

# Effects of Protein Phosphatase and Kinase Inhibitors on $\text{Ca}^{2+}$ and $\text{Cl}^-$ Currents in Guinea Pig Ventricular Myocytes

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## SUMMARY

It is well-established that in heart, both the L-type  $\text{Ca}^{2+}$  channel and the cystic fibrosis transmembrane conductance regulator  $\text{Cl}^-$  channel are regulated by cAMP-dependent phosphorylation. However, it is not clear whether both of these channels are regulated in concert by protein kinase A (PKA) or whether there are mechanisms that independently control the phosphorylation of these two PKA targets. The purpose of this study was to compare the effects of various protein phosphatase and protein kinase inhibitors on these two ionic currents ( $I_{\text{Ca}}$  and  $I_{\text{Cl}}$ ) in guinea pig ventricular myocytes to gain insight into these questions. We found that both the stimulation and washout of the effects of isoproterenol on  $I_{\text{Cl}}$  are about twice as fast as the effects on  $I_{\text{Ca}}$ , probably because the dephosphorylation reaction for  $I_{\text{Cl}}$  is faster than that for  $I_{\text{Ca}}$ . In contrast, inhibition of

protein phosphatases with  $10 \mu\text{M}$  microcystin stimulated both  $I_{\text{Ca}}$  and  $I_{\text{Cl}}$ , but the stimulation of  $I_{\text{Cl}}$  was much slower and smaller than the stimulation of  $I_{\text{Ca}}$ . The effect of microcystin was inhibited by staurosporine ( $K_i = 171.5$  and  $161 \text{ nM}$  for  $I_{\text{Ca}}$  and  $I_{\text{Cl}}$ , respectively), suggesting that the stimulation was due to a kinase. The kinase was not protein kinase C (PKC) because it was not inhibited by the specific pseudosubstrate inhibitor of PKC,  $\text{PKC}_{(19-31)}$ , and it was not PKA because it was not inhibited by adenosine 3',5'-cyclic phosphorothioate. These results suggest that although both the  $\text{Ca}^{2+}$  and  $\text{Cl}^-$  channels are regulated by cAMP-dependent phosphorylation, another protein kinase may also regulate these channels, and the kinetics of the response of the channels to phosphorylation can be modulated independently by protein phosphatases.

In mammalian cardiac myocytes,  $\beta$ -adrenergic agonists regulate a variety of ionic currents, including  $I_{\text{Ca}}$  and  $I_{\text{Cl}}$  (1-3). Both currents are stimulated by cAMP-dependent phosphorylation via the  $G_s$ /adenyl cyclase/cAMP/PKA cascade, but both of these currents may be regulated by phosphorylation of more than a single phosphorylation site.

In the case of  $I_{\text{Ca}}$ , Tsien *et al.* (4) proposed that two different phosphorylation sites were responsible for the  $\beta$ -adrenergic regulation of  $\text{Ca}^{2+}$  channel availability (N) and channel gating ( $P_o$ ). Support for this hypothesis has come from studies on rabbit ventricular myocytes in which different concentrations of okadaic acid selectively affect N and  $p_o$  (5) and from our studies on frog ventricular myocytes in which the amplitude of  $I_{\text{Ca}}$  is regulated by two phosphorylation sites that can be distinguished by the phosphatases that dephosphorylate them (6). One site is dephosphorylated by phosphatase 2A, and the other site is dephosphorylated by a phosphatase with a low sensitivity to the phosphatase inhibitors microcystin, okadaic acid, and calyculin A.

The regulation of  $I_{\text{Cl}}$  is even more complicated (3). The regulation of this channel, like that of the  $\text{Ca}^{2+}$  channel, involves two phosphorylation sites. One site is dephosphorylated by protein phosphatase 2A, and the other is dephosphorylated by a phosphatase with a low sensitivity to microcystin and okadaic acid, which may be protein phosphatase 2C (7). In addition, the channel is regulated by ATP binding and hydrolysis by its two nucleotide binding domains. The singly phosphorylated channel exhibits brief openings that correspond to hydrolysis of ATP by the first nucleotide binding site. The doubly phosphorylated channel exhibits a much longer channel open time that involves ATP binding to the second nucleotide binding site and stabilization of channel open state.

Although it is clear that both channels are regulated by PKA, there is evidence that other protein kinases may also be involved in their regulation as well. For example, PKC can regulate cardiac  $\text{Ca}^{2+}$  channels (8), and the CFTR  $\text{Cl}^-$  channel may also be regulated by PKC (3). Furthermore, we have recently shown that in frog ventricular myocytes there is a novel protein kinase, which we have termed PKX, that can

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**ABBREVIATIONS:** PKA, protein kinase A; CFTR, cystic fibrosis transmembrane conductance regulator;  $E_{\text{Cl}}$ ,  $\text{Cl}^-$  equilibrium potential;  $E_{\text{rev}}$ , reversal potential; Fsk, forskolin;  $I_{\text{Ca}}$ , L-type  $\text{Ca}^{2+}$  current;  $I_{\text{Cl}}$ , cAMP-activated  $\text{Cl}^-$  current; Iso, isoproterenol; myr-PKC $_{(19-31)}$ , myristoylated derivative of the pseudosubstrate protein kinase C inhibitor; PKC, protein kinase C; PKX, protein kinase X; TPA, 12-O-tetradecanoylphorbol-13-acetate; EGTA, ethylene glycol bis( $\beta$ -aminoethyl ether)-N,N',N'-tetraacetic acid; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid;  $R_p$ -cAMPS, adenosine 3'-5'-cyclic phosphorothioate.

phosphorylate one of the phosphorylation sites that regulates  $I_{Ca}$  (6).

The fact that both  $I_{Ca}$  and  $I_{Cl}$  seem to be regulated by two phosphorylation sites and that multiple protein kinases can regulate these channels raises the question of whether these channels are regulated in concert or independently by phosphorylation. For example, when cAMP levels increase in the cell, do both channels become phosphorylated with the same kinetics, or can the phosphorylation of each channel be adjusted independently? One way that phosphorylation could be adjusted would be by alterations in the activity of the protein phosphatases responsible for dephosphorylation. For these reasons, we decided to compare the regulation of  $I_{Ca}$  and  $I_{Cl}$  by  $\beta$ -adrenergic agonists and inhibitors of protein phosphatases and kinases in guinea pig ventricular myocytes.

## Materials and Methods

**Isolation of cardiac myocytes.** Single guinea pig ventricular myocytes were isolated by enzymatic dissociation. Female guinea pigs, weighing 300–400 g, were anesthetized with sodium pentobarbital (50 mg/kg). The chest was opened under artificial respiration, and the aorta was cannulated for perfusion with Tyrode's solution before the heart was removed from the animal. After the perfusate was changed to nominally  $Ca^{2+}$ -free Tyrode's solution for ~5 min, a nominally  $Ca^{2+}$ -free Tyrode's solution with 0.1–0.11 mg/ml collagenase (Yakult Co., Tokyo, Japan) was recirculated for ~9 min using a Langendorff apparatus. After the enzyme was washed out, the hearts were then stored in high- $K^+$  and low- $Cl^-$  solution at room temperature (22°) for 10 min. The isolated cells were obtained by gentle agitation of small pieces of ventricle and stored in the high- $K^+$  and low- $Cl^-$  solution for 1 hr. They were then kept in Tyrode's solution at room temperature before use.

