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Mechanism of Ca^{2+}-dependent Inactivation of L-type Ca^{2+} Channels in GH_3 Cells: Direct Evidence Against Dephosphorylation by Calcineurin

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Abstract. Dephosphorylation of Ca^{2+} channels by the Ca^{2+}-activated phosphatase 2B (calcineurin) has been previously suggested as a mechanism of Ca^{2+}-dependent inactivation of Ca^{2+} current in rat pituitary tumor (GH_3) cells. Although recent evidence favors an inactivation mechanism involving direct binding of Ca^{2+} to the channel protein, the alternative ''calcineurin hypothesis'' has not been critically tested using the specific calcineurin inhibitors cyclosporine A (CsA) or FK506 in GH_3 cells. To determine if calcineurin plays a part in the voltage- and/or Ca^{2+}-dependent components of dihydropyridine-sensitive Ca^{2+} current decay, we rapidly altered the intracellular Ca^{2+} buffering capacity of GH_3 cells by flash photolysis of DM-nitrophen, a high affinity Ca^{2+} chelator. Flash photolysis induced a highly reproducible increase in the extent of Ca^{2+} current inactivation in a two-pulse voltage protocol with Ca^{2+} as the charge carrier, but had no effect when Ba^{2+} was substituted for Ca^{2+}. Despite confirmation of the abundance of calcineurin in the GH_3 cells by biochemical assays, acute application of CsA or FK506 after photolysis had no effect on Ca^{2+}-dependent inactivation of Ca^{2+} current, even when excess cyclophilin or FK binding protein were included in the internal solution. Prolonged preincubation of the cells with FK506 or CsA did not inhibit Ca^{2+}-dependent inactivation. Similarly, blocking calmodulin activation with calmidazolium or blocking calcineurin with fenvalerate did not influence the extent of Ca^{2+}-dependent inactivation after photolysis. The results provide strong evidence against Ca^{2+}-dependent dephosphorylation as the mechanism of Ca^{2+} current inactivation in GH_3 cells, but support the alternative idea that Ca^{2+}-dependent inactivation reflects a direct effect of intracellular Ca^{2+} on channel gating.

Key words: GH_3 — Calcium-dependent inactivation — Cyclosporine — FK506 — Calcineurin — Phosphatase — Calcium channel

Introduction

Ca^{2+} entry into the cell through surface membrane Ca^{2+} channels plays a pivotal role in a diverse array of cellular functions including neuroendocrine secretion (Scherubl et al., 1993, 1994; Codignola et al., 1993; Hirning et al., 1988), synaptic plasticity (Stuart & Sakmann, 1994), excitation-contraction coupling (Lederer et al., 1990; Balke & Wier, 1991; Backx et al., 1993), and gene expression (Ahlijanian, Westenbroek & Catterall, 1990; Sheng et al., 1990; Murphy, Worley & Baraban, 1991; Misra et al., 1994). In addition to essential gating mechanisms (e.g., voltage- or ligand-dependent gating), multiple strategies have evolved by which cells can modulate this fundamental process. Examples include direct G-protein-mediated activation or inhibition of Ca^{2+} channels (Rosenthal et al., 1990), phosphorylation by protein kinases (Bean et al., 1984; McDonald et al., 1994), dephosphorylation by phosphatases (Hescheler et al., 1987; Frace & Hartzell, 1993; Ono & Fozzard, 1993), and Ca^{2+}-dependent inactivation (Brehm, Eckert & Tillotson, 1980; Lee, Marban & Tsien, 1985; Yue, Backx & Imredy, 1990; Imredy & Yue, 1992). Ca^{2+}-dependent inactivation, which refers to the reduction in inward Ca^{2+} current attributable to the rise in intracellular Ca^{2+} initiated by channel opening, is a negative feedback mecha-

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nism limiting Ca^{2+} entry during stimulation and is common to all L-type (dihydropyridine-sensitive) Ca^{2+} channels. While it is clear that elevated intracellular Ca^{2+} is required, two hypotheses have been put forth to account for Ca^{2+}-dependent inactivation of L-type Ca^{2+} currents in a variety of cells. The first, and more widely accepted, model involves direct inactivation by Ca^{2+}, i.e., calcium ions pass through the channel and bind to a receptor site somewhere on or near the channel causing closure of an inactivation ''gate.'' This effect would be accentuated by the very high flux of Ca^{2+} through the channel resulting in a high local concentration of Ca^{2+} at the inner mouth of the pore (Gutnick, Lux & Swandulla, 1989; Stern, 1992; Imredy & Yue, 1992). Alternatively, elevated intracellular Ca^{2+} could act via Ca^{2+}-regulated kinases or phosphatases leading to a change in the phosphorylation state of the channels. Based on studies of whole cell Ca^{2+} currents in Helix and Aplysia neurons, Chad and Eckert (1986) proposed that Ca^{2+} channel phosphorylation by cAMP-dependent protein kinase was absolutely required for the activation of high threshold (L-type) voltage-gated Ca^{2+} channels. Furthermore, they suggested that Ca^{2+} permeation through the channel could activate the Ca^{2+}/calmodulin-dependent phosphatase, calcineurin (phosphatase 2B), which in turn dephosphorylates and thereby inactivates the channel. A similar role for calcineurin-mediated dephosphorylation has been proposed as the mechanism for Ca^{2+}-dependent inactivation in rat pituitary adenoma (GH_3) cells (Kalman et al., 1988).

