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AN ENZYMATIC MECHANISM FOR CALCIUM CURRENT INACTIVATION IN DIALYSED *HELIX* NEURONES

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SUMMARY

1. 'Wash-out' and inactivation of the Ca current were examined in dialysed, voltage-clamped neurones of *Helix aspersa* under conditions that isolate the Ca current virtually free of other currents. EGTA or other internal Ca^{2+} chelators were routinely omitted from the dialysate.

2. The time-dependent loss, or wash-out, of Ca current was slowed by addition to the dialysing solution of agents, such as dibutyryl adenosine 3′-5′-cyclic monophosphate (dibutyryl cyclic AMP), Mg adenosine 5′-triphosphate (ATP) and the catalytic subunit of cyclic-AMP-dependent protein kinase, that promote protein phosphorylation and by EGTA. However, neither the phosphorylation-promoting agents nor internal EGTA prevented wash-out entirely, nor did they significantly restore previously 'washed-out' current.

3. With phosphorylating agents in the dialysing solution, the irreversible development of wash-out was greatly reduced by introduction of leupeptin, an inhibitor of protease activity. Thus, the irreversible component of wash-out appears to result from a Ca-dependent proteolytic process.

4. In the presence of leupeptin alone, Ca current amplitude continued to decline: however, the current could be largely or fully restored with addition of catalytic subunit, dibutyryl cyclic AMP, and Mg ATP to the dialysing solution. Thus, inhibition of proteolysis revealed a reversible component of wash-out that appears to result from dephosphorylation.

5. During perfusion with leupeptin, Mg ATP, dibutyryl cyclic AMP and catalytic subunit the Ca current remained stable for up to several hours without addition of internal Ca^{2+} buffer. The rate of inactivation of the current that occurs during a depolarizing step showed only a very gradual decline during this time.

6. Under these conditions, perfusion with calcineurin, a Ca–calmodulin-dependent phosphatase, caused a significant increase in the rate of Ca current inactivation. This inactivation was virtually eliminated by introduction of EGTA or by replacement of external Ca^{2+} with Ba^{2+}, which is consistent with the ion dependency for calmodulin-dependent activation of calcineurin.

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7. When ATP in the dialysate was replaced with ATP-γ-S (adenosine 5′-*O*-(thiotriphosphate)), an analogue that donates a thiophosphate group resistant to hydrolysis, the rate of inactivation slowed.

8. Since Ca-dependent inactivation during step depolarizations is enhanced by conditions that promote dephosphorylation, and Ca current wash-out is slowed by conditions that promote phosphorylation, inactivation and reversible wash-out appear to be related.

9. It was concluded that Ca-dependent inactivation of the Ca current results from the activation of a Ca-dependent phosphatase during entry and accumulation of Ca^{2+}, and that the inactivation subsequently is removed as phosphatase activity declines and dephosphorylated sites are rephosphorylated through the action of an endogenous protein kinase.

INTRODUCTION

Ca channels activated by depolarization can be placed into three categories based on mechanism of inactivation: (i) those showing virtually no inactivation, (ii) those undergoing voltage-dependent inactivation, and (iii) those undergoing inactivation through a mechanism dependent on elevation of intracellular Ca^{2+}. Ca-dependent inactivation is prevalent and has the following characteristics (Eckert & Chad, 1984). (i) The rate and extent of inactivation are current dependent. (ii) Inactivation depends on the ionic species carrying the current, Ca^{2+} being more effective than Sr^{2+}, which is more effective than Ba^{2+}. (iii) Inactivation kinetics are largely independent of membrane potential although secondary effects of potential may occur. (iv) Inactivation is diminished, and its rate of removal is accelerated by means that decrease free internal Ca^{2+}. All these characteristics can be seen in the inactivation of Ca current in neurones of *Aplysia* and *Helix* (Tillotson, 1979; Eckert & Tillotson, 1981; Plant & Standen, 1981; Plant, Standen & Ward, 1983; Eckert & Ewald, 1983*a*, *b*).

Ca currents exhibit 'wash-out' in dialysed cells (Byerly & Hagiwara, 1982; Doroshenko, Kostyuk & Martynyuk, 1982) and rapidly disappear in isolated outside-out membrane patches (Fenwick, Marty & Neher, 1982; Armstrong & Eckert, 1985), suggesting a loss or degradation of normal intracellular constituents during dialysis. Wash-out is accelerated by repeated activation of the Ca current and by other factors that promote or allow an elevation of internal Ca^{2+} concentration ($[Ca^{2+}]_i$); it is retarded by the intracellular application of Ca^{2+} chelating agents such as EGTA (Byerly & Hagiwara, 1982; Fenwick *et al.* 1982; Forscher & Oxford, 1985). The Ca^{2+} sensitivity of both Ca current inactivation and wash-out suggests that the two processes may be related.

Several observations implicate protein phosphorylation and dephosphorylation in the regulation and, perhaps, inactivation of Ca channels. (i) Wash-out can be slowed by agents that promote enzymatic phosphorylation, such as adenosine 3′,5′-cyclic monophosphate (cyclic AMP), Mg adenosine 5′-triphosphate (ATP), and/or the catalytic subunit of cyclic-AMP-dependent protein kinase (Doroshenko *et al.* 1982; Doroshenko, Kostyuk, Martynyuk, Kursky & Vorobetz, 1984; Yazejian & Byerly, 1984; Chad & Eckert, 1984*a*; Forscher & Oxford, 1985; Armstrong & Eckert, 1985).

(ii) Ca channels in cardiac cells undergo phosphorylation in response to β-adrenergic agonists, and this effect, which is mediated by cyclic-AMP-dependent protein kinase, increases the number of channels available for voltage-dependent activation (Osterrieder, Brum, Hescheler, Trautwein, Flokerzi & Hofman, 1982; Reuter, 1983; Bean, Nowycky & Tsien, 1984; Kameyama, Hofmann & Trautwein, 1985). (iii) β-Adrenergic agonists slow the rate of inactivation of Ca current in myocardial cells (Bean *et al.* 1984). (iv) Experimental enhancement of protein kinase C activity increases the size of the Ca current in some *Aplysia* neurones (DeRiemer, Strong, Albert, Greengard & Kaczmarek, 1985).

Observation (i) suggests that some Ca channels found in nerve cells, like those of cardiac cells, may require metabolic maintenance in the form of phosphorylation to keep the channels in a state from which they readily can open in response to depolarization. Thus, if the Ca channel (or an associated regulatory site) requires phosphorylation to undergo activation, dephosphorylation, by definition, would leave the channel in an inactivated state.

Using internally dialysed *Helix* neurones, we have examined the possibility that Ca-dependent inactivation and removal of inactivation are based on dephosphorylation/rephosphorylation mechanisms. These experiments were made possible by a solution of the irksome problem of irreversible Ca current wash-out, which appears to result in part from Ca-activated proteolytic activity, and in part from a loss of phosphorylating machinery during cell dialysis. Preliminary reports have appeared elsewhere (Chad & Eckert, 1984*a*, 1985*a*, *b*, *c*).

METHODS

The outer connective tissue was removed from circumoesophageal ganglia of *Helix aspersa* and the inner sheaths over the suboesophageal ganglia were split to expose the neurones. The ganglia were placed in normal saline (Table 1) containing 1 % (w/v) protease (Sigma type XIV) for 90 min at room temperature (~21 °C). The saline was continuously agitated to improve the cleansing of the cell surfaces. Transmission electron microscopy of neurones treated with this procedure revealed surface membranes with little or no glia remaining attached (A. Hermann, personal communication). After enzymatic treatment, the ganglia were thoroughly washed with several changes of saline and then kept at 4 °C for up to 2 h in normal saline containing 7 mM-glucose until use.