**Solutions.** The composition of the Tyrode's solution was 144 mM NaCl, 0.33 mM  $NaH_2PO_4$ , 4.0 mM KCl, 1.8 mM  $CaCl_2$ , 0.53 mM  $MgCl_2$ , 5.5 mM glucose, and 5.0 mM HEPES-NaOH buffer, pH adjusted to 7.4 with NaOH. Nominally  $Ca^{2+}$ -free Tyrode's solution simply omitted  $CaCl_2$ . High- $K^+$  and low- $Cl^-$  solution contained 70 mM glutamic acid, 15 mM taurine, 30 mM KCl, 10 mM  $KH_2PO_4$ , 0.5 mM  $MgCl_2$ , 11 mM glucose, 0.5 mM EGTA, and 5.0 mM HEPES-NaOH buffer, pH adjusted to 7.4 with KOH. The internal solution for recording  $I_{Ca}$  contained 85 mM CsCl, 10 mM EGTA, 20 mM tetraethylammonium Cl, 10 mM MgATP, 2 mM  $MgCl_2$ , 5 mM pyruvic acid, 5 mM Tris<sub>2</sub>-creatine phosphate, 0.1 mM Tris-GTP, 5.5 mM glucose, and 10 mM PIPES, pH to 7.15 with CsOH. External solution contained 130 mM NaCl, 20 mM CsCl, 1.8 mM  $CaCl_2$ , 0.5 mM  $MgCl_2$ , 5.5 mM glucose, and 5 mM HEPES, pH to 7.3 with CsOH. In some experiments, 0.02 mM ouabain was added to suppress Na-K pump currents (9). In Fig. 5, the composition of the 158 mM  $Cl^-$  external solution was the same as the standard external solution except that 2 mM  $CoCl_2$  replaced the 1.8 mM  $CaCl_2$ . The 28.6 mM  $Cl^-$  solution was the same as the 158 mM  $Cl^-$  solution except that NaCl was replaced with Na-aspartate. The bath was continuously superfused with control solution at a rate of ~2 ml/min, and the patch-clamped cell could be exposed to different test solutions within 1 sec by placing the cell at the mouth of one of a group of capillary tubes attached to reservoirs flowing at ~100  $\mu$ l/min.

The internal solution was changed as previously described (6). Briefly, a fine polyethylene tube was inserted to within 250  $\mu$ m of the tip of the patch pipette. At the onset of the experiment, the other end of the tubing was placed in a reservoir containing control internal solution while the gigaohm seal was being made and broken. Measurements of control currents were made while control solution was being perfused into the pipette. Then, the negative pressure was released, and the tubing was switched to another reservoir containing the experimental drug dissolved in the control solution. The new

solution was then aspirated into the patch pipette by again applying negative pressure to the patch pipette holder.

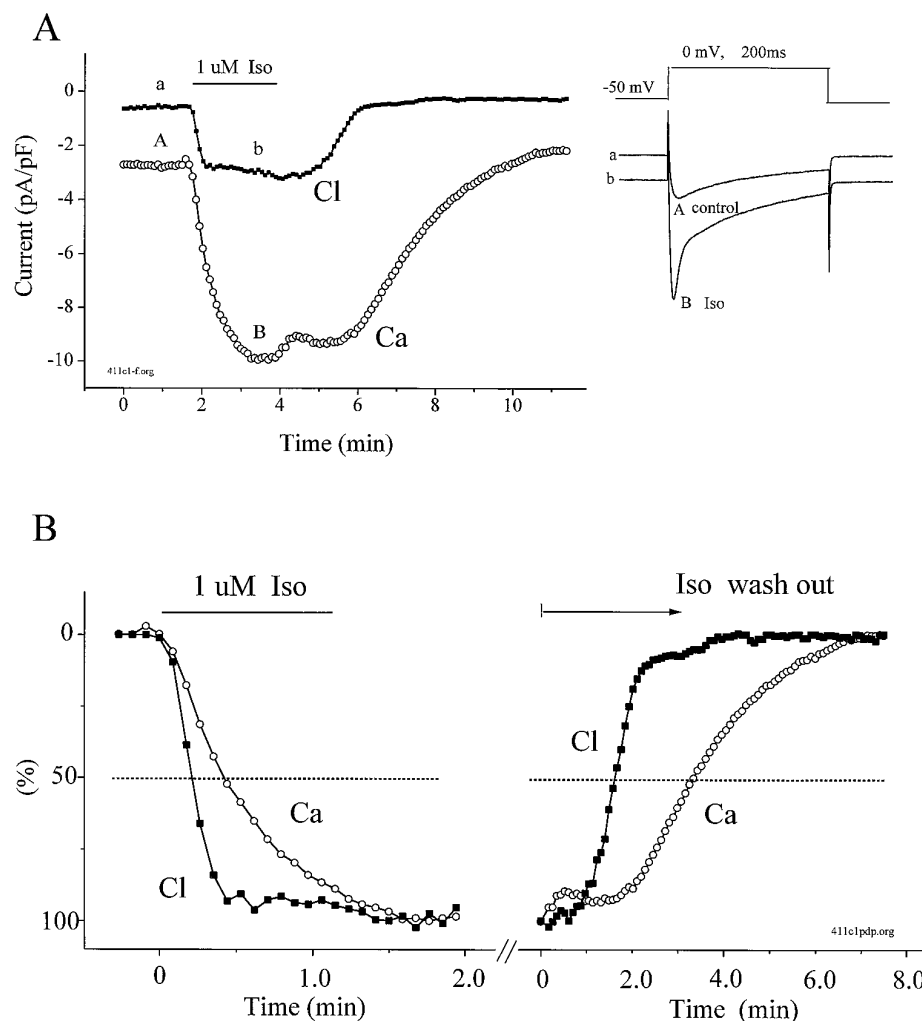
**Drugs.** Reagents included cAMP-dependent protein kinase inhibitor peptide  $R_p$ -cAMPS (gift of Dr. Ira Cohen, State University of New York, Stony Brook, NY); microcystin-LR (GIBCO BRL, Gaithersburg, MD); K252a, staurosporine, and Fsk (Calbiochem, San Diego, CA); calyculin A (LC Laboratories, Woburn, MA); TPA (Sigma Chemical, St. Louis, MO); and myr-PKC<sub>(19–31)</sub> (Promega, Madison, WI). Stock solutions of staurosporine were prepared at 1 mM in dimethylsulfoxide. Microcystin was made at a concentration of 0.5 mM in 10% methanol. Fsk was 10 mM in 100% ethanol. Stock solutions were stored at –20°.

**Recording methods.** Whole-cell currents were recorded using a patch-clamp amplifier (EPC-7; List Medical Instruments, Darmstadt, Germany). Cells were patch-clamped with borosilicate patch pipettes with a resistance of 1–2 M $\Omega$ . Total series resistance was usually <3 M $\Omega$ .  $I_{Ca}$  was elicited by voltage pulses delivered by a programmable digital stimulator (Challenger DB; W. Goolsby, Kinetic software and Emory University, Atlanta, GA). Routine pulses were from –50 to 0 mV. The pulse was 200 msec in duration, and  $I_{Ca}$  was measured as the peak inward current minus the current at the end of the pulse. Because the reversal potential of  $I_{Cl}$  ( $E_{Cl}$ ) was measured to be –7 mV and calculated to be –10 mV and because  $I_{Cl}$  at –50 mV was small relative to  $I_{Ca}$ , contamination of  $I_{Ca}$  by  $I_{Cl}$  at 0 mV should be negligible.  $I_{Cl}$  was measured as the steady state current at –50 mV. Experiments were conducted at room temperature unless otherwise stated.

## Results

**Kinetics of stimulation of  $I_{Ca}$  and  $I_{Cl}$  by Iso.** Initially, we were interested in comparing the time course of the effects of  $\beta$ -adrenergic stimulation on  $I_{Ca}$  and  $I_{Cl}$  in guinea pig ventricular myocytes to gain insights into possible differences in the regulation of these two currents (Fig. 1). Myocytes were voltage-clamped using the whole-cell configuration of the patch-clamp technique.  $K^+$  currents were blocked by internal tetraethylammonium and internal and external cesium. The inward  $Na^+$  current was blocked by holding the membrane potential at –50 mV. Myocytes were routinely depolarized from a holding potential of –50 to 0 mV for 200 msec at a frequency of 0.1 sec<sup>-1</sup>. The depolarization elicited a transient inward current that was completely blocked by  $Ca^{2+}$  channel blockers.  $I_{Ca}$  was measured as the difference between the peak inward current and the current at the end of the depolarizing pulse.  $I_{Cl}$  was measured as the steady state current at –50 mV. The current at –50 mV that is stimulated by Iso is  $I_{Cl}$  under these conditions because the reversal potential ( $E_{rev}$ ) of this current obtained by voltage ramps was near  $E_{Cl}$  (data not shown). Because  $I_{Ca}$  was measured near the  $E_{rev}$  for  $I_{Cl}$ , there was little contamination of  $I_{Ca}$  by  $I_{Cl}$ .