While the role of Ca^{2+}-regulated phosphatase action as a mechanism for Ca^{2+}-dependent inactivation of Ca^{2+} channels in cardiac (Frace & Hartzell, 1993; Imredy & Yue, 1994) and neuronal cells has been contested (Fryer & Zucker, 1993), in GH_3 cells, the mechanism of Ca^{2+} channel inactivation has not been reexamined with newly available calcineurin-selective inhibitors. Using rapid photolytic destruction of an intracellular Ca^{2+} buffer (DM-nitrophen) for probing the extent of Ca^{2+}-dependent inactivation in GH_3 cells, we tested the effects of interrupting Ca^{2+}-mediated dephosphorylation with Cyclosporine A or FK506, immunosuppressant drugs which are potent and specific inhibitors of calcineurin. The results of these experiments, as well as experiments using other inhibitors of Ca^{2+}-mediated dephosphorylation pathways (calmidazolium and fenvalerate), provide the strongest evidence to date against the hypothesis that inactivation of L-type Ca^{2+} currents is mediated by calcineurin.

Materials and Methods

Calcineurin Inhibitors

CsA was provided by Sandoz Pharmaceuticals and FK506 by Fujisawa. CsA and FK506 were solubilized in γ-cyclodextrin (RBI, Natick, MA; final γ-cyclodextrin concentration was 2.25% w/v; (Pitha et al., 1988)). CsA and FK506, as their immunophilin complexes, are specific high affinity inhibitors of calcineurin (Liu et al., 1991; Swanson et al., 1992). Recombinant human cyclophilin B was purified from an *E. coli* strain overexpressing the protein as described (Price et al., 1991). Recombinant human FK506-binding protein (FKBP) was purified to homogeneity from an *E. coli* strain overexpressing the protein by anion exchange and gel filtration chromatography (Harding et al., 1989). Because of CsA and FK506 are highly lipophilic and thus membrane permeant, these drugs were administered either in the bath solution during the experiment or they were added to the culture medium for preincubation prior to electrophysiologic study. In some experiments, cells were dialyzed with purified cyclophilin B or FK-BP-12, added to the pipette solution.

Calmidazolium chloride (RBI, Natick, MA), a potent inhibitor of calmodulin (Gietzen, 1983; Mazzei et al., 1984), was solubilized in DMSO at 5 mM and then diluted to 0.1 mM with buffer. Fenvalerate (LC Laboratories, Woburn, MA) was solubilized in DMSO to a final concentration of 0.01 mM. Fenvalerate has been shown to inhibit 70% of calcineurin phosphatase activity in vitro at a concentration of 10–100 μM (Enan & Matsumura, 1992).

Cell Culture

GH_3 cells, a mammalian cell line isolated from rat pituitary tumor (Tashjian, Jr. et al., 1968), were maintained in RPMI supplemented with 15% horse serum, 2.5% fetal calf serum, 1% L-glutamine, and 1% penicillin/streptomycin. Cultures were fed every other day.

Electrophysiology

The whole-cell configuration of the patch clamp technique (Sakmann & Neher, 1995) was used to record macroscopic L-type Ca^{2+} currents. Tissue culture dishes (35 mm) were transferred to the stage of an inverted microscope and superfused with external solution at a rate of 1–2 mL/min. The external bath solution had the following composition (in mM): $CaCl_2$ 25, tetraethylammonium (TEA) chloride 106, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) 10, CsCl 5, glucose 10, $MgCl_2$ 1 and pH 7.4 (with TEA-OH) at room temperature. In some experiments, 25 mM $CaCl_2$ was replaced with 25 mM $BaCl_2$. Patch-clamp electrodes (3–6 MΩ) were filled with an internal solution of the following composition (in mM): CsCl 80, HEPES 50, TEA 20, MgATP 5, DM-nitrophen (sodium salt) 5 and pH 7.2 with CsOH.

All experiments were performed at room temperature (22–23°C) using an Axopatch 200A patch-clamp amplifier (Axon Instruments, Foster City, CA), interfaced to a personal computer. Voltage commands and data collection were controlled using custom-written software. For whole-cell current recordings, the cell capacitance and series resistance were compensated and measured during 10 mV depolarizing pulses from a holding potential of −40 mV. In general, 60–80% of the series resistance was compensated. Whole-cell Ca^{2+} current records were filtered at 2 kHz and sampled at 10 kHz. Currents were leak-subtracted by a P/4 method (Sakmann & Neher, 1995). Data were stored in the computer for later analysis using custom-written software (IonView, B. O'Rourke).

