reach peak amplitude and, at −10 mV to 0 mV (absolute), is 20–50 times smaller than I_0A. I_0C was measured as the peak outward current 300–400 ms from depolarization onset. Elimination of I_0A with 2 mM 4-AP or of I_0K with 100 mM TEA only slightly reduces the outward current measured 300–400 ms from command onset (Alkon et al., 1984). Current values included in the tables were taken either immediately before or 10 min after a Ca^{2+} load.

To assess $I_{Ca^{2+}}$ in the absence of K^+ currents, external K^+ was raised to 300 mM. This raised the equilibrium potential for K^+ flux to ~0 mV (absolute) where close to maximal activation of $I_{Ca^{2+}}$ occurs. $I_{Ca^{2+}}$ measurements were made at this K^+ reversal potential. For each measurement of $I_{Ca^{2+}}$ the exact reversal potential was determined for the

early peak I_0A K^+ current (and thus all K^+ currents) in 300 mM K^+-ASW. During a depolarizing command step (1.0 s) to this reversal potential (usually ~0 mV absolute) the maximum inward current (see Fig. 5), $I_{Ca^{2+}}$, could then be measured in the absence of any net K^+ current. After the depolarizing command the inward tail current (measured 15 ms after depolarization offset) largely reflected a reversed K^+ current that arose from conductances activated during the depolarizing command.

Iontophoretic Injection

Each injection (of control or test solution) was accomplished with two microelectrodes inserted into the type B soma. Negative current (−2.0 nA for 2 min) was passed through a microelectrode containing either active or inactive enzyme solution. An equal but opposite current was passed through a second microelectrode filled with 3 M KCl. Thus, isopotential conditions were maintained under current clamp conditions during injection. The voltages recorded by each of the two microelectrodes were carefully measured before and after injection. Usually these voltage levels did not change and were confirmed by the potential shifts recorded at the end of the experiment on cell withdrawal. When a substantial voltage shift (≥5 mV) was recorded by the enzyme-filled microelectrode (following injection) and not the KCl-filled microelectrode, i.e., when the two microelectrodes recorded different voltage shifts after injection, the cell was discarded. There were, however, no changes of voltage consistently recorded by both microelectrodes as a function of injection (see Table I). Similarly, changes of holding current for voltage clamp recording (before and after injection) rarely occurred but when they exceeded 1.0 nA, the experiment was ended.

RESULTS

Enzyme Injection Reduces Voltage-dependent Outward K^+ Currents

Previous voltage clamp studies (Alkon et al., 1982*a*; 1984) demonstrated that the two major voltage-dependent outward K^+ currents in the voltage range from −60 to 0 mV (absolute) are I_0A, an early rapidly activating and inactivating current blocked by 1–2 mM 4-aminopyridine and a more slowly activating and inactivating current, I_0C, dependent on $Ca^{2+}{}_i$ and thus blocked by EGTA injection, Ca^{2+}-channel blockers (Co^{2+}, Cd^{2+}, Ni^{2+}) and substitution of Ba^{2+} for Ca^{2+} in the external perfusion medium (Alkon et al., 1984). These currents could be measured in ASW as peak values, I_0A, 20–30 ms to maximum amplitude and I_0C, 300–400 ms to maximum amplitude. All measurements of current amplitudes described here were made after correction for leak current. It should also be made clear that no consistent or specific effects of any treatments used on the leak current were observed. A single injection of CaM kinase II often at first had little or no effect on I_0A or I_0C (Table I, Fig. 1 *A*, *B*). After such an injection, however, a prolonged depolarizing step (to −5 mV for 225 s) paired with a 2.0 s light step (occurring 5.0 s after the depolarization onset) was followed by I_0A and I_0C reduction (Fig. 1 *C*, Table I) which persisted for the duration of the recording (1–1.5 h). Depolarization associated with light presentation has previously been shown to cause substantial and prolonged elevation of intracellular Ca^{2+} (Connor and Alkon, 1984). This Ca^{2+} load is known to cause a prolonged but reversible reduction of both I_0A and I_0C (Alkon et al., 1982*a*, *b*; 1984). Before enzyme injection, reduction of I_0A and I_0C following a Ca^{2+} load recovered over 1–5 min (Fig. 2). After enzyme injection, I_0A and I_0C were reduced by a Ca^{2+} load to levels from which they did not recover (Figs. 1, 2; Tables I, II). Heat-inactivated enzyme injection, however, did not pro-