In Fig. 1A, 1  $\mu$ M Iso increased  $I_{Ca}$  3.5-fold and increased  $I_{Cl}$  to 2.2 pA/pF.  $I_{Ca}$  was stimulated more slowly than  $I_{Cl}$ . Fig. 1B shows the time course of change of the normalized currents. The time to half-maximum stimulation was 12.0 sec for  $I_{Cl}$  and 24.6 sec for  $I_{Ca}$  (Fig. 1B). On washing out Iso,  $I_{Ca}$  also decreased more slowly than  $I_{Cl}$ . The time to half-maximum deactivation was 1.6 min for  $I_{Cl}$  and 3.1 min for  $I_{Ca}$ . On average, the half-times of activation and inactivation for  $I_{Ca}$  were 25.8  $\pm$  0.6 sec and 3.6  $\pm$  0.2 min, respectively (five cells), and the half-times of activation and inactivation for  $I_{Cl}$  were 12.0  $\pm$  0.2 sec and 2.1  $\pm$  0.2 min, respectively (six cells). In Fig. 1B, it seems that  $I_{Cl}$  in this cell may have washed out



**Fig. 1.** Effect of Iso on  $I_{Ca}$  (○) and  $I_{Cl}$  (■) in guinea pig ventricular myocytes. **A**, Time course of the effect of Iso; 1  $\mu$ M was added to the superfusion (bar). Representative current traces are shown for control (A, a) and after Iso (B, b). **B**, Comparison of onset and washout of the effect of Iso on  $I_{Ca}$  and  $I_{Cl}$ . Currents were normalized to the maximum current stimulated by Iso.

with two kinetic components, one fast and one slow. This was not a routine observation.

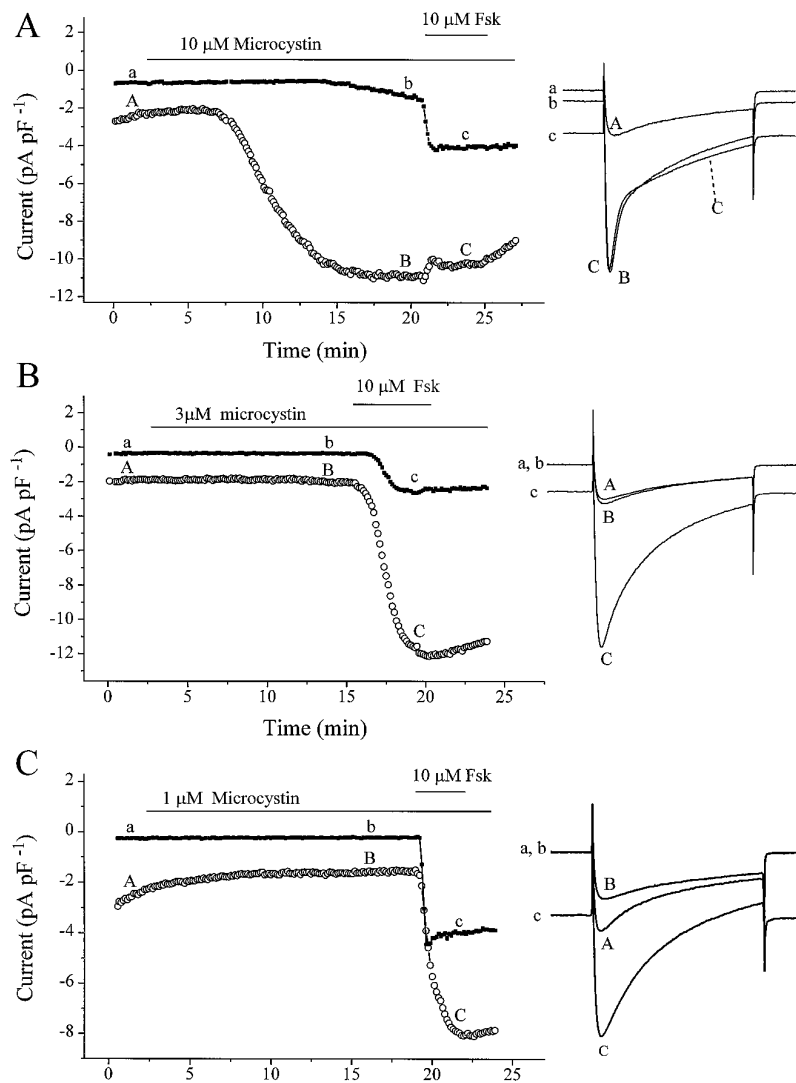
**Effects of protein phosphatase inhibitors on  $I_{Ca}$  and  $I_{Cl}$ .** To investigate further the phosphorylation reactions controlling these currents, we examined the effects of protein phosphatase inhibitors. Fig. 2A shows the effects of internal perfusion of the myocyte with the protein phosphatase inhibitor microcystin. The bar indicates the period when microcystin was added to the internal solution. On changing the internal solution, there was a  $\sim$ 3-min lag period before  $I_{Ca}$  increased. This was partly due to the dead volume of the internal perfusion system.  $I_{Ca}$  increased from 2.2 to 11.0 pA/pF in 13 min in response to 10  $\mu$ M microcystin. However,  $I_{Cl}$  did not begin to increase until after  $I_{Ca}$  had nearly reached its maximum (11 min after changing the internal solution); then,  $I_{Cl}$  increased very slowly. At 18 min after the addition of 10  $\mu$ M microcystin,  $I_{Cl}$  was 1.2 pA/pF. Microcystin (10  $\mu$ M) stimulated both  $I_{Ca}$  and  $I_{Cl}$ , but  $I_{Ca}$  always increased more rapidly than  $I_{Cl}$ . The subsequent application of Fsk stimulated  $I_{Cl}$  markedly but had little effect on  $I_{Ca}$ .

Fig. 2, B and C, shows the effects of lower concentrations of microcystin. These lower concentrations of microcystin (1 or 3  $\mu$ M) had no effect on  $I_{Cl}$  and had only minimal effects on basal  $I_{Ca}$ . However, subsequent application of 10  $\mu$ M Fsk produced a large, rapid increase in both currents. Both currents remained partially elevated after Fsk washout. Thus,

lower concentrations of microcystin seemed to inhibit a phosphatase responsible for dephosphorylation of sites phosphorylated by PKA but did not significantly stimulate the currents in the absence of PKA activity.

The very slow stimulation of  $I_{Cl}$  by microcystin shown in Fig. 2A was in contrast to the rapid stimulation of  $I_{Cl}$  relative to  $I_{Ca}$  by Iso in Fig. 1. The relative difference in the kinetics of stimulation of  $I_{Ca}$  and  $I_{Cl}$  by Iso and microcystin suggests that there are interesting differences in how phosphorylation regulates these channels.