Ca^{2+}-dependent inactivation of L-type Ca^{2+} currents was assessed using a standard two-pulse voltage protocol, as previously described (Brehm et al., 1980; Kalman et al., 1988; Lee et al., 1985). This protocol permits one to assess the availability of Ca^{2+} channels to open in response to a fixed voltage step (test pulse) from −40 to +20 mV following a conditioning pulse (or prepulse) of varying amplitude. A brief repolarizing interpulse permits channels in the open state to

return to the closed state without allowing inactivated channels to recover. The fraction of current activated in the test pulse therefore depends on events which occurred during the conditioning pulse. If inactivation was purely voltage-dependent, one would expect a progressive decrease in the amplitude of the test pulse current as the prepulse potential becomes more depolarized. However, with Ca^{2+} as the charge carrier, it is generally found that the current-voltage relation for the test pulse is U-shaped and depends on the extent of prepulse Ca^{2+} entry, thus the protocol examines the extent of Ca^{2+}-dependent inactivation. Specifically, the two-pulse protocol consisted of a 180 msec prepulse from a holding potential of −40 mV to a range of voltages from −30 to +60 mV in 10 mV increments. The prepulse was followed by a brief (10 msec) repolarization and then a 40 msec test pulse to +20 mV, which elicited maximal inward Ca^{2+} current. Previous work has indicated two classes of voltage-gated Ca^{2+} channels in GH_3 cells (Kalman et al., 1988). The first has a low threshold for activation (midpoint ~ −40 mV), undergoes voltage-dependent inactivation, and is insensitive to dihydropyridines. The other class has a high threshold for activation (midpoint ~ −20 mV), undergoes primarily Ca^{2+}-dependent inactivation and is sensitive to dihydropyridines (L-type). To study Ca^{2+}-dependent inactivation of L-type Ca^{2+} currents, a holding potential of −40 mV was used, which effectively inactivated the low-voltage activated currents.

Flash Photolysis

It has previously been shown that the Ca^{2+}-dependent component of inactivation can be largely eliminated by intracellular equilibration with Ca^{2+} buffers (Kohr & Mody, 1991; Gutnick et al., 1989; Plant, Standen & Ward, 1983; Fryer & Zucker, 1993). To substantially reduce the cell-to-cell variability in the extent of Ca^{2+}-dependent inactivation of whole-cell Ca^{2+} currents, we used the high-affinity photosensitive Ca^{2+} chelator DM-nitrophen (K_D, Ca^{2+} ≈ 5 nM; (Kaplan & Ellis-Davies, 1988)) to begin each experiment with the cells in the same functional state (i.e., with Ca^{2+}-dependent processes at a minimum). Flash photolysis was then used to destroy the DM-nitrophen so as to rapidly decrease intracellular Ca^{2+} buffering capacity (photolyzed DM-nitrophen has a very low affinity for Ca^{2+}; K_D,Ca^{2+} ≈ 3 mM (Kaplan & Ellis-Davies, 1988)), thus producing a rapid increase in Ca^{2+}-dependent inactivation. DM-nitrophen was introduced into GH_3 cells by adding 5 mM DM-nitrophen to the pipette solution. DM-nitrophen also chelates Mg^{2+}; however, little change in intracellular Mg^{2+} would be expected under these conditions since a large excess of Mg^{2+} was present (5 mM MgATP) and the binding affinity for Mg^{2+} is ~500-fold lower than for Ca^{2+} ($K_{D,Mg^{2+}}$ ≈ 2.5 μM; (Kaplan & Ellis-Davies, 1988). The calculated increase in free cytosolic Mg^{2+} from 3.3 μM to 288 μM due to complete photolysis of DM-nitrophen under our experimental conditions is well below that reported to exert a physiological effect on whole-cell Ca^{2+} currents (in the 5–10 mM range) (Hartzell & White, 1989). Flashes of ultraviolet light were produced by focusing the light output of a xenon arc flash lamp driven by a strobex power supply (Model 238B Chadwick-Helmuth, CA) onto the cell using an ellipsoidal reflector, two dichroic mirrors, and the objective lens, as previously described (Backx et al., 1991).

Phosphatase Assay

To confirm that calcineurin was present and could be inhibited by the compounds employed in the electrophysiological experiments, assays of phosphatase activity in the GH_3 cells were carried out by measuring the rate of dephosphorylation of a synthetic phosphopeptide substrate. A 19-residue polypeptide (DLDVPIPGRFDRRVSVAAE; R_{II} peptide) corresponding to the sequence of bovine cardiac cAMP-dependent kinase type II regulatory subunit was synthesized (Protein& Peptide Core facility, Mayo Clinic) and purified by reverse phase HPLC chromatography. R_{II} peptide was phosphorylated with [^{32}P]-ATP (Amersham, Arlington Heights, IL) by the catalytic subunit of cAMP-dependent protein kinase (Sigma, St. Louis, MO), as previously described (Hubbard & Klee, 1991). Bovine calcineurin was purified to homogeneity as described previously (Klee et al., 1983).

Calcineurin was assayed using [^{32}P]-R_{II} peptide as substrate according to standard methods (Hubbard & Klee, 1991; Swanson et al., 1992). Briefly, assays were carried out in a buffer containing 20 mM TrisCl (pH 7.5), 100 M KCl, 6 mM $MgCl_2$, 0.05 mM dithiothreitol, 0.1 mg/mL bovine serum albumin, 0.1 mM $CaCl_2$, 0.1 μM calmodulin, and 0.5 μM okadaic acid where necessary (*see below*). GH_3 cell lysates were preincubated with buffer for 10 min at 30°C and reactions were initiated upon addition of [^{32}P]-R_{II} peptide. After 10 min, the reactions were terminated by adding 0.5 mL of 0.1 M potassium phosphate buffer (pH 7.0) containing 5% trichloroacetic acid. Unreacted labeled peptide was adsorbed to a Dowex cation-exchange resin and the released [^{32}P]-orthophosphate quantified by scintillation counting of the supernatant. Since the extracts contain the serine/threonine protein phosphatases 1, 2A, 2B (calcineurin), and 2C (PP1, PP2A, PP2B, PP2C, respectively), the fraction of total phosphatase activity due to calcineurin activity was determined as described (Rusnak et al., 1996) by first adding okadaic acid to inhibit PP1 and PP2A (Bialojan & Takai, 1988) and then blocking calcineurin with an immunosuppressant drug complex (CsA-cyclophilin or FK506-FKBP; 1.0 μM) leaving only PP2C still active. The fraction of total phosphatase activity due to calcineurin was then calculated.