TABLE I
EFFECTS OF CaM KINASE II IN ASW

Cell	Membrane potential		I_A at −5 mV*			I_C at −5 mV*			Light-induced $I_C\downarrow(t_{1/2})$‡		I_{Na}	
	mv		*nA*			*nA*					*nA*	
	Before	After	Before	After	After Ca^{++} load	Before	After	After Ca^{++} load	Before	After	Before	After
1	−41	−38	22.0	19.4	17.5	7.8	4.1	2.3	—	—	−37.0	−37.0
2	−46	−46	26.9	26.5	25.3	12.0	11.8	9.2	6.9	17.3	−38.0	−39.0
3	−39	−38	—	—	—	—	—	—	6.1	9.2	—	—
4	−46	−42	27.0	22.3	—	15.1	13.3	—	6.2	9.4	—	—
5	−59	−58	25.8	22.9	16.8	9.0	8.2	5.7	6.5	10.6	−5.5	−6.0
6	−38	−38	25.0	27.0	19.0	19.7	18.5	12.5	7.5	13.2	−2.0	−2.5
7	−45	−49	32.0	31.7	23.1	15.3	13.5	8.4	—	—	—	—
8	−40	−40	24.3	20.5	17.5	7.1	6.0	5.4	—	—	—	—
9	−42	−46	24.7	25.0	24.8	18.0	15.2	10.5	—	—	−12.0	−22.0
10	−45	−48	28.3	28.0	24.4	14.7	13.3	6.8	—	—	−15.5	−19.0
Mean	−44.1	−44.3	26.2	24.8	21.1‖	13.2	11.5	7.6§	6.6	11.9¶	−18.3	−20.9
SEM	5.70	6.10	2.67	3.70	3.45	4.23	4.34	3.02	0.51	3.04	14.23	13.86

*Peak current values.
‡Time in seconds for light-induced decrease of I_C to recover from one-half maximum value.
§Significantly different from pre-type II—Ca^{++}-dependent protein kinase injection value at $P < 0.02$ by two-tailed *t*-test.
‖ $P < 0.01$.
¶ $P < 0.0005$.

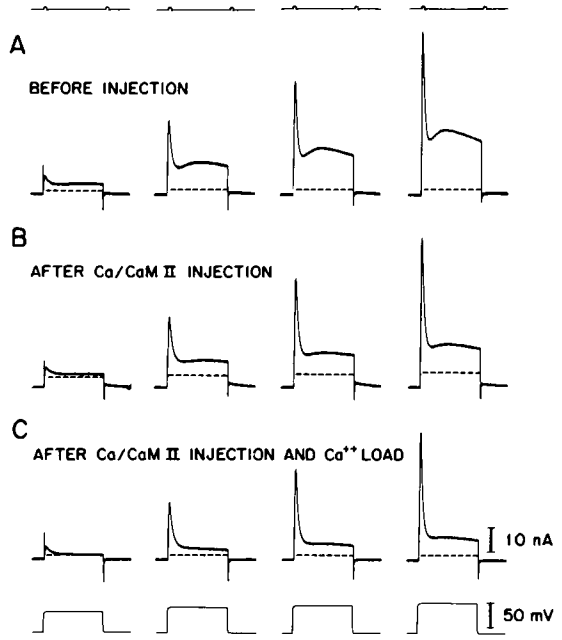

FIGURE 1 Effect of CaM kinase II injection on voltage-dependent outward currents across the membrane of the isolated type B photoreceptor cell soma. The currents (*A*) are elicited by commands, from left to right, to −20 mV, −10 mV, −5 mV, and 0 mV absolute, respectively, from a holding potential of −60 mV for 1 s. I_0A and I_0C are reduced after CaM kinase II injection (−2.0 nA for 2 min before *B*) although often such reduction does not occur without a Ca^{2+} load. These currents are markedly reduced (*C*) following a Ca^{2+} load by depolarization for 25 s to −5 mV from −60 mV paired with 5 s delayed light stimulation (10^4 ergs/cm^2·s) for 1 s. This reduction persisted for the duration of the recording. Dashed line indicates the level of the nonvoltage-dependent or leak current. No consistent or specific effects on the leak current were observed. *Top* trace shows 1 s time calibration.