Fig. 3 shows the dose-response curves for the effect of microcystin alone on  $I_{Ca}$  (Fig. 3A) and  $I_{Cl}$  (Fig. 3B). The effects of microcystin on both  $I_{Ca}$  and  $I_{Cl}$  were measured  $\sim$ 2–5 min after the effect of microcystin on  $I_{Ca}$  had plateaued. Because the effect of microcystin on  $I_{Cl}$  is much slower than the effect on  $I_{Ca}$ , the dose-response curve for  $I_{Cl}$  was not obtained in a steady state condition and may actually underestimate the steady state effect of microcystin. However, it was not practical to wait until the effect on  $I_{Cl}$  reached a plateau. Despite this limitation, the data demonstrate that the effect of microcystin was quantitatively less on  $I_{Cl}$  than it was on  $I_{Ca}$  over the same time period. Both currents were activated by microcystin at a threshold concentration of  $\sim$ 3  $\mu$ M. Data were fitted to the Hill equation (lines). The best-fit parameters were  $I_{Ca}$ ,  $K_{1/2} = 4.6 \mu$ M,  $I_{max} = 5.5$  pA/pF; and  $I_{Cl}$ ,  $K_{1/2} = 7.0 \mu$ M,  $I_{max} = 1.46$  pA/pF. The



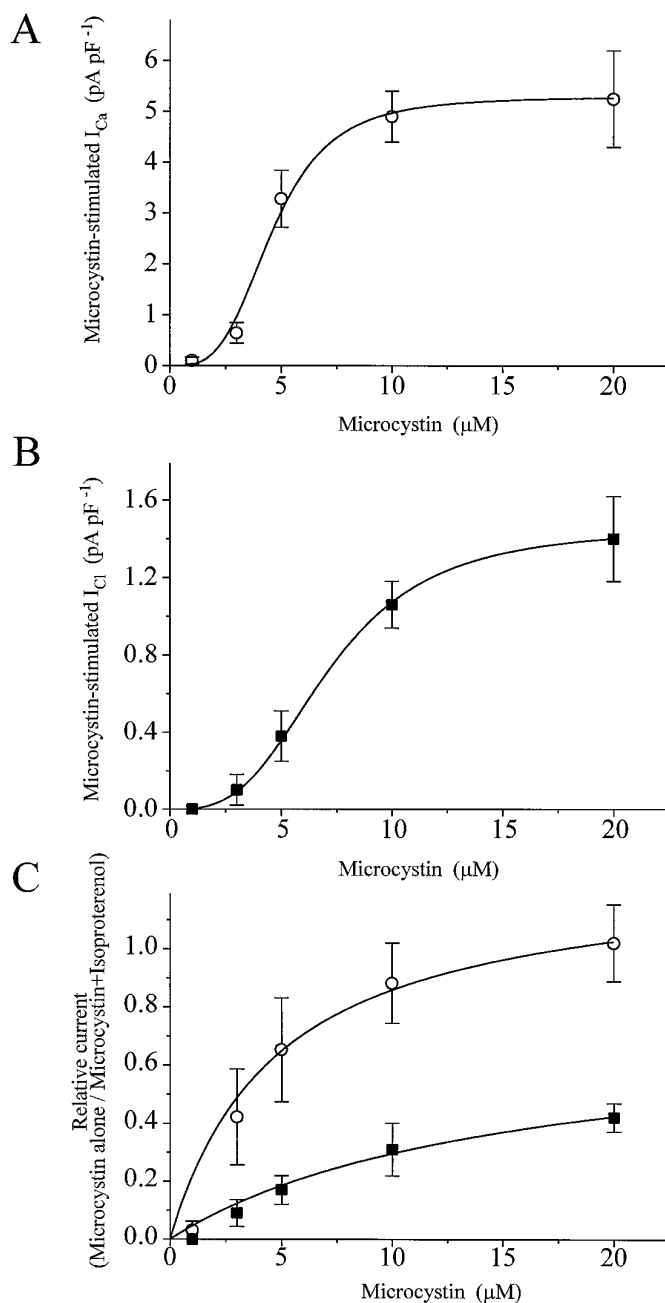
**Fig. 2.** Effects of the protein phosphatase inhibitor microcystin on  $I_{Ca}$  and  $I_{Cl}$ . A, 10  $\mu$ M microcystin was applied by internal perfusion, and 10  $\mu$ M Fsk was applied to the superfusion as indicated. Internal perfusion was begun at the time indicated, but  $\sim$ 3 min was required for the new solution to reach the tip of the pipette. Microcystin increased both  $I_{Ca}$  ( $\circ$ ) and  $I_{Cl}$  ( $\blacksquare$ ), but  $I_{Cl}$  increased more slowly than  $I_{Ca}$ . B, 3  $\mu$ M microcystin increased  $I_{Ca}$  a small amount but had no effect on  $I_{Cl}$  (trace B). The subsequent application of 10  $\mu$ M Fsk produced a large and rapid increase in both currents (trace C). C, Effect of internal perfusion with 1  $\mu$ M microcystin. Neither  $I_{Ca}$  nor  $I_{Cl}$  was stimulated by microcystin alone.

effects of microcystin on  $I_{Ca}$  and  $I_{Cl}$  are compared in Fig. 3C. At 20  $\mu$ M, microcystin alone was able to stimulate  $I_{Ca}$  as much as microcystin plus Iso, but in the same time period, the same concentration of microcystin alone was able to stimulate  $I_{Cl}$  only  $\sim$ 40% compared with the stimulation of  $I_{Cl}$  produced by microcystin plus Iso. This suggests that whatever kinases are responsible for the increases in  $I_{Ca}$  produced by microcystin alone are not as effective in stimulating  $I_{Cl}$ .

The  $I_{Ca}$  stimulated by microcystin had the same biophysical properties as the basal  $I_{Ca}$ . Fig. 4A shows that the shape of the peak current-voltage relationship of  $I_{Ca}$  was unaffected by microcystin, but the current was increased in amplitude at all potentials. The activation curve and inactivation curve also were not affected (Fig. 4, B and C).

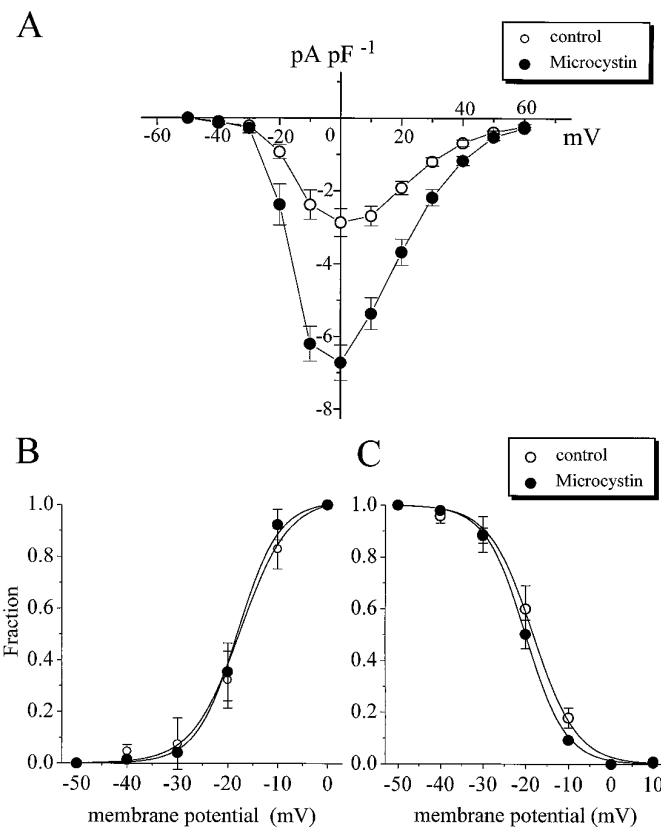
**The microcystin-stimulated current at  $-50$  mV is carried by  $Cl^-$ .** For work described in the preceding paragraphs, we assumed that the current at  $-50$  mV stimulated by high concentrations of microcystin (Fig. 2A) was  $I_{Cl}$ . To verify this, we measured the current-voltage relationship of the microcystin-induced current. The current-voltage relationship was determined by voltage ramps from  $-50$  to  $+50$  mV at 0.34 V/sec (Fig. 5). In these experiments, we used the standard external solution except that 2 mM  $CoCl_2$  replaced 1.8 mM  $CaCl_2$  to eliminate  $I_{Ca}$ . Fig. 5A shows current-voltage