Results

CsA- and FK-506-sensitive Calcineurin Phosphatase Activity in GH_3 Cell Lysates

Although Western blot analysis has shown that calcineurin is abundant in GH_3 cells (Farber, Wilson & Wolff, 1987), we sought to confirm that calcineurin activity was present in our cultures and that the immunosupressant drugs could effectively inhibit calcineurin. In the presence of Ca^{2+} and calmodulin, 38.8 ± 2.9% (mean ± SD of 4 to 8 assays) of total phosphatase activity in GH_3 cell lysates was inhibited by 1 μM CsA + 1 μM cyclophilin B and 58.5 ± 2.9% of the total activity was inhibited by 1 μM FK506 + 1 μM FKBP-12 (Fig. 1). Almost all of the remaining phosphatase activity was inhibited by okadaic acid (0.5 μM), which selectively inhibits serine-threonine phosphatases 1 and 2A at this concentration (Bialojan & Takai, 1988). Thus, 40–60% of the total phosphatase activity in GH_3 cells reflects type 2B (calcineurin).

Ca^{2+} Dependent Inactivation of L-type Ca^{2+} Currents

With Ca^{2+} as the charge carrier, the extent of inactivation of the test pulse current during the two pulse voltage protocol showed a U-shaped voltage dependence characteristic of L-type Ca^{2+} currents (Figs. 2–4). The iden-

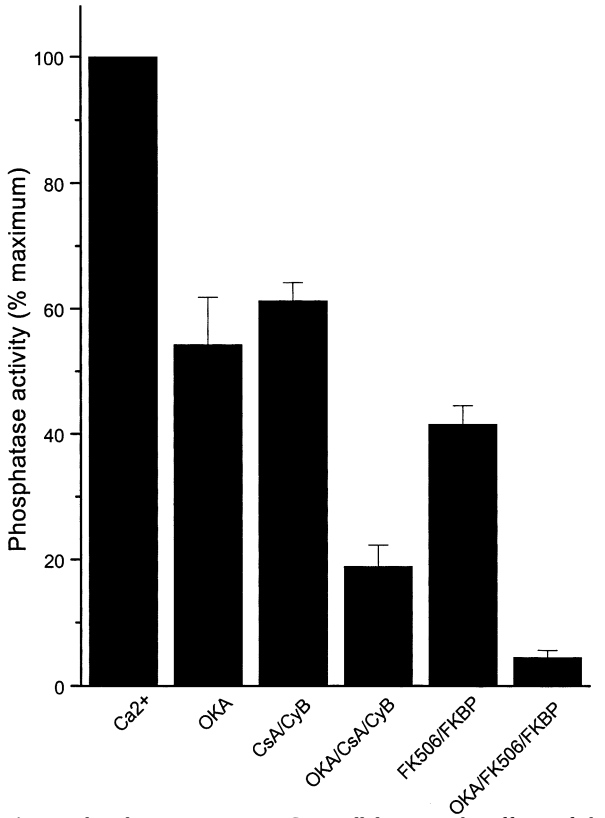

Fig. 1. Phosphatase activity in GH_3 cell lysates. The effects of the phosphatase inhibitors are expressed as percent of maximal phosphatase activity stimulated by 0.1 mM $CaCl_2$, and 0.1 μM calmodulin (mean ±SD; $n = 4$–8). Calcineurin (CsA- or FK506-sensitive activity) accounted for 40–60% of the total phosphatase activity, while phosphatases 1 and 2 A (OKA-sensitive activity) accounted for most of the remaining 50%. OKA, okadaic acid (0.5 μM); CsA, cyclosporine A (1.0 μM); CyB, cyclophilin B (1.0 μM); FKBO, FK binding protein-12 (1.0 μM).

tification of the Ca^{2+} currents as L-type was also confirmed by their sensitivity to the dihydropyridine antagonist nitrendipine (1 μM; *not shown*). Figure 2 shows that maximal inactivation of the test current was observed with a prepulse potential of +20 mV (peak Ca^{2+} entry), and that less inactivation was observed at membrane potentials equal to or greater than +30 mV, in accord with the decrease in Ca^{2+} entry over this range of potentials (Fig. 2). Photolysis of DM-nitrophen decreased the prepulse current (probably through a direct blocking effect on the channels (Morad et al., 1988)) and caused a marked increase in the degree of inactivation of the test pulse Ca^{2+} current. This increase in the extent of inactivation, even though Ca^{2+} entry was reduced at all prepulse potentials, reflects the strong influence of Ca^{2+} buffering on Ca^{2+}-dependent channel inactivation (Fig. 2). In confirmation of the Ca^{2+}-specific nature of the inactivation mechanism, photolysis of DM-nitrophen had no effect on prepulse or test pulse currents when the same voltage protocol was repeated with Ba^{2+} as the charge carrier (Fig. 2).