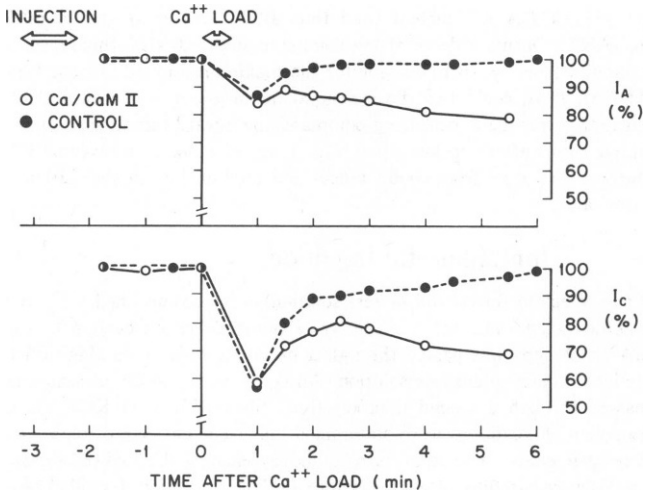

FIGURE 2 Time course of I_0A (*upper* panel) and I_0C (*lower* panel) reduction following a Ca^{2+} load. I_0A and I_0C were measured as the peak outward currents ~20 ms (I_0A) and 300–400 ms (I_0C) from the onset of a command depolarization to −5 mV absolute (holding potential = −60 mV). Ca^{2+} loads were given before (closed circles) and after iontophoretic injection of CaM kinase II. I_0A and I_0C amplitudes before the Ca^{2+} load are normalized as 100%. Note that CaM kinase II injection prevents recovery of I_0A and I_0C reduction after a Ca^{2+} load.

duce these effects (Fig. 3, Table II). Within the range of voltage steps used the voltage-dependence of the currents did not appear altered (Fig. 4) although the magnitudes of currents elicited at any given voltage were reduced by enzyme injection.

Enzyme injection, when followed by a Ca^{2+}-load, did not only cause reduction of I_0A and I_0C to peak amplitudes significantly different from values before injection (Table I). Enzyme and Ca^{2+}-reduced peak amplitudes were also significantly different from values obtained for other cells injected with inactivated enzyme or control buffer solution (Table II). I_0A and I_0C also did not remain significantly reduced from control levels after injection of inactivated enzyme or control buffer solution followed by a Ca^{2+} load (Table II). Finally, repeated enzyme injections each followed by a Ca^{2+} load reduced I_0A and I_0C to progressively lower levels.

Enzyme Injection Reduces Voltage-dependent Inward Ca^{2+} Currents

A previous voltage-clamp study (Alkon et al., 1984) demonstrated the presence of a single voltage-dependent

TABLE II
COMPARISON OF EFFECTS BETWEEN CaM KINASE II AND INACTIVATED ENZYME

	ΔI_A^* at −5 mV		ΔI_C^* at −5 mV		$\Delta I_{Ca^{++}}^*$ at E_K		ΔI_{tail}^* at E_K		$\Delta I_C^* \downarrow (t_{1/2})$‡	
	nA		*nA*		*nA*		*nA*			
	Inactivated enzyme	Enzyme	Inactivated enzyme	Enzyme	Inactivated enzyme	Enzyme	Inactivated enzyme	Enzyme	Inactivated enzyme	Enzyme
Mean	−2.4	−5.1§	−0.8	−5.4¶	−0.06	−0.8‖	−1.5	−9.9¶	0.6	5.3‖
SEM	1.45	3.05	1.46	2.26	0.42	0.43	1.62	9.57	0.67	2.72
n	4	8	4	8	5	7	4	7	4	5

*Δ is the difference between before injection and after Ca^{++} load following injection.
‡Time in seconds for difference of light-induced decrease of I_C to recover from one-half maximum value.
§$P < 0.05$.
‖$P < 0.02$.
¶$P < 0.01$.

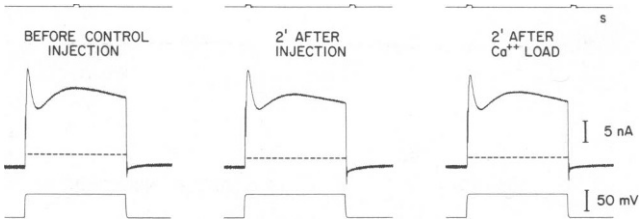

FIGURE 3 Effect of heat inactivated CaM kinase II injection on voltage dependent outward currents across the type B soma membrane. Iontophoretic injection (−2.0 nA for 2 min) of heat-inactivated (65° for 30 min) enzyme had no effect on I_0A and I_0C even after a Ca^{2+} load (*right* trace). Dashed line indicates level of nonvoltage-dependent or leak current. Injection of control buffer solution also produced no effect.

inward current carried by Ca^{2+} ($I_{Ca^{2+}}$ could be observed with 4-aminopyridine (1–3 mM), tetraethylammonium ion (100 mM) and Ba^{2+} substituted for Ca^{2+} in the external bathing medium. It can also be measured as it was here, in 300 mM K^+_o-ASW when the membrane is voltage-clamped at the reversal potential for K^+ flux (−5 to 0 mV absolute). It was previously shown by tail current measurements as well as measurements of the I_0A reversal potential that elevation of external [K^+_o] to 300 mM raises the K^+ equilibrium potential to a voltage range that allows close to maximal activation of $I_{Ca^{2+}}$ (Alkon et al., 1984). $I_{Ca^{2+}}$ was of small amplitude (1–3 nA) and was sustained, i.e., it showed no appreciable inactivation. A single injection of CaM kinase II was followed by a small but significant reduction of $I_{Ca^{2+}}$ (Fig. 5, Tables II, III). Depolarization for 25 s paired with light (as above) caused some additional reduction. This result did not occur when inactivated enzyme was injected (Table III). It should be noted that enzyme injection was accomplished out of voltage clamp under isopotential conditions (see Methods). In 300 mM K^+_o the potential of the type B cell is −5 to 0 mV. Thus for the 2 min during the enzyme injection the cell was exposed to depolarization and (via $I_{Ca^{2+}}$ a Ca^{2+} load. This might account for the enzyme injection having a reducing effect on $I_{Ca^{2+}}$ even prior to the Ca^{2+} load produced under voltage-clamp. The large inward tail current in 300 mM K^+_o, which is largely if not entirely K^+