relations before and after internal perfusion with 10  $\mu$ M microcystin. The microcystin-stimulated current was obtained by subtracting the before-perfusion value from the after-perfusion value (Fig. 5B). The current-voltage relationship was essentially linear with a small amount of outward rectification. The current reversed at  $-7.3$  mV, which was very close to  $E_{Cl}$  ( $-9.6$  mV). To verify that the current was a  $Cl^-$  current, we changed the bath  $[Cl^-]_o$ . The mean current-voltage relations of the microcystin-induced current with 28.6 and 158 mM  $[Cl^-]_o$  were determined as the difference between the average of five records obtained in the absence and presence of microcystin (Fig. 5C). The reversal potential was  $-7.1 \pm 1.9$  mV with 158 mM  $[Cl^-]_o$  and shifted to  $+36.2 \pm 3.9$  mV with 28.6 mM  $[Cl^-]_o$ . These values were almost identical to the calculated  $E_{Cl}$  value in each condition ( $E_{Cl} = -9.6$  and  $+34.8$  mV in 158 and 28.6 mM  $[Cl^-]_o$ , respectively, with  $[Cl^-]_i = 109$  mM). The dotted lines indicate the current-voltage relationship calculated from the Goldman-Hodgkin-Katz equation (10) assuming a simple  $Cl^-$  conductance. From these data, we conclude that the microcystin-stimulated background current was carried by  $Cl^-$ . Because intracellular  $Ca^{2+}$  is highly buffered, it is unlikely this is a  $Ca^{2+}$ -activated  $Cl^-$  conductance and thus it is probably the CFTR  $Cl^-$  channel.



**Fig. 3.** Dose-response curve for the effect of microcystin on  $I_{Ca}$  (A) and  $I_{Cl}$  (B). Data points represent mean (5–13 cells) increase in  $I_{Ca}$  and  $I_{Cl}$  produced by a single internal application of microcystin. Data were fitted to the Hill equation (lines). The best-fit parameters were  $I_{Ca}$ ,  $K_{1/2} = 4.6 \mu\text{M}$ ,  $I_{max} = 5.5 \text{ pA/pF}$ ; and  $I_{Cl}$ ,  $K_{1/2} = 7.0 \mu\text{M}$ ,  $I_{max} = 1.46 \text{ pA/pF}$ . C, Microcystin-stimulated current relative to current stimulated in the presence of microcystin and Fsk together.  $\circ$ ,  $I_{Ca}$ ;  $\blacksquare$ ,  $I_{Cl}$ . The best-fit parameters were  $I_{Ca}$ ,  $K_{1/2} = 4.8 \mu\text{M}$ ,  $I_{max} = 1.27 \text{ pA/pF}$ ; and  $I_{Cl}$ ,  $K_{1/2} = 15.0 \mu\text{M}$ ,  $I_{max} = 0.74 \text{ pA/pF}$ .

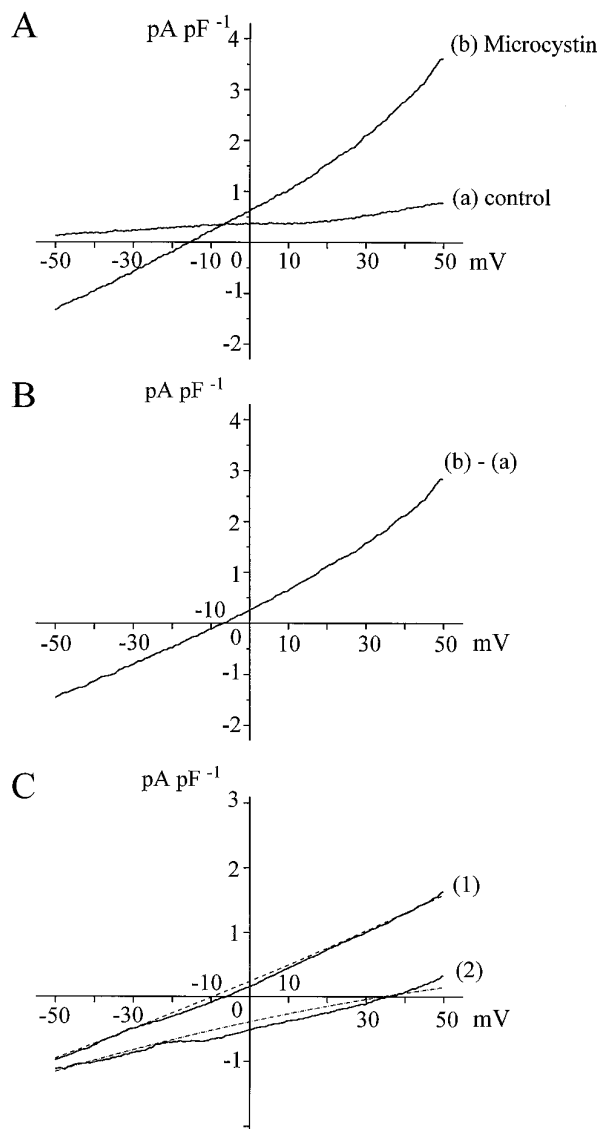
**Effects of microcystin at 36°.** The results in Fig. 2 differ somewhat from those of Hwang *et al.* (7), who showed that after washout of Fsk in the presence of microcystin,  $I_{Cl}$  decreased to a level  $\sim 25\%$  of the maximally stimulated current. In contrast, we found that the effect of Fsk on  $I_{Cl}$  in the presence of 10  $\mu\text{M}$  microcystin was nearly irreversible (Fig. 2A). One difference between our experiments and their experiments was that ours were performed at room tempera-



**Fig. 4.** Effects of microcystin on current-voltage relationship, activation, and inactivation curves of  $I_{Ca}$ . A, Peak current-voltage relationships before ( $\circ$ ) and after ( $\bullet$ ) addition of 10  $\mu\text{M}$  microcystin. Values are mean of five cells. B, Activation curves. The values were normalized to the maximum  $I_{Ca}$ . Continuous curves, fits to the Boltzmann relation. The half-maximal activation voltage ( $V_{1/2}$ ) was  $-17.5 \text{ mV}$  for control and  $-18.0 \text{ mV}$  after the addition of 10  $\mu\text{M}$  microcystin. C, Inactivation curve obtained by a double-pulse protocol before (circles) and after ( $\bullet$ ) addition of 10  $\mu\text{M}$  microcystin. The double-pulse protocol consisted of a 200-msec test pulse to 0 mV preceded by various conditioning prepulses of 2-sec duration between  $-50$  and  $+10 \text{ mV}$  separated by a 3-msec repolarization to  $-80 \text{ mV}$ . Values for  $V_{1/2}$  were  $-18.2 \text{ mV}$  for control and  $-20.0 \text{ mV}$  after addition of 10  $\mu\text{M}$  microcystin.

ture, whereas theirs were performed at 36°. When we repeated our experiments at 36° (Fig. 6, A and B), the stimulatory effects of 10  $\mu\text{M}$  microcystin on  $I_{Ca}$  and  $I_{Cl}$  were qualitatively the same as at room temperature. Subsequent exposure to Fsk caused an rapid increase in  $I_{Cl}$ , but in contrast to the results at room temperature, washout of Fsk was followed by a  $\sim 75\%$  decrease in  $I_{Cl}$  (Fig. 6, A and B). These results confirm those of Hwang *et al.* (7) and suggest that the difference between our result and theirs is due to higher phosphatase activity at 36°, which results in a partial dephosphorylation of  $I_{Cl}$ .