For isolation of single cells, ganglia were pinned to the Sylgard base of the experimental chamber, and a suction pipette with a tip diameter of 20–30 μm was used to remove individual neurones. The suction pipette contained an internal perfusion inlet, positioned close to the tip, through which the pipette solution could be changed, negative pressure at the outlet producing the flow (cf. Lee, Akaike & Brown, 1978). A conventional two-electrode voltage-clamp technique was used with the perfusion barrel as the current electrode, and a conventional micro-electrode (10 MΩ) as the voltage electrode. Control of membrane potential, as judged by smoothness of current–voltage relations, was at an optimum when the axon was severed from the neurone close to the hillock, leaving a nearly spherical cell body.

Ca currents were isolated by modifications of intra- and extracellular ionic compositions. Internal K^+ was replaced by Cs^+, and tetraethylammonium (TEA) and 3,4-diaminopyridine (DAP; Table 1) were used to blocked voltage-activated K channels. The experimental chamber was perfused with 50 mM-Ca (Table 1) unless otherwise stated. External TEA was used to block both voltage and Ca-dependent K channels. Leak correction was carried out by summation of currents resulting from hyperpolarizing and depolarizing voltage steps. Large hyperpolarizing pulses (~ 60 mV) produce a time-dependent inward current (Chesnoy-Marchais, 1983) that can introduce error into the leakage-corrected Ca currents. To avoid activation of the chloride conductance, a 'P/3' pulse protocol was used in producing all the current traces shown. Three hyperpolarizing steps, each a

TABLE 1. Ionic composition of internal and external solutions

	Extracellular					
	Normal	10 Ca	50 Ca	50 Ba	50 Sr	Intracellular
Na^+	80	—	—	—	—	—
K^+	4	—	—	—	—	—
Mg^{2+}	5	5	5	5	5	—
Ca^{2+}	7	10	50	—	—	—
Ba^{2+}	—	—	—	50	—	—
Sr^{2+}	—	—	—	—	50	—
HEPES	5	50	30	20	20	100
pH*	7·3	7·3	7·3	7·3	7·3	7·8
Cs^+	~ 1·3	~ 17	~ 8	~ 5	~ 5	~ 60
Asp^-	—	—	—	—	—	20
Cl^-	108	80	135	135	135	10
TEA	—	50	25	25	25	10
DAP	—	2·5	2·5	2·5	2·5	—

Values in mM. *Titrated to pH with base. Asp^-, aspartate; TEA, tetraethylammonium; DAP, 3,4-diaminopyridine.

TABLE 2. Additives for intracellular perfusion

Agent	Concentration in dialysing solution
Leupeptin (Sigma)	100 μM
Mg ATP (Sigma)	7 mM
ATP-γ-S (Boehringer–Mannheim)	7 mM
Dibutyryl cyclic AMP (Sigma)	5 mM
Dithiothreitol (Sigma)	30 mM
Cyclic-AMP-dependent catalytic subunit of protein kinase	20 μg ml^{-1}*
Calcineurin	40 μg ml^{-1}*
Calmodulin	10 μM

Standard values except where otherwise specified in text.

Sources of catalytic subunit, calcineurin and calmodulin are listed in the Acknowledgments.

* Values as wt. protein ml^{-1}.

third of the depolarizing step, were given, and the resultant currents summed with the current recorded during the depolarization.

Several steps were taken to avoid proton currents (Byerly, Meech & Moody, 1984). The internal pH, pH_i, was adjusted to 7·8 with 100 mM-HEPES buffer, and the external pH, pH_o, to 7·3. A high concentration of buffer was chosen because of the difficulty of controlling pH_i in the dialysed cell (Byerly *et al.* 1984), especially because of the likelihood that bound protons may be liberated during entry and accumulation of Ca^{2+}. The alkalinization of the cell interior with the buffer was also intended to produce a sufficient positive shift in current–voltage relations for activation of the proton current (Byerly *et al.* 1984) so that pulses to +10 mV, the standard value used in these experiments, would not result in activation of the proton conductance. Further, DAP and 4-aminopyridine (4-AP) were used in the bath throughout in concentrations of 2·5 mM to further reduce the proton conductance (Byerly *et al.* 1984).

Stimulus protocols were programmed, and data were digitized (12-bit, 5 kHz), stored and subsequently analysed with a DEC 11–23 computer. The standard protocol utilized a holding potential, V_h, of −40 mV and 100 ms steps to +10 mV. The sequence of test pulse plus P/3 pulses was repeated every 60 s.

The various metabolites, enzymes and enzyme inhibitors (Table 2) were added as needed to the standard dialysate (Table 1) immediately before use. Catalytic subunit of cyclic-AMP-dependent protein kinase together with calcineurin was sometimes pre-incubated with 30 mM-dithiothreitol

(Sigma) for 10 min at room temperature (~21 °C) to minimize loss of activity resulting from oxidation of thiol groups (Rubin, Erlichman & Rosen, 1974). No EGTA or other Ca buffer was used in the internal solution, except where indicated. The extracellular Ca^{2+} concentration was 50 mM unless stated otherwise.

The experiments were carried out at temperatures of 17–19 °C, maintained ± 0·2 °C for a given experiment. Data were used only from cells that remained mechanically stable, and were discarded if the cell showed any sign of gradually being pulled into the pipette. To quantify the rate of inactivation occurring during a step depolarization, the difference in amplitude between the size of the current peak and the current at a fixed later time, generally 100 ms after onset of the voltage step, was divided by the peak current (Chad, Eckert & Ewald, 1984). This quotient is termed the inactivation index.