Inhibition of Ca^{2+}/Calmodulin-activated Pathways

If calcineurin is required for the Ca^{2+}-dependent component of channel inactivation, the first step in the process would be the binding and activation of calmodulin by Ca^{2+}. Therefore, we tested whether the calmodulin inhibitor calmidazolium (10 μM) could reduce Ca^{2+} current inactivation after photolysis of the DM-nitrophen. In the absence of calmidazolium, the maximum percentage inactivation of the test current was 20 ± 4% at baseline and 44 ± 7% after photolysis (Fig. 3). The addition of calmidazolium to the bath after photolysis had no effect on the maximum percent inactivation, with the average being 49 ± 6% ($n = 3$).

While blocking all Ca^{2+}/calmodulin-dependent pathways had no effect on Ca^{2+}-dependent inactivation, we sought further confirmation that calcineurin was not involved in the mechanism by using specific inhibitors of the phosphatase. Despite the abundance of calcineurin phosphatase activity present in the GH_3 cells demonstrated above, immunosuppressant inhibition of calcineurin had no effect on the augmentation of Ca^{2+}-dependent inactivation induced by photolysis of DM-nitrophen (Figs. 4 and 5). Ten μM CsA was incapable of altering the extent of inactivation when added to the bath after photolysis, even with the extra precaution of including cyclophilin B (5 μM) in our intracellular solution (% maximal inactivation: 16 ± 6% at baseline, 35 ± 7% after photolysis, 31 ± 6% in CsA; Fig. 4). Furthermore, the augmentation of Ca^{2+}-dependent inactivation after flash photolysis was not prevented by preincubation of cells with either FK506 (1 hr pretreatment with 1.0 μM FK506 in the culture medium plus intracellular dialysis with 5 μM FK binding protein) or CsA (1.0 μM CsA in culture medium for 24 hr) (Fig. 5). In control cells ($n = 9$), peak inactivation increased from 43 ± 7% to 60 ± 9% ($P < 0.05$) after photolysis of DM-nitrophen, whereas in cells preincubated with FK506 ($n = 4$ cells) inactivation increased from 46 ± 11% to 58 ± 10% ($P < 0.05$) and in cells preincubated with CsA ($n = 6$ cells), inactivation increased from 37 ± 6% to 54 ± 10% ($P < 0.05$).

Further evidence that calcineurin does not mediate Ca^{2+}-dependent inactivation was obtained by examining the effects of fenvalerate, a pyrethroid insecticide which inhibits calcineurin through an immunophilin-independent mechanism (Enan & Matsumura, 1992). Preincubation of cells with 20 μM fenvalerate did not prevent the augmentation of Ca^{2+}-dependent inactivation induced by flash photolysis of DM-nitrophen; peak inactivation increased from 46 ± 10% to 67 ± 7% ($P < 0.05$; $n = 5$) in these experiments.

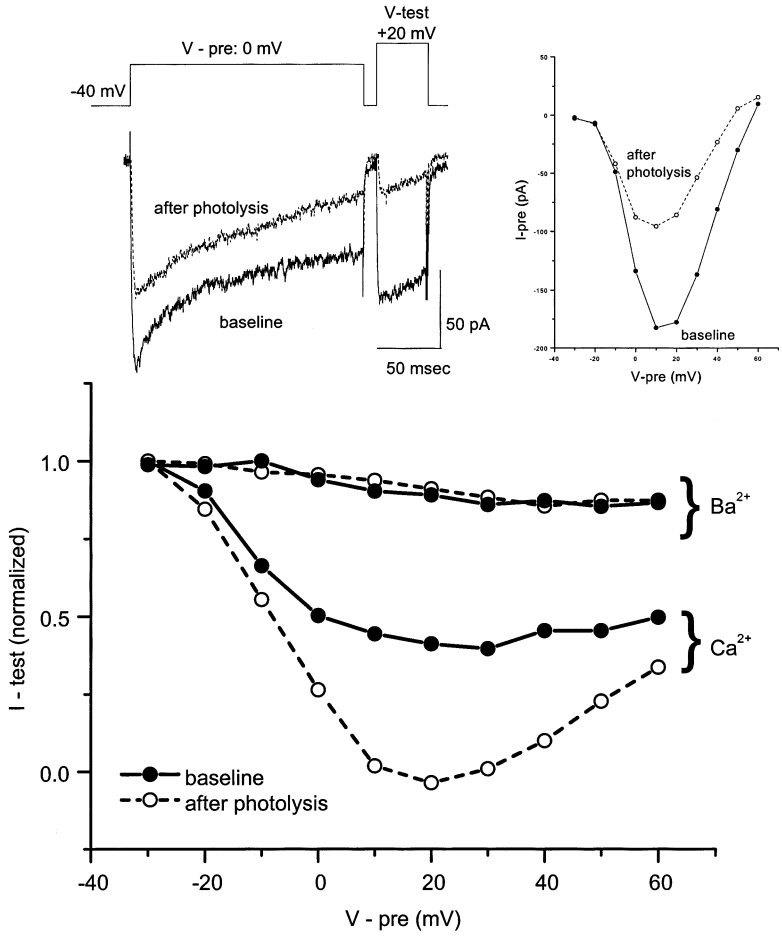

Fig. 2. Augmentation of Ca^{2+}-dependent inactivation of whole cell Ca^{2+} currents by flash photolysis of DM-nitrophen. Top left, two-pulse voltage protocol and corresponding current traces from a representative cell with 25 mM Ca^{2+} as the charge carrier. Top right, current-voltage relationship for Ca^{2+} currents before and after photolysis of DM-nitrophen. Despite reduced peak Ca^{2+} influx after photolysis, Ca^{2+}-dependent inactivation was enhanced as a result of reduced Ca^{2+} buffer capacity in the cell, as evidenced by the pronounced decrease in normalized test-pulse currents shown in the lower panel. Photolysis had no effect on the inactivation of Ba^{2+} currents, which undergo strictly voltage-dependent inactivation.