**Inhibitory effects of microcystin and Fsk on  $I_{Ca}$ .** The experiments at 36° revealed a very interesting aspect of  $I_{Ca}$  regulation that was less evident but also present at room temperature. Although  $I_{Ca}$  was stimulated by internal perfusion with 10  $\mu\text{M}$  microcystin at 36°, subsequent exposure to Fsk produced a complex response in  $I_{Ca}$ . Initially,  $I_{Ca}$  rose quickly, but after  $\sim 30 \text{ sec}$ , it began to decline. This decline continued even after Fsk was washed out. These results suggested that at 36°, Fsk may have triggered an event that caused a decrease in  $\text{Ca}^{2+}$  channel activity. Additional evi-



**Fig. 5.** Current-voltage relationship of the microcystin-stimulated background current. A, Current-voltage relationships obtained by triangular ramp pulses ( $-50$  to  $+50$  mV,  $dV/dt = 0.34$  V/sec) before (a) and after (b) application of  $10 \mu\text{M}$  microcystin. B, Current-voltage relationship of the microcystin-stimulated background current obtained after subtracting a from b in A. C, Effect of changing external  $[\text{Cl}^-]_o$  on current-voltage relations for the microcystin-stimulated background current. 1,  $158 \text{ mM } [\text{Cl}^-]_o$ . 2,  $28.6 \text{ mM } [\text{Cl}^-]_o$ .  $[\text{Cl}^-]_i$  was  $109 \text{ mM}$ .  $E_{rev}$  at  $158 \text{ mM } [\text{Cl}^-]_o$  was  $-7.1 \pm 1.9 \text{ mV}$ .  $E_{rev}$  at  $28.6 \text{ mM } [\text{Cl}^-]_o$  was  $+36.2 \pm 3.9 \text{ mV}$ . Values are mean of four or five cells. Dotted lines, current-voltage relation calculated from the Goldman-Hodgkin-Katz equation assuming a pure  $\text{Cl}^-$  conductance.

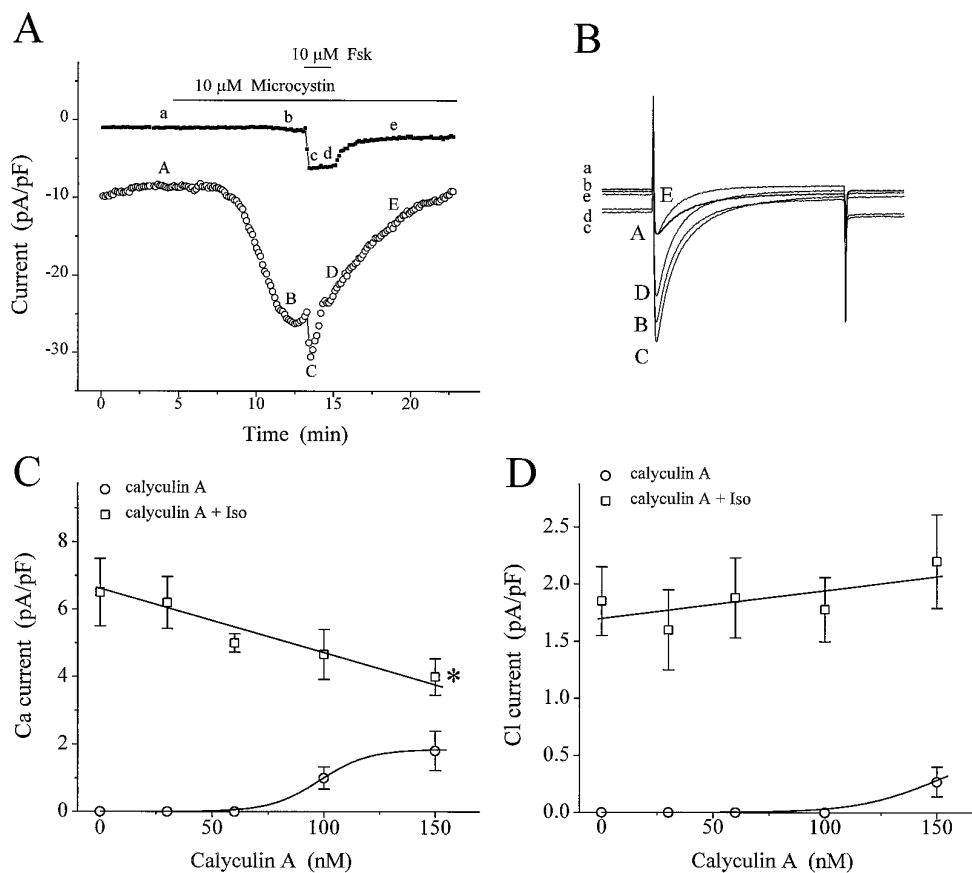
dence for this inhibitory effect of Fsk can be seen at room temperature (Fig. 2A);  $I_{Ca}$  in the presence microcystin alone was stable for  $\sim 5$  min, but after exposure to Fsk,  $I_{Ca}$  began to decrease, although  $I_{Cl}$  did not change.

To examine this effect in more detail, we examined the effect on  $I_{Ca}$  and  $I_{Cl}$  of another protein phosphatase inhibitor, calyculin A. Fig. 6C shows the effects of calyculin A and calyculin A plus Iso on  $I_{Ca}$ , and Fig. 6D shows the effects on  $I_{Cl}$ . Iso stimulated  $I_{Cl}$  to approximately the same degree, regardless of the concentration of calyculin A present. The amplitudes of the  $\text{Cl}^-$  currents in the presence of any concentration of calyculin and Iso were not significantly differ-

ent than the amplitude in the absence of calyculin ( $>5\%$ ,  $t$  test). In contrast, the stimulation of  $I_{Ca}$  by Iso decreased as the calyculin A concentration increased over the same range. The amplitude of the  $\text{Ca}^{2+}$  current in the presence of Iso and  $150 \text{ nM}$  calyculin was significantly different than the amplitude in the absence of calyculin ( $<1\%$ ,  $t$  test). These results suggest the possibility that there is an inhibitory phosphorylation that is potentiated by protein phosphatase inhibitors that selectively affects  $I_{Ca}$ . The fact that  $I_{Cl}$  is not affected shows that the effect is not a nonspecific effect.

**Effects of protein kinase inhibitors on the response of  $I_{Ca}$  and  $I_{Cl}$  to microcystin.** We hypothesize that microcystin stimulates  $I_{Ca}$  and  $I_{Cl}$  because there is a basally active protein kinase whose activity is counteracted by a basally active protein phosphatase. When the phosphatase is inhibited by microcystin, the phosphoprotein can accumulate and the current amplitude increases. In our studies on frog cardiomyocytes, we showed that the effect of microcystin required ATP and was blocked by protein kinase inhibitors (6, 11). To test whether the same mechanism could explain the effect of microcystin in guinea pig, we examined the ability of staurosporine to block the effects of microcystin (Fig. 7). Staurosporine, which is a nonspecific protein kinase inhibitor (12, 13), did block the effect of microcystin on  $I_{Ca}$  and  $I_{Cl}$ . Staurosporine was applied extracellularly at concentrations of  $100$ – $300 \text{ nM}$  before internal perfusion with  $20 \mu\text{M}$  microcystin was begun. At  $300 \text{ nM}$ , staurosporine decreased basal  $I_{Ca}$  and nearly completely inhibited the increase in  $I_{Ca}$  and  $I_{Cl}$  produced by microcystin (Fig. 7A). Even though  $300 \text{ nM}$  staurosporine nearly completely blocked the response of  $I_{Ca}$  and  $I_{Cl}$  to microcystin, subsequent application of Fsk produced large increases in both currents. Fig. 7B shows the dose-response curve for the effect of staurosporine on the microcystin-stimulated current. The  $EC_{50}$  values for inhibition of the effect of staurosporine on  $I_{Ca}$  and  $I_{Cl}$  were  $171.5$  and  $161 \text{ nM}$ , respectively. On average, in the presence of  $300 \text{ nM}$  staurosporine, microcystin-stimulated  $I_{Ca}$  and  $I_{Cl}$  were  $0.48 \pm 0.13$  and  $0.46 \pm 0.22 \text{ pA/pF}$ , respectively. In comparison,  $I_{Ca}$  and  $I_{Cl}$  values in the presence of  $300 \text{ nM}$  staurosporine,  $20 \mu\text{M}$  microcystin, and  $10 \mu\text{M}$  Fsk were  $5.5 \pm 0.79$  and  $3.4 \pm 0.43 \text{ pA/pF}$ , respectively. These results suggest that staurosporine inhibits a protein kinase that is responsible for stimulation of  $I_{Ca}$  and  $I_{Cl}$  by microcystin but that staurosporine at these concentrations has only a small effect on PKA.