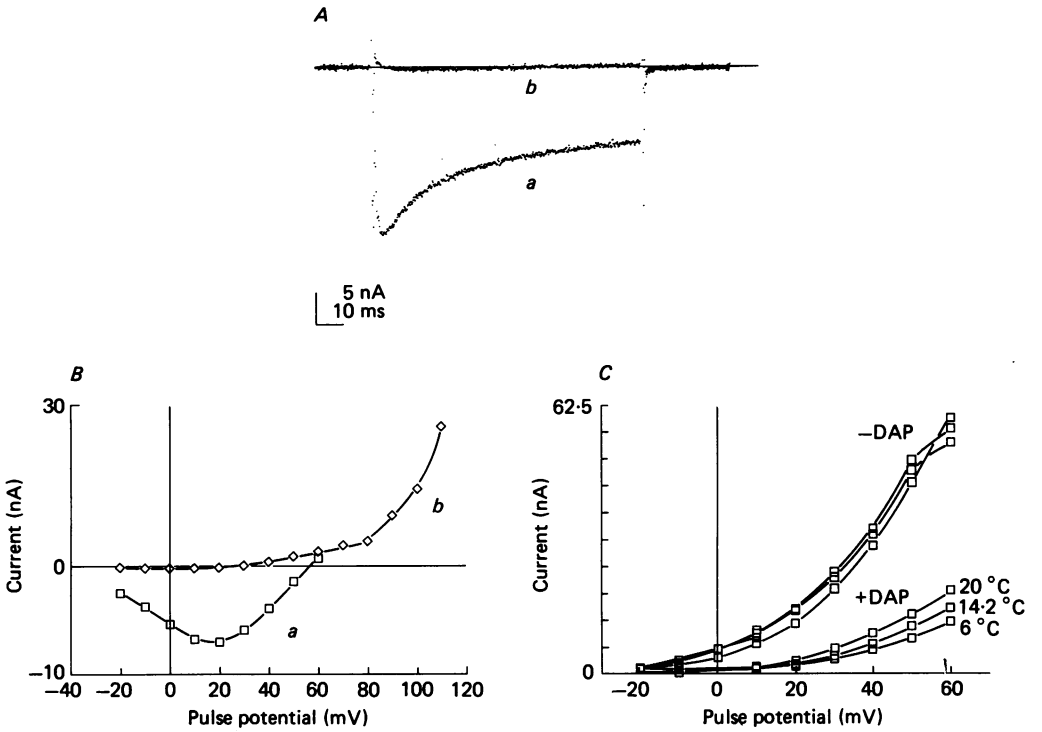

Fig. 1. *A*, current recorded from dialysed *Helix* neurone. Voltage steps were to +10 mV from a holding potential of −40 mV. *a*, before and *b*, after wash-out of Ca current (2·5 mM-DAP). *B*, currents measured after 100 ms pulses plotted against step voltage, *a*, before and *b*, after wash-out of Ca current (2·5 mM-DAP, temperature, 19 °C). *C*, currents measured at 100 ms plotted against potential after wash-out of Ca current (+DAP, 2·5 mM). The outward current, presumably carried primarily by H^+ (Byerly *et al.* 1984), became larger with increased temperature. Removal of DAP (−DAP, 20 °C) led to an increase in outward current.

RESULTS

Isolation and stabilization of Ca current

It was first necessary to consider possible time-variant outward currents, generally carried by K^+ or H^+, that might obscure the true time course of the Ca current. Membrane currents recorded during steps to +10 mV before, *a*, and after, *b*, Ca current wash-out are shown in Fig. 1 *A*. After wash-out there was no significant inward or outward current at that potential. Thus, currents resistant to wash-out such as

voltage-dependent Na, K and H currents are presumed to have been absent prior to wash-out of the Ca current. Under conditions that activate a proton current, I_H, in snail neurones, I_H remains after Ca current wash-out, indicating that this current is not dependent on Ca^{2+} entry (Byerly *et al.* 1984). Currents measured at the end of a 100 ms step during the initial stages of an experiment, namely before wash-out of the Ca current has occurred, were inward for potential steps up to +50 mV at 18 °C (Fig. 1*Ba*). After wash-out, a rapidly activating outward current was observed at voltages above +30 mV, which increased markedly above +80 mV (Fig. 1*Bb*). This current became smaller with increased pH_i, and thus appears to have been I_H (Byerly *et al.* 1984). This conclusion is consistent with the sensitivity of that current to DAP and temperature (Fig. 1*C*). Under the conditions used below (pH_i 7·8, external DAP, 17–19 °C), the proton conductance should have been insignificant during steps up to +10 mV (Byerly *et al.* 1984). Furthermore, the reversal potential of the proton current, ~ +29 mV, calculated from the internal and external pHs (see Methods) was positive with respect to the +10 mV test potential; thus, barring large elevations in intracellular proton concentrations during depolarization, any remaining proton current elicited during steps up to +10 mV should have been inward.

Prior to wash-out there were no slow tail currents characteristic of K or H conductances activated by Ca^{2+} entry. A serotonin-sensitive K current, $I_{K(s)}$, is suppressed by the activity of a cyclic-AMP-dependent protein kinase (Siegelbaum, Camardo & Kandel, 1982; Shuster, Camardo, Siegelbaum & Kandel, 1985). We observed a small leakage current, perhaps due to Cs^+ passing through the K(s) channel, which was reduced by kinase activity. In this regard, the K(s) channel has been found to have a significant Cs^+ conductance in patch-clamp studies (C. Erxleben & J. Chad, unpublished). However, $I_{K(s)}$ shows little or no voltage-dependent kinetics, and so if present under our conditions, should have behaved as a simple time-invariant leakage conductance. Thus, the possible sources of contaminating current known to us appear to have been either too small or endowed with insufficient voltage sensitivity to influence appreciably either the amplitude of the kinetics of the inward current under our conditions.

Up to three types of Ca channels have been recognized in a given cell type (Carbone & Lux, 1984; Matteson & Armstrong, 1984; Nowycky, Fox & Tsien, 1984, 1985*a*; Armstrong & Eckert, 1985). These can be distinguished from one another by virtue of their differing voltage sensitivities of activation and/or inactivation. To determine if our results reflect the properties of more than one class of Ca channels, we held dialysed neurones at several holding potentials (−80, −60, −40 mV), and then stepped to a fixed test potential (+10 mV). The currents elicited in this way were identical in amplitude and time course (Fig. 2). Furthermore the three types of Ca channel reported differ in their sensitivities to the dihydropyridines (Nowycky *et al.* 1985*a*). The Ca currents recorded under our experimental conditions could be blocked by nitrendipine (5 μM). Thus, a single class of channels appears to have given rise to the current recorded in the following experiments.

The gradual loss, or wash-out, of the Ca current (Kostyuk & Krishtal, 1977; Byerly & Hagiwara, 1982; Fenwick *et al.* 1982) limits the usefulness of the dialysed cell preparation. Possible causes for the time-dependent loss of Ca current are: (i) the gradual loss of essential, diffusible intracellular molecules during cell dialysis, or (ii)

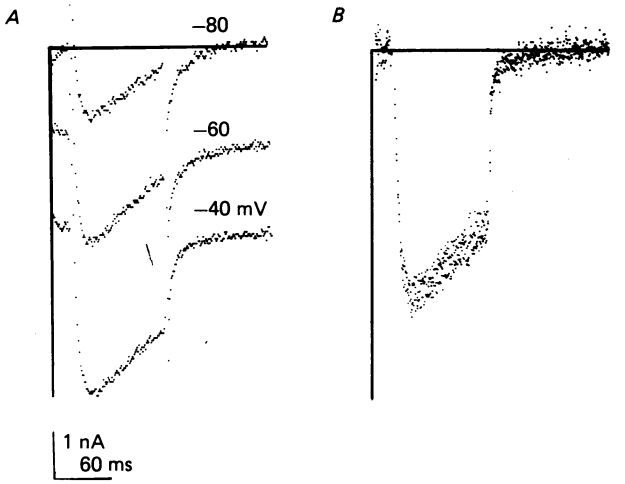

Fig. 2. Currents recorded at +10 mV from a dialysed neurone with different holding potentials (−80, −60, −40 mV). *A*, uncorrected currents; *B*, superimposed leak-corrected currents (see text) showing no significant dependence on hold potential.

the catabolic destruction of channels or other molecular components essential for Ca current activation. As noted above, supplementation of the dialysing solution with various combinations of ATP, Mg^{2+}, cyclic AMP and catalytic subunit of cyclic-AMP-dependent protein kinase can slow Ca current wash-out. The effect of added catalytic subunit under our conditions (i.e. no Ca^{2+} buffer in dialysate) can be seen in Fig. 3*A*. The wash-out was clearly slowed, although not halted, by the introduction into the perfusate of a phosphorylating mixture containing 7 mM-Mg/ATP, 5 mM-dibutyryl cyclic AMP, and 20 μg catalytic subunit ml^{-1}. Following removal of the phosphorylating mixture, the rate of wash-out returned to approximately its original value. Other experiments (data not shown) confirmed published observations (Fenwick *et al.* 1982; Byerly & Hagiwara, 1982; Forscher & Oxford, 1985) that inclusion of EGTA in the dialysate also slows wash-out.

It seems significant that the phosphorylating agents, while they slowed the rate of Ca current wash-out in our preparation, neither prevented nor significantly reversed the wash-out once it had progressed appreciably. Thus, there appears to be an irreversible component of Ca current loss during wash-out that was neither prevented nor reversed by measures that promote phosphorylation. This irreversible component appears to develop in a Ca-dependent manner, since it can be slowed by internal EGTA (data not shown).