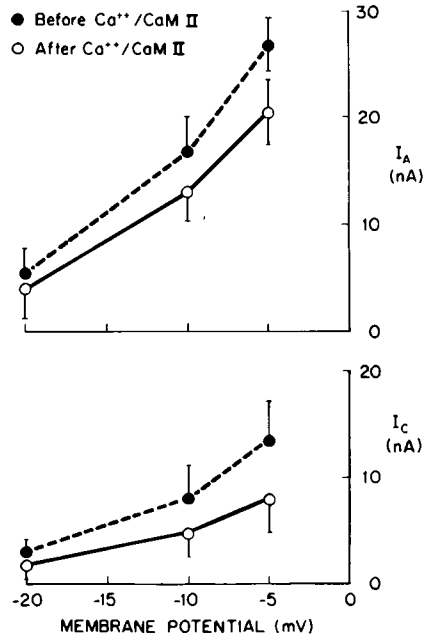

FIGURE 4 Effects of CaM kinase II on I_0A and I_0C current-voltage relations. Peak amplitudes (leak corrected) of I_0A (*upper* panel) and I_0C (*lower* panel) before (closed circles) and after CaM kinase II (open circles) injection are plotted as a function of voltage. All values were measured at least 2 min after a Ca^{2+} load. Note that the current voltage relation (in the limited voltage range shown) was unchanged by enzyme injection although the current amplitudes remain reduced following a Ca^{2+} load. Bars represent standard errors for n = 11 different cells.

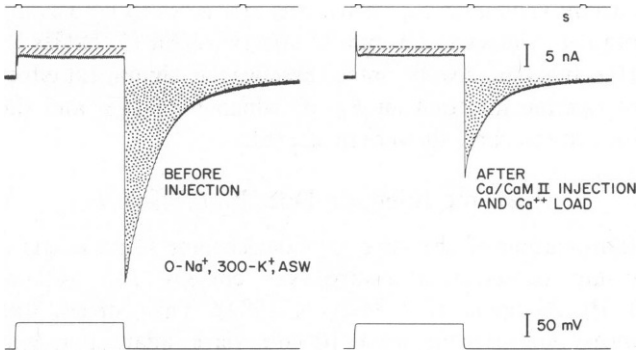

FIGURE 5 Effects of CaM kinase II injection on voltage-dependent Ca^{2+} current, $I_{Ca^{2+}}$, and inward tail currents that were elicited by depolarization to 0 mV absolute in 0-Na^+, 300-K^+, 10-Ca^{2+}-ASW. The small sustained inware current, $I_{Ca^{2+}}$ (represented by the hatched area below the leak level indicated by the dot-dash line) can be measured in isolation of K^+ currents at 0 mV absolute, the new reversal potential for K^+ flux. Voltage-dependent K^+ current is manifest as an inward tail current (indicated by dotted area) following offset of the command depolarization. Both $I_{Ca^{2+}}$ and the tail current were reduced (the former only slightly) after an iontophoretic injection of CaM kinase II under isopotential conditions. No consistent or specific effects on the leak current were observed.

TABLE III
EFFECTS OF CaM KINASE II IN 300 mM K^+-ASW

Cell	Membrane potential		$I_{Ca^{++}}$ at E_{K^+}		I_{tail} at E_K	
	mV		*nA*		*nA*	
	Before	After	Before	After	Before	After
11	−5	−2	−3.0	−2.8	−30.8	− 1.1
12	−2	−2	−2.3	−1.1	−36.5	—
13	0	0	−2.5	−1.5	− 4.3	− 1.5
14	2	1	−1.0	−0.3	− 8.3	—
15	3	4	−2.5	−1.0	−17.1	− 1.4
16	4	4	−1.3	−0.3	—	—
17	−1	−1	−1.7	−0.6	− 2.0	− 1.6
18	−3	−3	−1.0	−0.8	−24.5	−23.2
19	−2	−2	−1.0	0	−17.3	− 8.0
20	−2	0	−2.3	−1.5	−20.2	−10.0
Mean	−0.6	−0.1	−1.9	−1.0*	−17.9	− 6.7*
SEM	2.69	2.34	0.71	0.77	11.02	7.53

*$P < 0.05$.

current (Alkon et al., 1984), was also reduced by enzyme but not by inactivated enzyme injections (Fig. 5, Tables II, III). It should also be noted there was no significant effect of enzyme injection on E_{K^+} at which both $I_{Ca^{2+}}$ and the inward tail currents were measured.