Another interpretation that must be entertained is that staurosporine inhibits the action of microcystin nonspecifically via a mechanism independent of protein kinase inhibition. Although we cannot absolutely exclude this possibility, there are a number of reasons why this seems unlikely. First, in frog myocytes, in which microcystin produces a stimulation of  $I_{Ca}$  and an inhibition of the delayed rectifier  $\text{K}^+$  current  $I_K$ , only the stimulation of  $I_{Ca}$  is inhibited by staurosporine (6). Therefore, the action of staurosporine is not nonspecific. Second, it seems clear that a kinase is involved in the action of microcystin on  $I_{Ca}$  because the effect requires ATP and is blocked not only by staurosporine but also by other kinase inhibitors such as K252a and H7 (6). Furthermore, the inhibitory effect of staurosporine is only observed if it is applied before the stimulatory effect of microcystin has occurred. If staurosporine is applied after microcystin has already stimulated the current, staurosporine is unable to inhibit the current. This behavior is consistent with a mech-



**Fig. 6.** Inhibitory effects of microcystin and Fsk on  $I_{Ca}$  and  $I_{Cl}$  at 36° and room temperature. At 10  $\mu$ M, microcystin was perfused internally, and 10  $\mu$ M Fsk was added to the superfusion as indicated. A, Temperature = 36°. The amplitudes of  $I_{Ca}$  (○) and  $I_{Cl}$  (■) are plotted versus time. B, Sample traces from the experiment shown in A. C, Temperature = 22°. Dose-response curve for the effect of calyculin A on  $I_{Ca}$ . ○,  $I_{Ca}$  density stimulated by calyculin A alone. □,  $I_{Ca}$  density in the presence of calyculin and Iso. D, Dose-response curve for the effect of calyculin A on  $I_{Cl}$ . ○,  $I_{Cl}$  density stimulated by calyculin A alone. □,  $I_{Cl}$  density in the presence of calyculin and Iso. Values in C and D are mean of 4–10 cells. \*, Statistical significance from current in the absence of calyculin at 1% level (*t* test).

anism involving microcystin inhibiting a phosphatase and staurosporine inhibiting a kinase because one would expect staurosporine to have no effect once phosphorylation had taken place in the presence of an inhibitor of dephosphorylation (microcystin). It is difficult to propose a nonspecific mechanism of staurosporine action that has this behavior. Because a kinase is clearly involved in the response to microcystin and because the effect of microcystin is blocked by staurosporine, a known kinase inhibitor, we favor the simplest interpretation that staurosporine blocks the effect of microcystin by inhibition of a kinase.

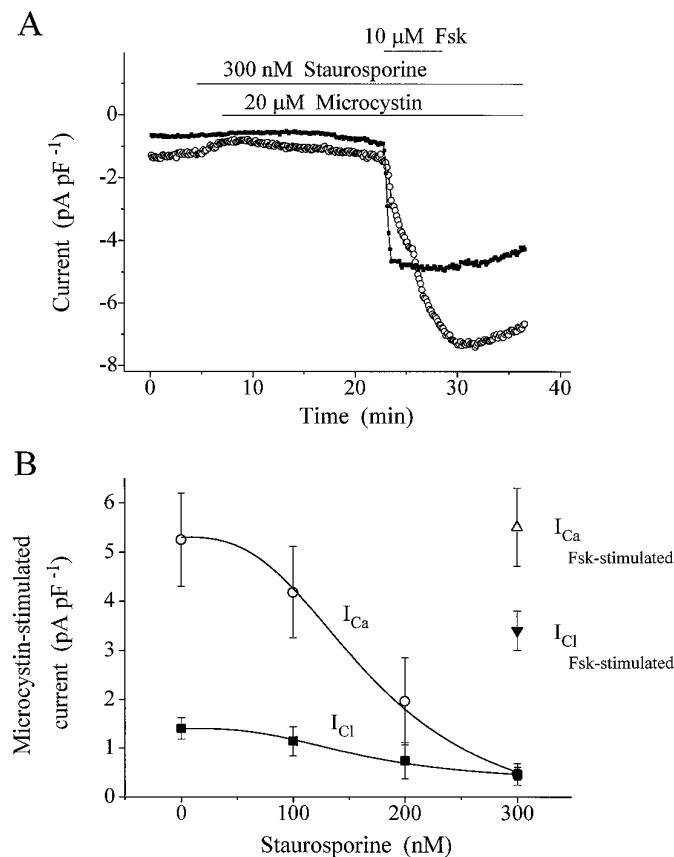
Staurosporine also reduced basal  $I_{Ca}$ . On average, 300 nM staurosporine reduced basal  $I_{Ca}$  by  $29.5 \pm 10.1\%$  (six cells). This decrease did not seem to be rundown because the effect of staurosporine was rapid. This suggests that the basal amplitude of  $I_{Ca}$  may be determined by a kinase that is basally active and inhibited by staurosporine.

**PKA inhibition has no effect on the response of  $I_{Ca}$  and  $I_{Cl}$  to microcystin.** To determine which protein kinases might be responsible for the stimulation of these currents by microcystin, we examined the effects of the competitive PKA inhibitor  $R_p$ -cAMPS on the responses of  $I_{Ca}$  and  $I_{Cl}$  to 10  $\mu$ M microcystin (Fig. 8A). In this experiment, the cells were first perfused internally with 2 mM  $R_p$ -cAMPS, which had very little effect on basal  $I_{Ca}$ . After ~7-min internal perfusion with  $R_p$ -cAMPS, neither  $I_{Ca}$  nor  $I_{Cl}$  responded to 1  $\mu$ M Iso, showing that PKA had been inhibited. However, the subsequent addition of 10  $\mu$ M microcystin to the internal perfusion produced a large stimulation of both  $I_{Ca}$  and  $I_{Cl}$ . To be certain that PKA had been completely inhibited, we also performed this experiment in a different order (Fig. 8B). In this cell,

$R_p$ -cAMPS completely blocked PKA because Fsk had no effect on either  $I_{Ca}$  or  $I_{Cl}$  when added after microcystin perfusion, but microcystin was still capable of increasing both currents. The mean values for 10  $\mu$ M microcystin-stimulated  $I_{Ca}$  and  $I_{Cl}$  in the presence of  $R_p$ -cAMPS were  $6.0 \pm 0.4$  and  $1.1 \pm 0.1$  pA/pF, respectively (six cells). The mean values for 10  $\mu$ M microcystin-stimulated  $I_{Ca}$  and  $I_{Cl}$  without  $R_p$ -cAMPS were  $4.9 \pm 0.5$  and  $1.0 \pm 0.12$  pA/pF, respectively (15 cells). These results show that the effects of microcystin on  $I_{Ca}$  or  $I_{Cl}$  were not mediated by phosphorylation catalyzed by PKA.

**PKC inhibition has no effect on the response of  $I_{Ca}$  and  $I_{Cl}$  to microcystin.** Staurosporine is a nonselective protein kinase inhibitor, and it inhibits PKC *in vitro* at submicromolar concentrations (13). This suggests the possibility that the basally active protein kinase that stimulates  $I_{Ca}$  and  $I_{Cl}$  in the presence of microcystin is PKC. PKC is known to activate  $I_{Ca}$  in guinea pig ventricular myocytes (14), but it is controversial whether PKC regulates  $I_{Cl}$  (3). Fig. 9A shows the effect of the PKC activator TPA (0.1  $\mu$ M) on  $I_{Ca}$  and  $I_{Cl}$ .  $I_{Ca}$  began to increase within 1 min after exposure to TPA and reached 11.3 pA/pF.  $I_{Cl}$  began to increase more slowly than  $I_{Ca}$  and reached a density of 2.3 pA/pF. Thus, it seems that both currents may be regulated by PKC. To determine whether the effect of microcystin could be explained by PKC-dependent phosphorylation, we examined the effects of a highly selective pseudosubstrate PKC inhibitor.