A known irreversible consequence of elevating cytoplasmic Ca^{2+} is the digestion of protein molecules by Ca-activated proteases such as calpain (Murachi, 1983). Cell perfusion has been reported to result in elevated levels of intracellular free Ca^{2+} that are not fully suppressed even when the perfusate contains 10 mM-EGTA (Byerly & Moody, 1984). Such elevated Ca^{2+} levels should activate any endogenous Ca-dependent proteases remaining in the cell, and Ca channels might, therefore, undergo proteolytic degradation during cell perfusion in response to elevation of $[Ca^{2+}]_i$. This process may be further augmented during cell dialysis by loss of natural protease inhibitors such as calpastatin (Murachi, 1983).

If a proteolytic loss of Ca channels contributes significantly to irreversible wash-out, introduction of a protease inhibitor should lead to a reduction in the wash-out of Ca current. Thus, to test the possibility that Ca-dependent proteases play a role in the loss of Ca current, we added the tripeptide leupeptin (acetyl-L-leucyl-L-leucyl-L-argininal), an inhibitor of Ca-dependent proteases (Aoyagi, Miyatu, Nanbo, Koyima, Matsuyaki, Ishizuka, Takeuchi & Umezawa, 1969; Sasaki, Kikuchi, Yomoto, Yoshimura & Murachi, 1984), to the dialysate at a concentration of 100 μM.

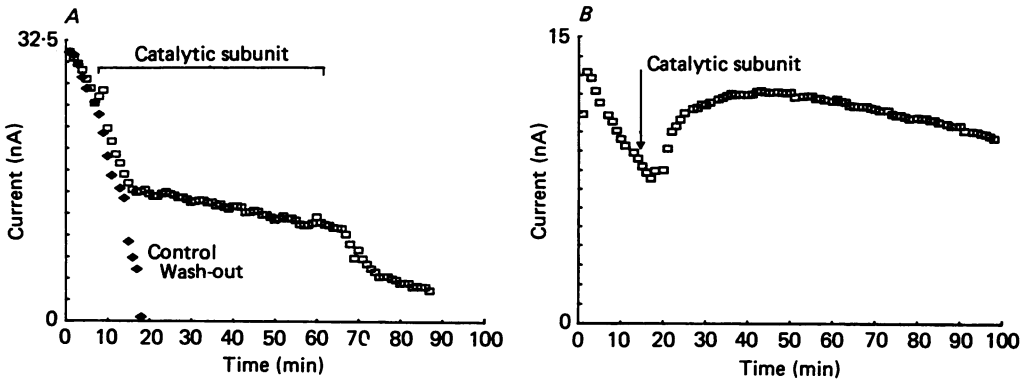

Fig. 3. Amplitudes of Ca currents obtained with steps to +10 mV plotted against time during dialysis. *A*, dialysis of cell with unsupplemented dialysate (◆) produced a rapid wash-out of Ca current. A different neurone (□), dialysed initially with dialysate alone, showed similar initial rate of wash-out. Addition of catalytic subunit (20 μg ml^{-1}) and Mg ATP, then caused a significant reduction in rate of wash-out. The delay between introduction of catalytic subunit and the response is due to the time required for the agent to reach the pipette tip and enter the cell. Ordinate calibrated for test cell, control cell had initial current of 13 nA, which was scaled to the other plot. *B*, in another neurone, leupeptin (100 μM) was added to the dialysate throughout, but had little effect on the initial rate of wash-out as compared to the experiments without leupeptin, as shown in *A*. Addition of Mg ATP, dibutyryl cyclic AMP, and catalytic subunit (Table 2) then produced a significant recovery of the Ca current, followed by a very much slower loss of current amplitude. Extracellular Ca^{2+} was 10 μM throughout in both *A* and *B*.

As compared with experiments performed without leupeptin (Fig. 3*A*), the addition of leupeptin appeared to have little effect on the rate of Ca current wash-out (Fig. 3*B*), and had no effect on the kinetics of the Ca current.

It is significant, however, that leupeptin greatly increased the effectiveness of phosphorylating agents in preventing wash-out, and indeed in producing full, or nearly full, restoration of Ca current amplitude following partial wash-out of the current. Thus, during dialysis with leupeptin, the introduction of catalytic subunit, Mg ATP and cyclic AMP typically arrested the steep time-dependent decline of Ca current amplitude, restoring it to nearly its original level (Fig. 3*B*). The current then remained stable within 10% for periods of up to 60 min or longer. In some experiments dithiothreitol was included with catalytic subunit to retard the oxidation of protein thiol groups and thus prevent loss of enzyme activity. In other experiments it was omitted, and the results were not perceptibly different, presumably because the catalytic subunit was provided in sufficient excess so that some loss of enzyme activity was not noticeable during the course of an experiment. Dithiothreitol by itself

also produced a weak slowing of wash-out, which may be attributable to a reduction in loss of enzyme activity of the native kinase remaining in the cell during dialysis. An additional possibility is that dithiothreitol also serves to protect thiol groups that may be associated with the Ca channel itself. This seems less likely, however, since infusion of excess catalytic subunit without inclusion of dithiothreitol was sufficient to prevent wash-out.

As noted earlier, the phosphorylating mixture when used in the absence of leupeptin and EGTA, generally produced only a slowing of wash-out, or at best a relatively small restoration of the current, and then only if wash-out had not progressed very far. This observation along with the effect of leupeptin suggests that wash-out arises from two fundamentally different processes: (i) an irreversible loss of Ca channel function that can be slowed by inhibition of proteolytic enzymes, and (ii) a reversible loss of channel function that can be prevented or reversed by an elevation of phosphorylating activity, providing proteolytic activity has been suppressed. The use of leupeptin to minimize the irreversible component conveyed two important advantages in the following experiments. First, it permitted stable, long-term experiments during cell perfusion. Secondly, it permitted the use of internal solutions free of Ca^{2+}-chelating agents, such as EGTA, that can interfere with Ca-dependent processes.

Ca current inactivation enhanced by Ca-dependent phosphatase

Several lines of evidence point to protein phosphorylation as playing a role in Ca channel regulation (see Introduction). Thus, inactivation may result from a Ca-dependent dephosphorylation of either the channel itself or an associated regulatory molecule. Because inactivation is Ca dependent, this hypothesis requires a Ca-dependent phosphatase. Such an enzyme, a Ca–calmodulin-dependent phosphatase, termed calcineurin, has been isolated from mammalian brain (Klee, Crouch & Krinks, 1979).

If a Ca-dependent dephosphorylation is responsible for inactivation of the Ca current, then addition of a Ca-dependent phosphatase such as calcineurin should produce an increased rate of inactivation in the dialysed neurone. This prediction was tested with a mixture of calcineurin (40 μg protein ml^{-1}) together with calmodulin (10 μM) introduced into the dialysate after some run-down of inactivation had taken place in the dialysed cell. The introduction of calcineurin (always in conjunction with calmodulin) produced a marked increase in the rate of Ca current inactivation as well as a small reduction in peak amplitude of the Ca current (Fig. 4*A*; see also 7). As noted above, a reduction in current amplitude normally results in a slowing of inactivation (Chad *et al.* 1984), therefore, the increased rate of inactivation occurred in spite of, rather than because of, the small drop in peak amplitude of the current. The introduction of either calmodulin alone or dithiothreitol alone did not alter the rate of inactivation. Likewise, addition of a Ca-insensitive dephosphorylating enzyme, alkaline phosphatase (Sigma, 250 μg protein ml^{-1}) to the dialysate, had no detected effect on the rate of inactivation.