Discussion

The important role of Ca^{2+} entry in triggering a wide variety of cellular processes has prompted much investigation into the mechanisms by which Ca^{2+} channels are regulated. Phosphorylation of L-type Ca^{2+} channels by cyclic-AMP-dependent protein kinase is perhaps the best described of these mechanisms (Tsien et al., 1986; Kameyama et al.). Direct phosphorylation of the channel protein itself has been demonstrated (Nunoki, Florio & Catterall, 1989; Hosey et al., 1989) and the functional effects of phosphorylation by protein kinase A include both an increase in the number of channels available for opening during a depolarizing stimulus (Tsien et al., 1986) and prolongation of the channel open time in a gating pattern referred to as ''mode 2'' (Yue, Herzig & Marban, 1990). Previous investigators have argued that phosphorylation of the Ca^{2+} channel is an absolute requirement for channel opening (Armstrong & Eckert, 1987), while more recent single-channel data suggest that dephosphorylated channels can open infrequently albeit briefly (Imredy & Yue, 1994). A second feature inherent to L-type Ca^{2+} channels limiting Ca^{2+} entry into the cell is the decrease in Ca^{2+} current with time (inactivation) during a maintained depolarizing stimulus. Ca^{2+} channel inactivation involves two mechanisms; a strictly voltage-dependent process and a Ca^{2+}-dependent process (Lee et al., 1985; Hadley & Lederer, 1991).

Because the gating pattern of L-type Ca^{2+} channels greatly depends on the phosphorylation state of the channel, it is natural to suppose that Ca^{2+} current inactivation may involve a dephosphorylation process. The abundance of the Ca^{2+}-activated phosphatase calcineurin in neuronal cells made this idea particularly attractive. Support for this hypothesis was obtained in molluscan neurons by Chad and Eckert (1986), and was reinforced by later studies in GH_3 cells (Kalman et al., 1988). In short, the evidence consisted of two findings: (i) a decrease in the rate of Ca^{2+} current inactivation by cAMP and (ii) acceleration of the inactivation rate by constitutively active calcineurin applied intracellularly. At the time of these proposals, conclusive evidence of the role of calcineurin in the normal process of Ca^{2+}-dependent inactivation could not be obtained for lack of a specific inhibitor of calcineurin (Armstrong, 1989).

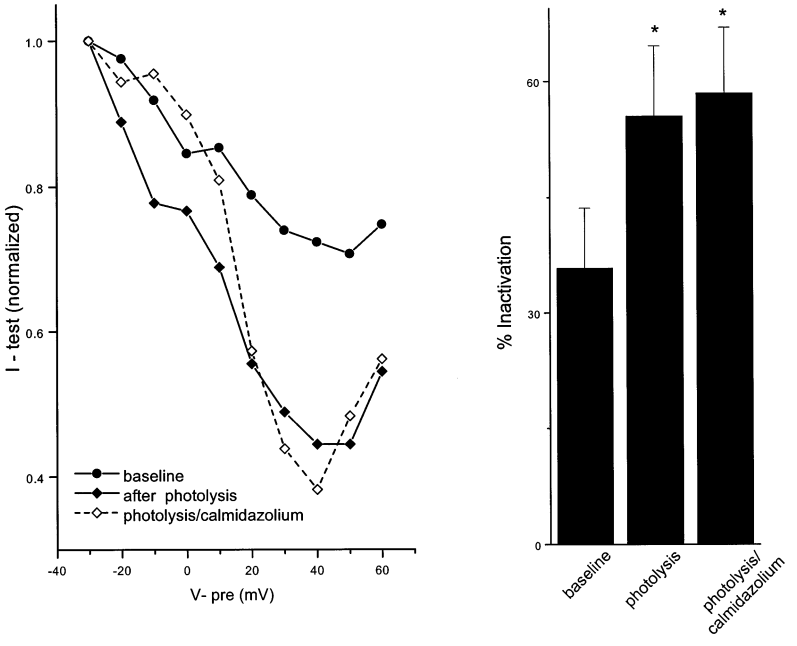

Fig. 3. Lack of effect of the calmodulin inhibitor calmidazolium on Ca^{2+} dependent inactivation of Ca^{2+} current sin GH_3 cells. Left panel, current-voltage relation in a representative cell at baseline, after photolysis of DM-nitrophen, and after bath application of 10 μM calmidazolium. Right panel, peak inactivation of the test-pulse Ca^{2+} current at a prepulse potential of $^{+}$20 mV (mean ±SE; $n = 4$). The augmentation of Ca^{2+}-dependent inactivation produced by flash photolysis of DM-nitrophen was not reversed by calmidazolium.