Enzyme Injection Does Not Affect I_{Na^+}

Illumination of the type B photoreceptor soma elicits a voltage-independent inward Na^+ current, I_{Na^+} (Alkon, 1979; Alkon et al., 1982*a*, *b*; 1984). This current was measured after at least 10 min dark adaptation and thereafter, in the dark, at 3 min intervals. Enzyme injection with or without a Ca^{++} load was not followed by any change of I_{Na^+} other than a slight but not significant increase which typically occurs during dark-adaptation (Fig. 6, Table I). The same increase was observed following injection of inactivated enzyme as well.

Enzyme Injection Prolongs Light-induced Reduction of I_0C

Results of other studies are consistent with the interpretation that illumination of the type B photoreceptor releases Ca^{2+} from internal stores. This light-induced elevation of $[Ca^{2+}{}_i]$ was thought to cause prolonged inactivation of steady-state I_0C elicited by a step depolarization (Alkon and Sakakibara, 1984; 1985). Ca^{2+}-mediated inactivation of I_0C appears as a light-induced "apparently" inward (really a reduced outward current) (Fig. 7). Enzyme injection (but not injection of inactivated enzyme) caused a significant prolongation of the light-induced reduction of I_0C (Figs. 7 *B*, 8; Tables I, III). It was previously shown that light does not affect the sustained $I_{Ca^{2+}}$ (Alkon and Sakakibara, 1985). Thus, the enzyme-induced prolongation of light-elicited reduction of I_0C is most likely due to a direct enzyme effect on I_0C (and not due to an indirect enzyme effect on $I_{Ca^{2+}}$).

Trifluoroperazine Increases I_0A and I_0C

Trifluoroperazine (TFP) is known to inhibit CaM kinase II, C-kinase, and possibly other enzymatic reactions. Although TFP is not a specific inhibitor of CaM kinase II, its actions on the Ca^{2+}-dependent kinase endogenous to the type B soma might be expected to produce effects opposite to those of enzyme injection. In fact, perfusion of the type B cell soma with 5 μM TFP consistently caused a marked increase of I_0A and I_0C (Fig. 9 *A*, *B*; Table IV) and a shortening of the light-induced reduction of I_0C (Fig. 10, Table IV). Within the range of voltage steps used, these increases of I_0A and I_0C did not appear to arise from a change of the voltage-dependence of each current although their amplitudes were increased (Fig. 11). Perfusion with TFP also consistently eliminated or greatly reduced the effects of CaM kinase II injection with Ca^{2+} loads on the outward K^+ currents (Fig. 9 *B*). All of these results are consistent with the other observations reported here indicating that the CaM kinase II catalyzes reaction(s) mediating or regulating inactivation of I_0A and I_0C.

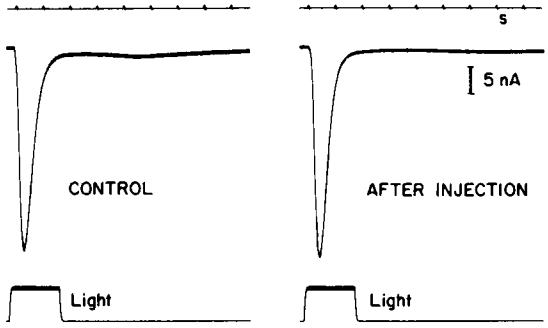

FIGURE 6 Effect of CaM kinase II injection on light-induced inward sodium current, I_{Na^+}. Two seconds light steps (10^4 ergs/cm^2·s) presented after 10 min dark adaptation at 3 min intervals, elicited inward currents at a holding potential of −60 mV. Note that no change in the early peak inward current is apparent after the injection of CaM kinase II.

DISCUSSION

Enzyme-specific reduction of I_0A, I_0C, and $I_{Ca^{2+}}$ was shown to occur following voltage-clamp conditions know to cause prolonged elevation of $Ca^{2+}{}_i$. After enzyme injection (but not injection of inactivated enzyme), for example, a light step presented during a 25 s command depolarization (providing the calcium load) produced I_0A and I_0C reduction that did not reverse during the recording period of (1–2 h). These effects of CaM kinase II appear to be an amplification of a regulatory mechanism already existing