In the intact cell, the injection of the Ca^{2+}-chelating agent EGTA greatly reduces the rate of inactivation, and speeds the removal of the small residual inactivation that remains. This constitutes important evidence that the inactivation is mediated

by intracellular Ca^{2+} (Brehm & Eckert, 1978; Eckert & Tillotson, 1981; Plant *et al.* 1983). We therefore tested the effectiveness of calcineurin/calmodulin with EGTA (1 mM) in the dialysate. Addition of EGTA to the dialysate greatly reduced the rate of inactivation, so any inactivation produced by the subsequent addition of calcineurin should have been readily detected. However, with EGTA in the dialysate calcineurin produced no increase in inactivation (Fig. 4*B*). Thus, the effect of calcineurin on inactivation appears to derive from its Ca-dependent phosphatase activity.

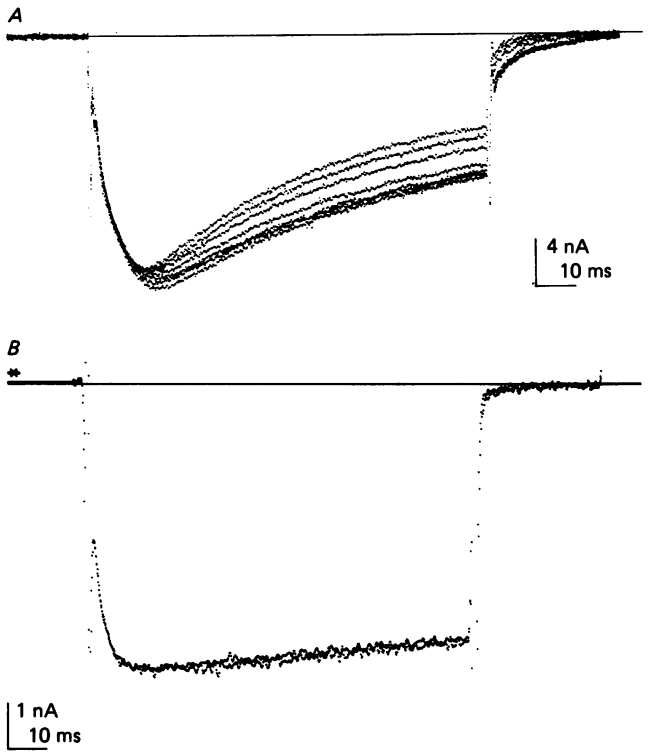

Fig. 4. Effect of internally applied phosphatases. *A*, addition of calcineurin (40 μg ml^{-1}) plus calmodulin (10 μM) to the dialysate caused an increase in rate of inactivation. Sweeps occurred at 120 s intervals during infusion of calcineurin. The largest current was recorded prior to introduction of calcineurin. The decline in peak current amplitude during this series was somewhat more rapid than the rate of loss of current prior to addition of the calcineurin. *B*, calcineurin effect is absent in the presence of EGTA. Two Ca currents are superimposed, one recorded before, and one 20 min following infusion of calmodulin/calcineurin in dialysate containing 1 mM-EGTA. Steps to +10 mV from a holding potential of −40 mV throughout.

Ionic dependence of enzymatically mediated inactivation

The rate of inactivation of current carried by the Ca channels depends on the species of ion carrying the current, the rate of inactivation being greatest when the current is carried by Ca^{2+}, somewhat less for Sr^{2+}, and least for Ba^{2+} (Brehm & Eckert, 1978; Tillotson, 1979; Eckert & Tillotson, 1981; Ashcroft & Stanfield, 1981, 1982). If the Ca-dependent inactivation exhibited by the intact cell and the

additional inactivation seen after addition of calcineurin to the dialysate in these experiments both arise from the phosphatase activity of calcineurin or a closely related calcineurin-like phosphatase, the inactivation and the phosphatase activity of enzyme would both be expected to exhibit similar ion-selectivity sequences. To test the ion selectivity of inactivation of the calcineurin-supplemented dialysed neurone, inward

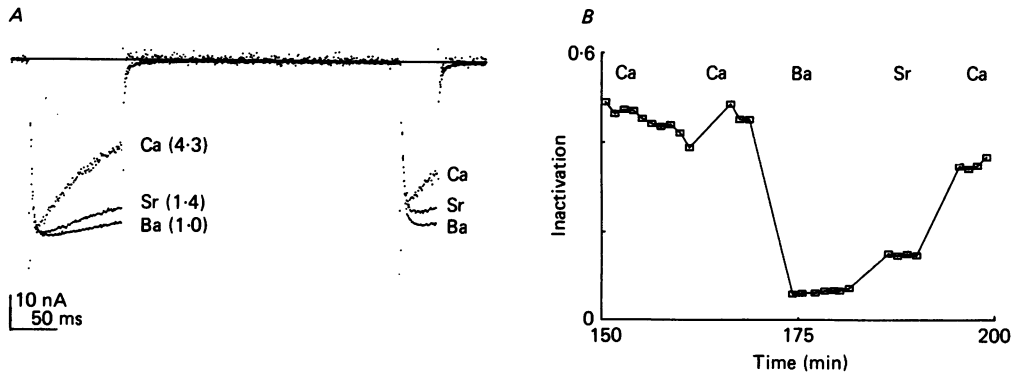

Fig. 5. Ionic dependence of inactivation in the dialysed neurone. Currents during double pulses (I and II) to +10 mV in 50 mM-Ca, Ba and Sr. *A*, currents displayed with pulse I peaks normalized to Ba using gain factors of Ca, 4·3; Sr, 1·4; and Ba, 1·0. *B*, inactivation index (see Methods) plotted against time in solutions of 50 mM-Ca, Ba or Sr. The inactivation sequence was Ca > Sr > Ba, the reverse of the sequence of peak amplitudes of the pulse I currents (shown in *A*).

currents were recorded during a double-pulse protocol in the presence of different external divalents (Fig. 5*A*). The strongest inactivation occurred when Ca^{2+} carried the current, and the weakest when Ba^{2+} carried the current (Fig. 5*B*). The differences in peak amplitude and in rate of inactivation exhibited by the three ionic currents appear to be causally unrelated, since the sequence for current strength, $Ba^{2+} > Sr^{2+} > Ca^{2+}$, is the inverse of the sequence for rate of inactivation, $Ca^{2+} > Sr^{2+} > Ba^{2+}$. For a given ion species, the rate of inactivation normally shows a positive relation to the amplitude of the current (Eckert & Chad, 1984). Furthermore, ion accumulation in the domain of a single channel (Chad & Eckert, 1984*b*) should be greater for Ba^{2+} than for Ca^{2+}, since Ba^{2+} carries the larger single-channel current (Lux & Nagy, 1981). Thus, if both Ca^{2+} and Ba^{2+} promote inactivation with equal potency, the rate of inactivation of the Ba current should have been greater than that of the Ca current. Finally, the selectivity sequence of divalent ion binding by calmodulin (Cheung, 1984), and for activation of calcineurin (C. Klee, personal communication) are both $Ca^{2+} > Sr^{2+} > Ba^{2+}$.