The present experiments critically test such a scheme in GH_3 cells using new techniques for rapidly altering internal Ca^{2+} buffering capacity and new tools for specifically inhibiting calcineurin.

Phosphatase Assays

Biochemical studies confirmed the abundance of Ca^{2+}-dependent phosphatase activity in GH_3 cells, with the calcineurin inhibitors blocking approximately half of the total phosphatase activity. This finding provides support for earlier indications by Western blot analysis (Farber et al., 1987) that this phosphatase is abundant in these cells and confirmed its specific and potent inhibition by CsA and FK506.

Ca^{2+}-dependent Inactivation of L-type Ca^{2+} Currents

The voltage- and Ca^{2+}-dependent components of inactivation could be clearly distinguished from each other experimentally by buffering cytosolic Ca^{2+}, substituting a different charge carrier for Ca^{2+}, or simply by inspecting the current-voltage relationship for the test pulse inactivation of Ca^{2+} currents in the two-pulse protocol. By first removing most of the Ca^{2+}-dependent inactivation component with the photosensitive Ca^{2+} buffer in the intracellular solution, variations in the basal extent of Ca^{2+}-dependent inactivation could be eliminated and each cell could then serve as its own control. Destruction of the Ca^{2+} buffer by flash photolysis then produced a distinct alteration in the current-voltage dependence of the test pulse Ca^{2+} current. The expected U-shaped relation is indicative of Ca^{2+}-dependent inactivation since the greatest degree of inactivation occurs when Ca^{2+} entry is maximal (near +20 mV) and is reversed as the driving force for Ca^{2+}-entry is reduced on further depolarization toward the Ca^{2+} reversal potential. The only other explanation for the upturn in the inactivation curve at positive potentials would be that the Ca^{2+} currents are facilitated by the larger depolarizations, a phenomenon that has been described on both the whole-cell (Kleppisch et al., 1994; Hryshko & Bers, 1990; Bourinet et al., 1994) and single-channel level (Pietrobon & Hess, 1990). Voltage-dependent enhancement of Ca^{2+} current is unlikely since there was no such facilitation with Ba^{2+} as the charge carrier. The possibility that Ca^{2+}-dependent facilitation at positive potentials by Ca^{2+}-calmodulin activation of a kinase (Xiao et al., 1994), could also be ruled out since there was no effect of the calmodulin inhibitor calmidazolium. Thus, the changes in the current-voltage relationship after flash photolysis could be attributed to increased Ca^{2+}-dependent inactivation. These changes were probably an underestimate of the maximum extent of Ca^{2+}-dependent inactivation since the peak inward Ca^{2+} current was partly reduced by the direct effects of photolysis.

Calcineurin Inhibition

The flash photolysis technique afforded us a highly reproducible measure of Ca^{2+}-dependent inactivation, allowing us to test unequivocally the role of calcineurin in this process. The specific calcineurin inhibitors CsA or

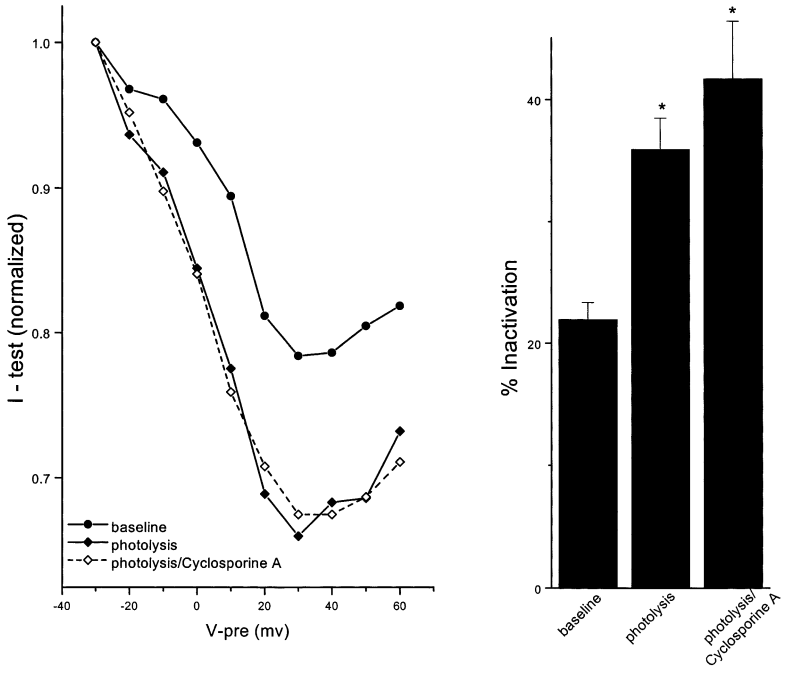

Fig. 4. Lack of effect of CsA on Ca^{2+}-dependent inactivation of Ca^{2+} currents in GH_3 cells. Left panel: current-voltage relation in a representative cell at baseline, after photolysis of DM-nitrophen, and after bath application of 10 μM CsA. In these experiments, cells were internally equilibrated with cyclophilin B (5 μM in pipette solution) before photolysis. Peak inactivation of the test-pulse Ca^{2+} current at a prepulse potential of $^{+}20$ mV (mean ± SEM; $n = 4$). CsA, added in the presence of cyclophilin, had no effect on the augmentation of Ca^{2+}-dependent inactivation produced by flash photolysis of DM-nitrophen.