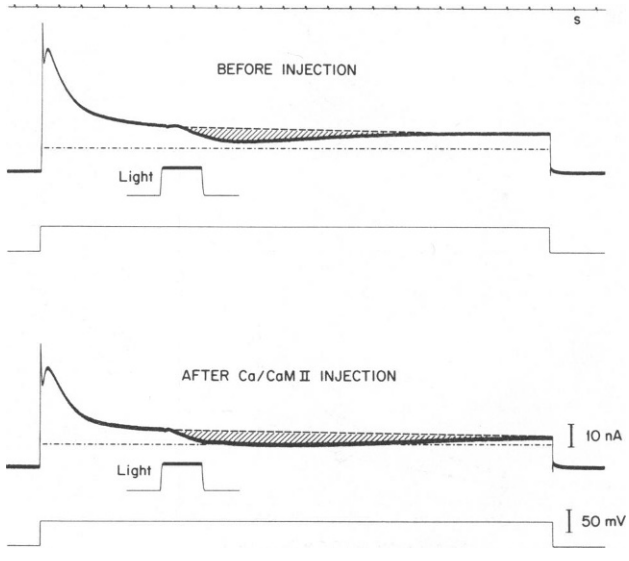

FIGURE 7 Effect of CaM kinase II injection on light-induced decrease of I_0C. The light step (10^4 ergs/cm^2·s) occurred 5 s after the onset of a 25 s depolarizing command step to −5 mV from a holding potential of −60 mV. Iontophoretic injection of CaM kinase II causes a prolongation of light-induced decrease of I_0C (indicated by shaded area). Dot-dash line indicates level of leak or nonvoltage-dependent current. Dashed line indicates steady-state level of I_0C in the absence of a light step. No consistent or specific effects on the leak current were observed.

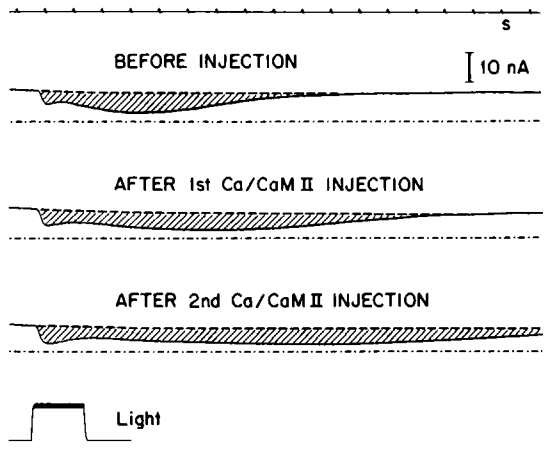

FIGURE 8 Cumulative effect of CaM kinase II injection on light-induced decrease of I_0C. Iontophoretic injection of CaM kinase II (−2.0 nA for 2 min) causes a prolongation of light-induced decrease of I_0C (indicated by the shaded area) and further prolongation can be seen after the second injection. Dot-dash lines indicate level of nonvoltage-dependent (or leak) current. See Fig. 7 for conditions of light stimulation.

within the type B cell — namely Ca^{2+}-mediated reduction of ionic currents. Thus, prior to injection, a Ca^{2+} load was shown to reduce I_0A (Alkon et al., 1982*b*), I_{Na^+} (Alkon et al., 1982*b*), I_0C (Alkon and Sakakibara, 1984, 1985; Alkon et al., 1984), and, in elevated external Ca^{2+}, $I_{Ca^{2+}}$ (Alkon et al., 1984). This Ca^{2+}-mediated reduction, however was always followed by recovery within 1–5 min. CaM kinase II injection prevented this recovery.

The specificity of Ca^{2+}-mediated reduction of ionic currents was suggested in the past (Alkon et al., 1982*a*) by the finding that iontophoresis of Mg^{2+} (like iontophoresis of Ca^{2+}) markedly reduced I_{Na^+} with little effect on I_0A or I_0C (unlike iontophoresis of Ca^{2+}). Thus, Ca^{2+}-mediated reduction of I_{Na^+} appeared to have differences from Ca^{2+}-mediated reduction of I_0A and I_0C. That I_{Na^+} was not reduced but I_0A, I_0C, and $I_{Ca^{2+}}$ were reduced by enzyme injection further indicates such differences.

The results of the present report are in agreement with previous work that demonstrated that reduction of I_0A and a late K^+ current (then not identified as I_0C) were produced by injection of phosphorylase kinase only when followed by a Ca^{2+} load (Acosta-Urquidi et al., 1984). In that study enzyme effects of $I_{Ca^{2+}}$ were not measured, as they were here. The fact that kinase injection followed by Ca^{2+} load did reduce $I_{Ca^{2+}}$ raises the possibility that I_0C was not directly reduced by the treatment, but only indirectly due to a decreased $I_{Ca^{2+}}$ (since I_0C depends on the magnitude of $I_{Ca^{2+}}$). However, the light-induced reduction of I_0C during the command depolarization was also significantly prolonged as a result of the enzyme treatment (Figs. 7 *B*, 8) and this effect, as mentioned above, cannot be attributed to effects of light and enzyme on $I_{Ca^{2+}}$. One other possible explanation seems equally unlikely — that the enzyme injection followed by Ca^{2+} load produces an increase of the $Ca^{2+}{}_i$ elevation due to intracellular release