Substitution of Ba^{2+} for Ca^{2+} in the bath produces a shift of about −15 mV in the normalized current–voltage plot of the inward current peak attributable to diminished surface-charge screening (Fig. 6*A*). This shift is not sufficient cause for the slowing of inactivation seen when Ca^{2+} is replaced with Ba^{2+}, for the inactivation remained slower even after compensation for the −15 mV shift (Fig. 6*B*). The Ba current elicited at −5 mV showed virtually no inactivation during a 100 ms step, even though the Ba current was much larger than the Ca current elicited at +10 mV. The

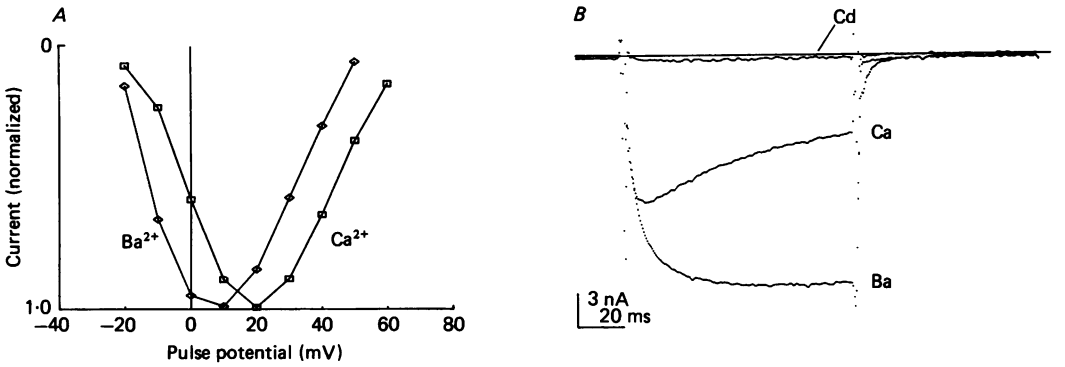

Fig. 6. Ionic dependence of inactivation is not due to surface charge effects. *A*, normalized current–voltage plots of inward current show an approximate 15 mV shift when external Ca^{2+} (50 mM) is replaced with Ba^{2+}. *B*, currents recorded with holding and stimulus potentials compensated to correct for surface charge effects. At −5 mV the Ba current was much larger than the Ca current at +10 mV but none the less showed virtually no inactivation during the voltage step. The addition of 0·1 mM-Cd to the external Ca solution eliminated the inward current.

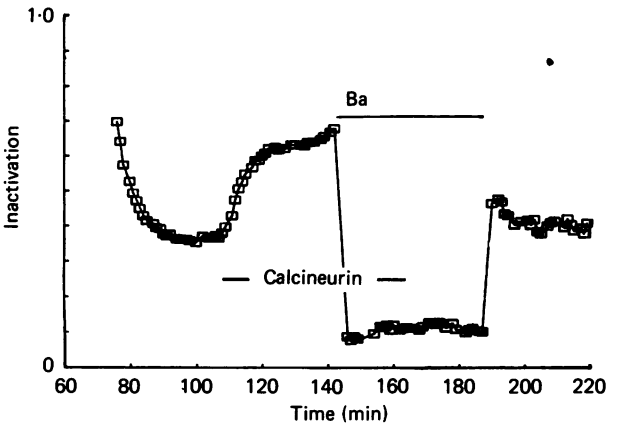

Fig. 7. Ionic dependence of calcineurin-enhanced inactivation. Degree of inactivation during a 100 ms step to +10 mV plotted against time. Dialysate contained catalytic subunit, dibutyryl cyclic AMP, Mg ATP and leupeptin. The inactivation index dropped during initial period shown because calcineurin plus calmodulin had been present and was removed at about 70 min. Addition of calcineurin plus calmodulin to the dialysate again at about 105 min caused a strong increase in the rate of inactivation. When external Ca^{2+} was subsequently replaced with equimolar Ba^{2+}, inactivation was reduced. Removal of calcineurin while Ba^{2+} remained in the bath had little effect, but upon restoration of external Ca^{2+} the inactivation partially recovered.

differences in kinetics cannot be attributed to the different voltages in this experiment, since Ca-dependent inactivation normally diminishes with increasing depolarization for a given current amplitude (Eckert & Ewald, 1983*b*; Chad *et al.* 1984).

If calcineurin introduced into the dialysed cell produces inactivation in a manner similar to inactivation in the intact cell, the increased rate of inactivation associated with calcineurin should be virtually eliminated when Ca^{2+} is replaced with Ba^{2+}. Fig. 7 shows a plot of the inactivation index plotted against time. In the control

solution, inactivation had fallen to a stable level. Addition of calcineurin and calmodulin to the dialysate was then followed by a return to a much higher rate of inactivation. Replacement of external Ca^{2+} with Ba^{2+} then produced a precipitous decrease in the rate of inactivation, again in spite of an increase in the amplitude of the current. The low rate of inactivation seen in Ba^{2+} was unchanged by subsequent removal of calcineurin from the dialysate, indicating that the calcineurin-mediated inactivation was virtually absent when Ba^{2+} carried the current. Inactivation subsequently increased to the earlier level after external Ba^{2+} was replaced by $Ca.^{+2}$

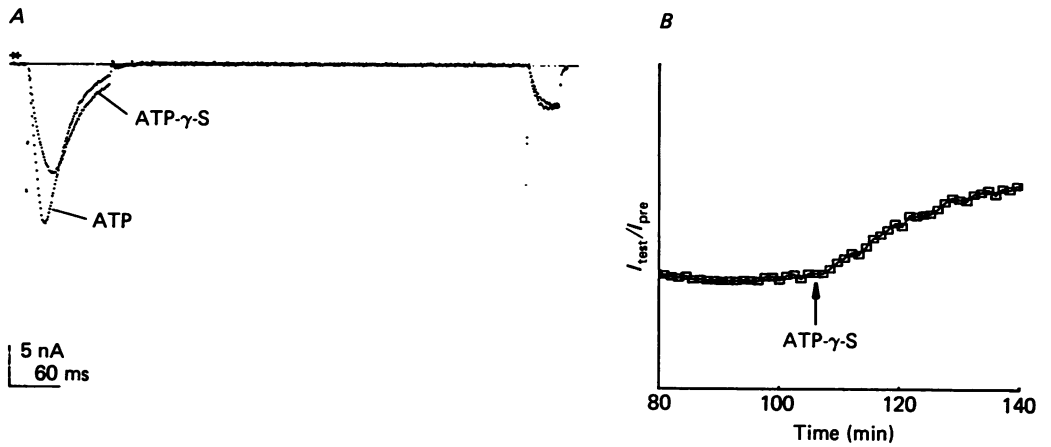

Fig. 8. Effect of replacing intracellular ATP with ATP-γ-S. *A*, currents elicited by double pulses to +10 mV, with 500 ms interval between pre-pulse and test pulse. Dialysate contained 20 μg catalytic subunit ml^{-1} throughout. The larger, rapidly inactivating current was recorded while the dialysate contained 7 mM-ATP. When this was replaced by ATP-γ-S (28 mM, with 8 mM-$MgCl_2$), there was a slowing of rise time and a reduction of amplitude along with a decrease in rate of inactivation. Note 'cross-over' of currents during pre-pulse due to a disproportionate slowing of inactivation with ATP-γ-S. *B*, ratio of test pulse current amplitude (I_{test}) to pre-pulse current amplitude (I_{pre}) plotted against time. Substitution of ATP-γ-S for ATP (arrow) was followed by a gradual increase in relative size of the test pulse, which is indicative of decreased inactivation.

Inactivation slowed by substitution of ATP-γ-S for ATP

If an increase in phosphatase activity increases Ca-dependent inactivation, it should be possible to reduce the rate of inactivation by interfering with the action of the phosphatase. The ATP analogue adenosine 5′-*O*(3-thiotriphosphate), ATP-γ-S (Eckstein, 1985), is useful for this purpose because the thiophosphate group donated by this nucleotide is resistant to enzymatic hydrolysis (Gratecos & Fischer, 1971), so that dephosphorylation proceeds more slowly than it does for the phosphate group donated by ATP. Thus, if Ca current inactivation depends upon a dephosphorylation, replacement of ATP in the dialysing solution with ATP-γ-S and consequent thiophosphorylation of the regulatory site should slow the rate of inactivation.