FK506, when given either acutely or 1–24 hr prior to electrophysiological testing, had no significant effect on the extent of Ca^{2+}-dependent inactivation. Similarly, there was no effect of directly inhibiting calmodulin with calmidazolium (thus preventing calcineurin activation) or inhibiting calcineurin with the structurally distinct compound fenvalerate. The calmidazolium result agrees with that of Imredy and Yue (1994), who showed no effect of calmodulin inhibition (or even total phosphatase inhibition) on the single-channel manifestation of Ca^{2+}-dependent inactivation in guinea pig myocytes. Furthermore, in Aplysia neurons, the same model system used by Chad and Eckert, recent evidence has contradicted the initial findings which prompted the dephosphorylation hypothesis. Fryer and Zucker (1993) found no effect of protein kinase A activators or phosphatase inhibitors on Ca^{2+}-dependent inactivation of Ca^{2+} currents.

The lack of influence of calcineurin inhibitors on Ca^{2+}-dependent inactivation does not diminish the importance of phosphorylation and dephosphorylation in the regulation of Ca^{2+} current. In fact, the phosphorylation state of the channels could alter the rate of inactivation of macroscopic Ca^{2+} currents indirectly. One possibility is that, by regulating the number of functional channels in the membrane, phosphorylation could increase the total amount of Ca^{2+} entering the cell during depolarization and thus increase the extent of inactivation. A second consideration is that an increase in phosphorylation could enhance Ca^{2+} loading of internal stores, leading to an increase in the rise in Ca^{2+} in the submembrane space in cells undergoing excitation-contraction or excitation-secretion coupling. Another

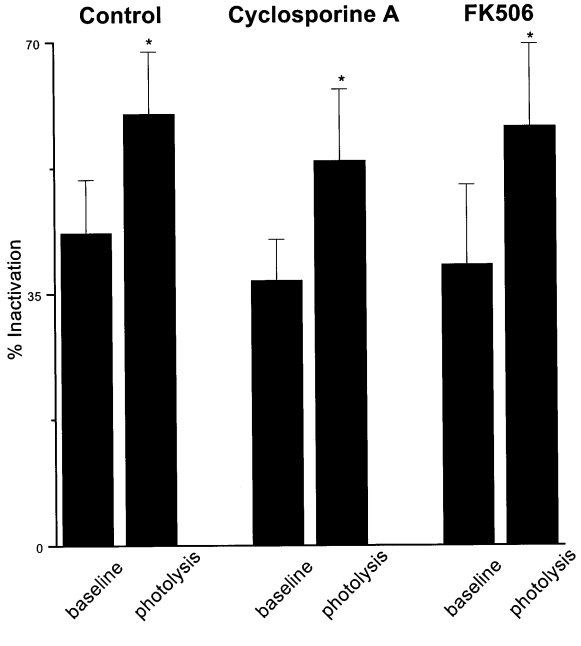

Fig. 5. Lack of effect of long-term preincubation of cells with CsA or FK506 on Ca^{2+}-dependent inactivation of Ca^{2+} currents in GH_3 cells. Summary data (mean ± SEM) showing peak inactivation of Ca^{2+} currents elicited by the test pulse (with the prepulse potential of $^{+}20$ mV) before (baseline) and after (photolysis) flash photolysis of DM-nitrophen in three different groups of GH_3 cells: control cells ($n = 9$), cells preincubated with 2 μM CsA for 24 hr ($n = 6$), and cells preincubated 1 μM FK506 for 1 hr ($n = 4$). In 3 of the CsA-pretreated cells, cyclophilin-B (1.0 μM) was added to the pipette solution for electrophysiological recordings. FKBP-12 (1.0 μM) was added to the pipette solution in all of the FK506-pretreated cells.

possibility is that the gating pattern of the channels could be drastically altered by phosphorylation. Shift of a significant proportion of Ca^{2+} channels into a long opening mode would have the effect of slowing the apparent rate of macroscopic current decay. This last mechanism may account for the slowing of Ca^{2+}-dependent inactivation observed in cardiac myocytes during β-adrenergic stimulation (Hadley & Lederer, 1991) and may contribute to alterations in inactivation rate induced by phosphatase inhibition.

Mechanism of Ca^{2+}-dependent Inactivation

In arguing against dephosphorylation as the biophysical basis of Ca^{2+}-dependent inactivation of Ca^{2+} channels, the present results are entirely consistent with recent structure-function studies of Ca^{2+} channels (Haack & Rosenberg, 1994; de Leon et al., 1996). Construction of Ca^{2+} channel chimeras by combining part of the sequence of a Ca^{2+} channel which does not possess the property of Ca^{2+}-dependent inactivation with part of the sequence of an L-type Ca^{2+} channel has implicated an EF-hand region of the Ca^{2+} channel α_1 subunit as the binding site mediating Ca^{2+}-dependent inactivation (de Leon et al., 1996). Thus, occupation of a Ca^{2+} binding site on the pore forming subunit of the channel by Ca^{2+} entering through the pore may be the sole mechanism for Ca^{2+}-dependent inactivation of L-type Ca^{2+} channels in GH_3 cells. Our results provide important evidence against channel dephosphorylation as a contributing factor in this process.

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