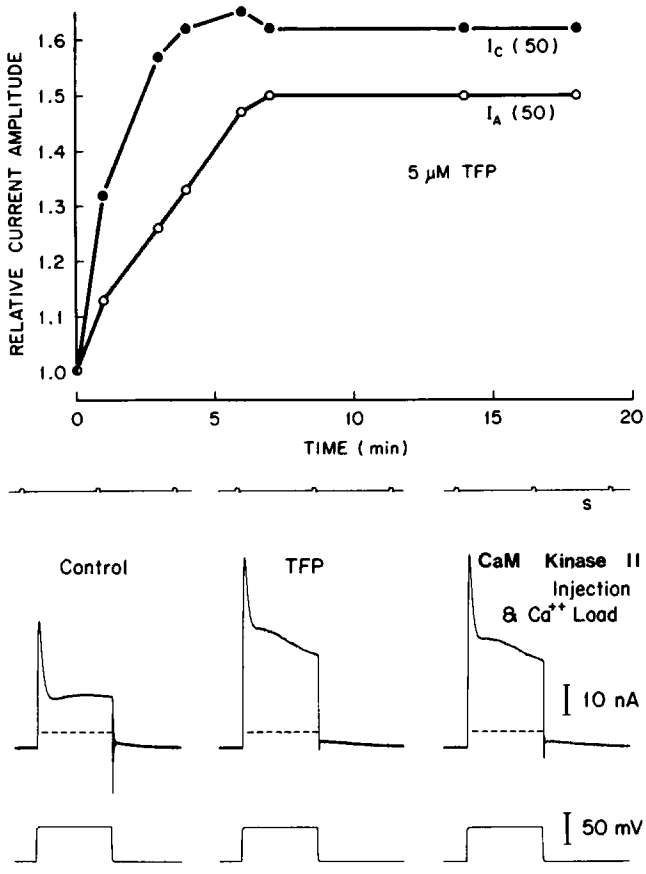

FIGURE 9 (*A*) Time course of I_0A and I_0C increase with 5 μM trifluoperazine (TFP). Current amplitudes (after leak correction of I_0A (indicated by open circle) and I_0C (filled circle) increase progressively within 7 min and stay constant thereafter. I_0A and I_0C values are normalized to current amplitudes before addition of TFP to the ASW. (Thus "1.0" represents no change.) I_0A and I_0C were measured as peak outward currents elicited by voltage steps to −10 mV from a holding potential of −60 mV. (*B*) TFP perfusion increases amplitudes of outward K^+ currents (I_0A and I_0C). TFP also markedly reduces or eliminates the effect of CaM kinase II injection on I_0A and I_0C with Ca^{2+} loads.

TABLE IV
EFFECTS OF TFP (5 μM)

Cell	I_A at −5 mV*		I_C at −5 mV*		Light-induced $I_C\downarrow$ ($t_{1/2}$)‡		I_{Na} at −5 mV	
	nA		*nA*				*nA*	
	Before	After	Before	After	Before	After	Before	After
21	22.8	34.5	13.1	19.5	8.8	7.0	9.2	6.6
22	28.0	34.8	13.3	17.5	12.3	3.1	5.0	6.5
23	17.3	17.5	8.8	9.8	7.5	7.7	4.6	3.0
24	25.5	29.5	8.2	12.3	8.1	6.5	3.5	3.8
25	18.0	28.4	5.6	16.5	4.2	4.3	2.5	3.5
Mean	23.6	31.8§	10.1	16.5§	8.2	5.7‖	5.0	4.7
SEM	3.71	2.88	3.28	2.63	2.59	1.73	2.29	1.55

*Peak current values.
‡Time in seconds for light-induced decrease of I_C to recover from one-half maximum value.
§$P < 0.05$.
‖$P < 0.02$.

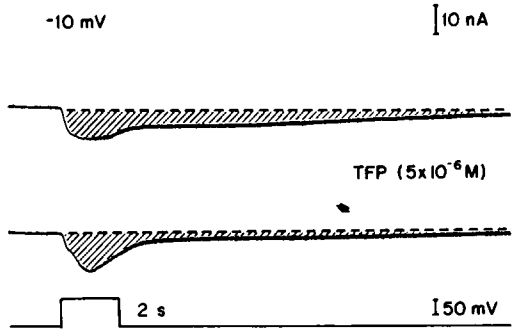

FIGURE 10 Effect of perfusion with TFP on light-induced decrease of I_0C. Addition of TFP (5 μM) to the external ASW decreases the duration of light-induced decrease of I_0C (indicated by shaded area). For conditions of light stimulation, see Fig. 7. Dashed line indicates level of steady-state outward current (in the absence of light) in response to command depolarization to −10 mV (−60 mV holding potential). Light step monitored on *bottom* trace.

by the light stimulus. If light-induced $Ca^{2+}{}_i$ release were enhanced, the light-induced I_0C (see Alkon, 1979; Alkon and Sakakibara, 1984, 1985) would also increase. Increased light-induced I_0C would oppose and cause an apparent reduction of the light-induced I_{Na^+}. However, enzyme-induced I_{Na^+} reduction was not observed. On the contrary, a slight increase due to dark adaptation was seen for cells injected with either enzyme or inactivated enzyme.