The predicted effect of replacing ATP with ATP-γ-S can be seen in Fig. 8*A*. The larger, rapidly inactivating pre-pulse and test pulse currents were recorded during perfusion with ATP and catalytic subunit. Replacement of ATP with ATP-γ-S produced three related effects: (i) the rate of rise and the peak amplitude of the Ca

current declined, (ii) the rate of inactivation during the depolarization became slower (Fig. 8*A*) and (iii) the ratio of test pulse peak current to pre-pulse peak current increased (Fig. 8*B*).

These effects of ATP-γ-S may be explained as follows: (i) it has been reported that cyclic-nucleotide-dependent kinases are significantly less efficient in rate of thiophosphorylation than in rate of phosphorylation (Palvimo, Linnala-Kankkunen & Mäenpää, 1985); thus, the reduction in amplitude of the Ca current may be related to slowed rate of thiophosphorylation of the regulatory site by ATP-γ-S. The slowed rate of Ca current activation seen in Fig. 8*A* suggests, however, that the thiophosphorylation of the channel may have changed its gating characteristics, and that this may be responsible for the smaller amplitude. An understanding of these effects requires further study. (ii) A reduction in Ca current amplitude normally contributes to a slowing of Ca-dependent inactivation, for inactivation develops in relation to the rate of Ca^{2+} entry and accumulation (Eckert & Tillotson, 1981; Plant & Standen, 1981; Chad *et al.* 1984), and thus the slowing of inactivation following introduction of ATP-γ-S might have resulted merely from the reduced current amplitude. However, the slowing of inactivation produced by ATP-γ-S significantly exceeded the slowing attributable to a drop in current amplitude. This is evidenced by the occurrence of a crossing of the decaying phase of the current recorded after introduction of ATP-γ-S over the decaying phase of the current before introduction of ATP-γ-S. Whereas a Ca current of diminished amplitude normally exhibits a diminished rate of inactivation, this alone never results in such crossing-over. Thus, a reduction in peak current amplitude is normally accompanied by a reduction in amplitude over the entire course of the current, consistent with the negative feedback principles implicit in current-dependent inactivation (Chad *et al.* 1984). This is true regardless of whether the size of the current is altered by depolarization to different voltages or by other means that change the current density. The progressive, disproportionate slowing of inactivation seen with ATP-γ-S is therefore indicative of an increased fraction of current having become resistant to inactivation. (iii) This interpretation is supported by the diminished difference in peak current amplitudes elicited by the pre-pulse and the test-pulse, which is another indication of diminished inactivation (Fig. 8*B*). The fraction of current resisting inactivation is presumably increased by the population of channels whose regulatory sites were thiophosphorylated by the ATP-γ-S.

DISCUSSION

Reversible and irreversible wash-out of Ca current

Our findings, obtained on dialysed cells without the use of intracellular Ca^{2+} chelators, provide evidence that the Ca current wash-out normally seen in dialysed cells arises from two separate processes, one reversible and one irreversible. The appearance of the irreversible component of wash-out can be suppressed by dialysis with leupeptin, an inhibitor of protease activity. This leaves a component of wash-out that can be virtually eliminated or reversed by measures promoting intracellular phosphorylation. Evidence discussed below indicates that Ca-dependent inactivation of the Ca current also results from a dephosphorylation. This, together with the

observation that both reversible wash-out and Ca current inactivation depend on an elevation of $[Ca^{2+}]_i$ suggests the reversible component of Ca current wash-out may be an expression of Ca current inactivation.

Since leupeptin inhibits protease activity, its ability to slow the irreversible process contributing to wash-out suggests that the irreversible component arises from proteolytic activity. Leupeptin alone produces little or no apparent change in the over-all rate of wash-out, perhaps because dephosphorylation occurs more rapidly than proteolysis, thereby determining the over-all rate of Ca current wash-out. The rate of irreversible wash-out can be seen when measures are taken to minimize the reversible wash-out (Fig. 3*A*).

Elevated levels of intracellular Ca^{2+} that reportedly occur during dialysis (Byerly & Moody, 1984) could contribute in two separate ways to the reversible and the irreversible components of wash-out. Since the irreversible component is suppressed by leupeptin, and since elevated $[Ca^{2+}]_i$ promotes irreversible wash-out, that component of wash-out would appear to arise from Ca-dependent proteolysis. The reversible component appears to arise from Ca-dependent dephosphorylation, since it can be minimized by the addition of phosphorylating agents and to some degree by internal EGTA, and is accelerated by addition of a Ca-dependent phosphatase. The Ca^{2+} sensitivity of both these processes presumably accounts for observations that lowering of $[Ca^{2+}]_i$ with EGTA slows Ca current wash-out during whole-cell patch clamping and cell dialysis (Fenwick *et al.* 1982; Byerly & Hagiwara, 1982; Forscher & Oxford, 1985).

Phosphorylation, in addition to counteracting development of the reversible component of wash-out, may also serve to slow development of the irreversible component, since phosphorylation has been found to protect some proteins against enzymatic hydrolysis (Holzer & Heinrich, 1980). Addition of ATP and catalytic subunit to the dialysis solution may also help slow wash-out by promoting the extrusion or sequestration of free Ca^{2+} from the cell interior, thereby counteracting an elevation of $[Ca^{2+}]_i$ (Yazejian & Byerly, 1984). A similar argument might be made for effects of phosphorylating and dephosphorylating agents on Ca channel inactivation without invoking an enzymatic mechanism of inactivation. However, changes in $[Ca^{2+}]_i$ regulation by these agents cannot adequately explain the present findings. Thus, the change in kinetics of inactivation produced by substitution of ATP-γ-S for ATP seem incompatible with a simple change in $[Ca^{2+}]_i$ or Ca^{2+} buffering, but suggest, instead, a direct effect of thiophosphorylation on channel properties. In addition, the actions of calcineurin seem incompatible with an indirect effect on the Ca channel through altered Ca^{2+} buffering. For example, the major effect of calcineurin develops *during* the flow of Ca current, with only minimal change in current peak amplitude. If calcineurin merely produced a steady-state rise in $[Ca^{2+}]_i$ it should cause a significant loss of current amplitude (i.e. wash-out). The dynamic character of the calcineurin effect is apparent in Fig. 7, in which it is seen that introduction of external Ba^{2+} in place of Ca^{2+} produced a sudden, full decrease in rate of inactivation. Finally, steady dialysis with EGTA is significantly less effective in preventing Ca current wash-out than dialysis with phosphorylating agents (Doroshenko *et al.* 1982, 1984; also our unpublished observations), which suggests an action other than mere lowering of $[Ca^{2+}]_i$.

Hypothesis for an enzymatic mechanism of inactivation

Addition of the Ca-dependent phosphatase calcineurin caused an increase in the rate of inactivation. The component of increased inactivation was Ca^{2+} dependent, and disappeared when Ba^{2+} carried the current, or the cell was perfused with EGTA. Addition of calcineurin to the dialysate might produce these actions through several means, for instance by (i) altering Ca^{2+} buffering, so as to affect the free Ca^{2+} levels experienced by the internal Ca^{2+} receptor mediating inactivation: (ii) interfering with an undetected outward current that affected the rate of inward current decay in spite of precautions taken to eliminate the problem; or (iii) increased phosphatase activity causing an increase in Ca-dependent inactivation of Ca channels. The first possibility seems unlikely, since calmodulin binds Ca^{2+} and should thus lower $[Ca^{2+}]_i$, which in turn should decrease Ca-dependent inactivation (Eckert & Chad, 1984). The second possibility is unlikely because K channels, including those known to be phosphorylation modulated (Deterre, Paupardin-Tritsch, Bockaert & Gerschenfeld, 1981; Siegelbaum *et al.* 1982; DePeyer, Cachelin, Levitan & Reuter, 1982; Shuster *et al.* 1985; Ewald, Williams & Levitan, 1985), were pharmacologically blocked as described above. Activation of the $I_{K(s)}$ channel exhibits virtually no voltage dependence (Siegelbaum *et al.* 1982; Shuster *et al.* 1985). In additiohn, dephosphorylation of the Ca-dependent K channels, assuming they were incompletely blocked by the TEA present, should produce a decrease in outward current, since phosphorylation up-modulates these channels in *Helix* (DePeyer *et al.* 1982), and would therefore produce an effect on net current opposite that of calcineurin.

The simplest explanation of the action of calcineurin is that it increases the rate of Ca-dependent inactivation through a Ca-dependent increase in rate of dephosphorylation. This, along with other modifications of the Ca current seen in response to dephosphorylating and phosphorylating agents (Figs. 4 and 7) and in response to thiophosphorylation (Fig. 8), suggests an enzymatic basis both for Ca-dependent inactivation of the Ca current and for the subsequent removal of the inactivation.

In the enzymatic hypothesis (Fig. 9), the Ca channel readily enters an activated state in response to membrane depolarization, provided an associated regulatory site is phosphorylated. Conversely, the Ca channel exhibits a reduced probability of activation (i.e. it is in an inactivated state) when the regulatory site is dephosphorylated. Prior to Ca^{2+} entry, the regulatory sites are presumed to be in a dynamic balance between phosphorylation and dephosphorylation through steady-state competition of kinase and phosphatase activities. During flow of Ca current, $[Ca^{2+}]_i$ is postulated to rise in microscopic domains centred on active Ca channels (Chad & Eckert, 1984*b*). We propose that these local elevations in $[Ca^{2+}]_i$ lead to a local increase in Ca-dependent phosphatase activity and hence to an increased rate of dephosphorylation. Accordingly, the Ca-dependent dephosphorylation of regulatory sites associated with Ca channels is responsible for the Ca-dependent inactivation of the Ca^{2+} conductance. Following repolarization and the deactivation of Ca channels, $[Ca^{2+}]_i$ declines, hence phosphatase activity declines, and rephosphorylation by protein kinase of the dephosphorylated regulatory sites proceeds. The decline of phosphatase activity and the rephosphorylation are reponsible, according to this view, for the time-dependent removal of inactivation following repolarization and cessation of Ca^{2+} entry.

The ability of a particular mammalian-derived phosphatase (i.e. calcineurin) or kinase (i.e. catalytic subunit of cyclic-AMP-dependent protein kinase) to catalyse the reactions promoting inactivation and removal of inactivation is suggestive only, and we must ask if related forms of these enzymes are in fact present in molluscan neurones. In this regard, soluble cyclic-AMP-dependent protein kinases have been identified in *Aplysia* and *Helix* (Bandle & Levitan, 1977; Novak-Hofer, Lemof, Villemain & Levitan, 1985). Physiological regulation of Ca channels in molluscan neurones may none the less normally be carried out by presently unidentified endogenous kinases. Calcineurin-like immunoreactivity has also been found in *Aplysia* neurones (Saitoh & Schwartz, 1983; K. Baimbridge, unpublished).

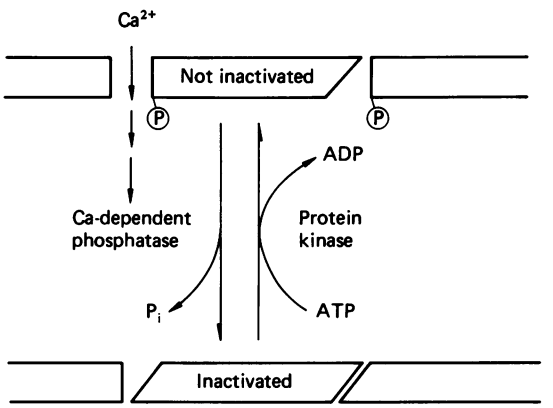

Fig. 9. Hypothesis for enzymatic Ca-dependent inactivation and removal of inactivation. Top, Ca channel opens in response to depolarization if the regulatory site is phosphorylated. The subsequent rise in $[Ca^{2+}]_i$ activates a Ca–calmodulin-dependent phosphatase, which leads to an increased rate of dephosphorylation. Bottom, dephosphorylation leaves the channel in a state of diminished probability of activation (i.e. inactivated). Rephosphorylation returns the channel to a 'not inactivated' state from which it again can open freely in response to depolarization. As an alternative, the dephosphorylated state may exhibit altered kinetics resulting in a reduced probability of the channel being open in response to depolarization.

Recent work on isolation of Ca channels from the transverse tubules of skeletal muscle, based on dihydropyridine binding (Curtis & Catterall, 1984, 1985), has demonstrated the presence of a multi-subunit complex in which one subunit is particularly sensitive to proteolysis, and one or more subunits are substrates for phosphorylation by cyclic-AMP-dependent protein kinase. These observations raise the possibility that phosphorylation inferred from our experiments may occur directly on the channel proteins themselves, and is consistent with the inferred sensitivity of the Ca current to proteolytic activity in dialysed neurones.

Of the three types of Ca channels observed in chick dorsal root ganglion neurones (Nowycky *et al.* 1985*a*), the slow 'L' channel remains active under conditions similar to those used in the present experiments. This channel exhibits different modes of gating activity (cf. Hess, Lansman & Tsien, 1984) that can be affected by the binding of dihydropyridines (Nowycky, Fox & Tsien, 1985*a*). By analogy, dephosphorylation of a regulatory site associated with the Ca channel in snail neurones may produce a change in state of the channel, such as a reduced probability of opening or a shorter mean duration of opening in response to depolarization.

Relation of the enzymatic model to the binding site model of inactivation

At first consideration, the enzymatic model presented here and the binding site model proposed earlier (Standen & Stanfield, 1982; Chad *et al.* 1983, 1984) appear to be contradictory, for the latter in its simplest form has Ca^{2+} binding to a regulatory site, producing inactivation of channels with 1 : 1 stoicheiometry. The kinetics of activation of calcineurin by Ca^{2+} are evidently complex, for calcineurin has two subunits, one of which binds calmodulin and also binds four atoms of Ca with an apparent dissociation constant (K_d) of less than 10^{-6} M (Klee *et al.* 1979). Calmodulin itself binds four Ca atoms at two pairs of sites with K_ds of 10^{-5} M and 10^{-6} M (Klee, Crouch & Richman, 1980). However, if intracellular free Ca^{2+} near the channels rises into the micromolar range, the kinetics of phosphatase activity will be dominated by Ca^{2+} binding to the lower-affinity sites of the calmodulin, which would give the appearance of a simple 1 : 1 stoicheiometry. Modelling studies indeed suggest that in the vicinity of the active channel the activity of Ca^{2+} rises into the micromolar range or above (Chad & Eckert, 1984*b*). The naturally occurring Ca-dependent phosphatase must be relatively immobile, for inactivation shows little decline, if any, as compared to the wash-out of the Ca current. Thus, the phosphatase may be bound, perhaps to the membrane, in the vicinity of the site that regulates the inactivation of the Ca channel. Indeed, biochemical evidence suggests that calcineurin is bound to the cell membrane via an NH_2-terminal myristyl group (Aitken, Chen, Santikarn, Williams, Calder, Smith & Klee, 1982).

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