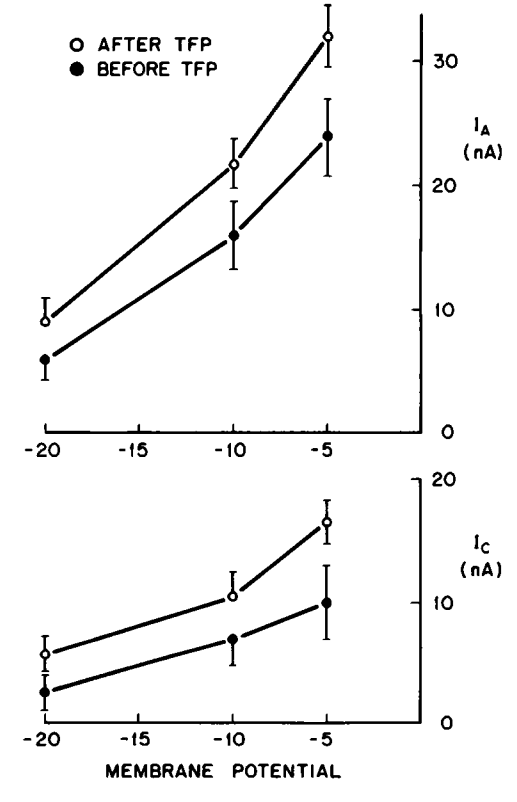

FIGURE 11 Voltage-dependence of I_0A (*upper*) and I_0C (*lower*) before and after perfusion of TFP. Points are means and bars are standard errors of five individual cells. Note that although the magnitudes of I_0A and I_0C (elicited by depolarizing step to −10 mV from −60 mV holding potential) are reduced by TFP (5 μM) the voltage-dependence within this voltage range is unchanged.

Thus from these results, inactivation of I_0A, I_0C, and $I_{Ca^{2+}}$ by elevation of $Ca^{2+}{}_i$ can be modulated by CaM kinase II. The results of studies on endogenous Ca^{2+}-dependent protein phosphorylation (Neary et al., 1984) provide evidence that *Hermissenda* CaM kinase can be stimulated by Ca^{2+}-levels within what has been previously determined as physiologic levels. Moreover, the molluscan CaM kinase II cross-reacts with an antibody to mammalian neuronal CaM kinase II (DeRiemer et al., 1984), suggesting that the two enzymes could share substrate specificity. Modulation of the type B soma currents need not, of course, depend exclusively on Ca^{2+}/CaM phosphorylation. This may be only one of many steps within biochemical pathways that regulate the membrane currents. Recently, in fact, evidence has been obtained that activation of Ca^{2+}/phospholipid-dependent protein kinase (C-kinase) (Alkon et al., also can enhance Ca^{2+}-mediated reduction of I_0A and I_0C. The possibility exists, therefore, that Ca^{2+}/CaM-dependent and C-kinase dependent phosphorylation may act synergistically to cause larger and more sustained reduction of I_0A and I_0C (as has been observed for aldosterone secretion (Kojima et al., 1984) and platelet activation (Kaibuchi et al., 1983). Finally, it should be emphasized that the enzymatic manipulations reported on here and in other studies are only approximations at what may be occurring physiologically during, for instance, such processes as learning or sensory adaptation. As our experience with such manipulations increases, those which are most physiologic may become apparent. In another study, for example, iontophoresis of inositol trisphosphate (which is thought to mobilize $Ca^{2+}{}_i$) also reduced I_0A and I_0C (but not I_{Na} or $I_{Ca^{2+}}$), but in the absence of a Ca^{2+} load (Sakakibara et al., 1985). Such results as well as those of several other reports including the present one certainly implicate Ca^{2+}-stimulated phosphorylation in ionic current regulation. But understanding of the series of interacting biochemical reactions that directly affect membrane channels, the rate limiting steps, and the subcellular loci of critical molecular constituents must await further experimentation.

Note added in proof: Conditioning-specific reduction of $I_{Ca^{2+}}$ (in addition to I_0A and I_0C) has recently been observed (Collins et al., unpublished observations), consistent with involvement of CaM Kinase II which here affects I_0A, I_0C, and $I_{Ca^{2+}}$.

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