For these experiments, we used the myristoylated derivative of the peptide pseudosubstrate inhibitor PKC<sub>(19–31)</sub>; the myristoylated derivative has been reported to be a more effective inhibitor of PKC because it is targeted to the membrane (15). In Fig. 9B, 100  $\mu$ M myr-PKC<sub>(19–31)</sub> was applied

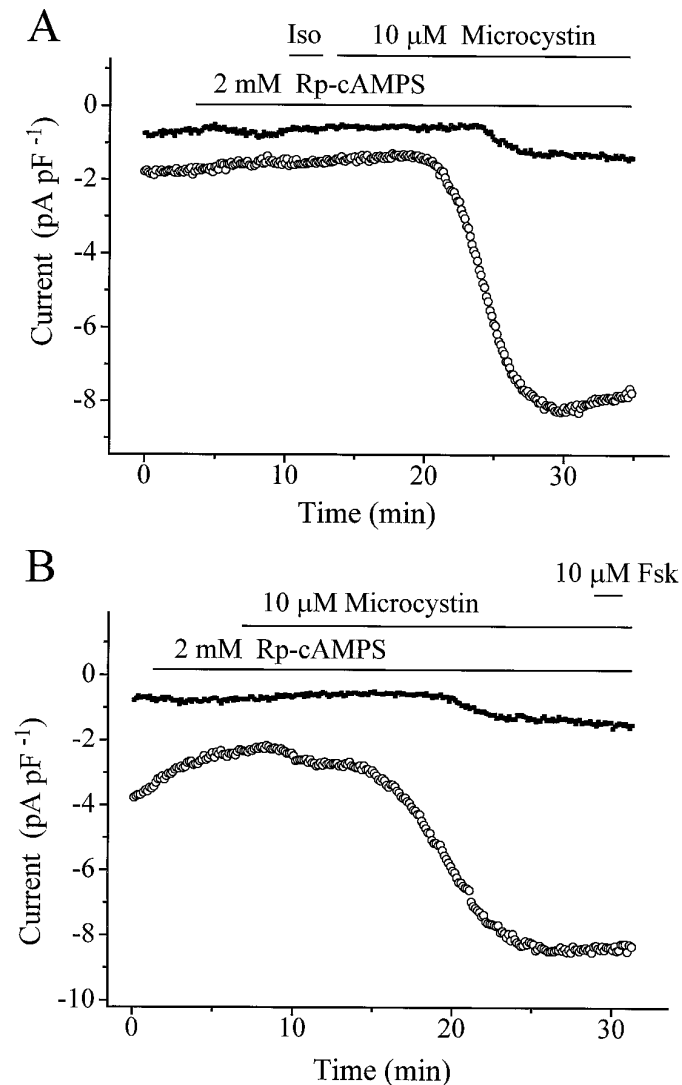


**Fig. 7.** Effect of the protein kinase inhibitor staurosporine on the response of  $I_{Ca}$  and  $I_{Cl}$  to microcystin.  $\circ$ ,  $I_{Ca}$ ;  $\blacksquare$ ,  $I_{Cl}$ . A, 300 nM staurosporine and 10  $\mu$ M Fsk were applied by superfusion, and 20  $\mu$ M microcystin was applied by internal perfusion during the times indicated. Staurosporine decreased basal  $I_{Ca}$  and inhibited the effect of microcystin on  $I_{Ca}$  and  $I_{Cl}$ . Subsequent application of Fsk produced a large increase in  $I_{Ca}$  and  $I_{Cl}$  even in the presence of staurosporine. B, Dose-response curve for the effect of staurosporine on microcystin-stimulated  $I_{Ca}$  and  $I_{Cl}$ . Data were fitted to the power logistic equation (lines).  $EC_{50}$  values were 171.5 nM for  $I_{Ca}$  and 161 nM for  $I_{Cl}$ . Response of  $I_{Ca}$  ( $\Delta$ ) and  $I_{Cl}$  ( $\blacktriangledown$ ) to 10  $\mu$ M Fsk in the presence of 300 nM staurosporine and 20  $\mu$ M microcystin (five cells).

internally with 2 mM  $R_p$ -cAMPS. After a 6-min perfusion with myr-PKC<sub>(19-31)</sub> and  $R_p$ -cAMPS, 0.1  $\mu$ M TPA had no effect on  $I_{Ca}$  or  $I_{Cl}$ . However, 10  $\mu$ M microcystin increased both  $I_{Ca}$  and  $I_{Cl}$  to 7.1 and 1.8 pA/pF, respectively. These results show that stimulation of  $I_{Ca}$  and  $I_{Cl}$  by microcystin is not mediated by PKC.

## Discussion

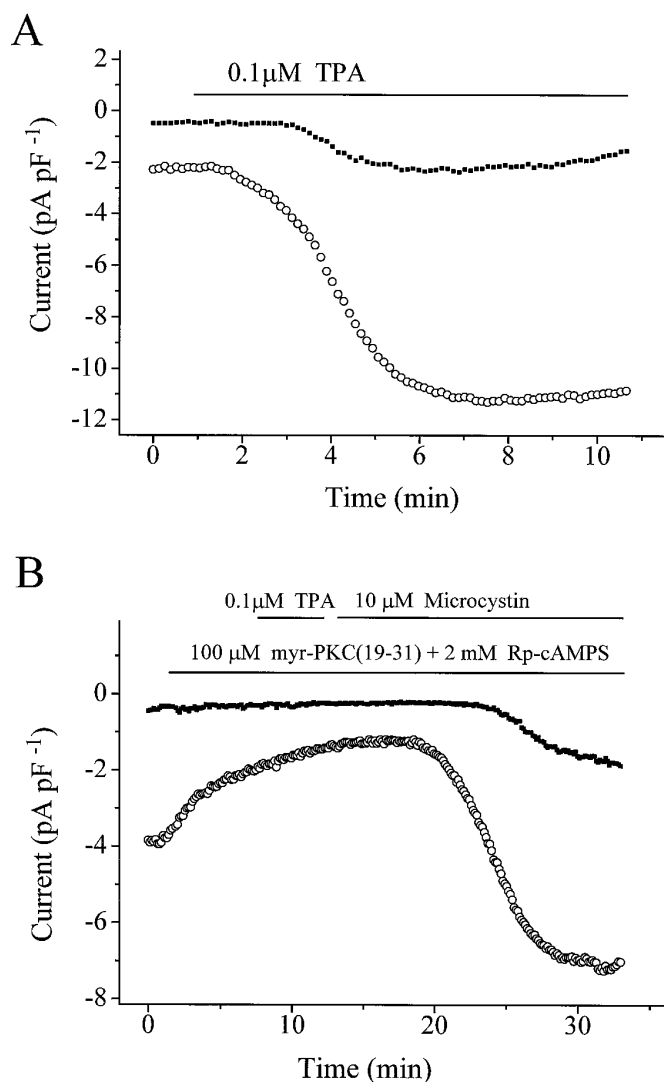
**$I_{Ca}$  and  $I_{Cl}$  are regulated independently by phosphorylation.** These data show that although both  $I_{Ca}$  and  $I_{Cl}$  are regulated by PKA phosphorylation, their regulation is not necessarily coordinate. For example, the increases in amplitude of  $I_{Ca}$  and  $I_{Cl}$  produced by Iso occur with different kinetics.  $I_{Cl}$  increases more rapidly than  $I_{Ca}$  in response to stimulation by Iso; the washout of the Iso effect is also faster. If we assume that the rate-limiting step regulating the amplitudes of  $I_{Ca}$  and  $I_{Cl}$  is a phosphorylation reaction,  $D \rightleftharpoons P$ , then the rate of increase in the current will be proportional to  $\alpha + \beta$  and deactivation will be proportional to  $\beta$ , where  $\alpha$  is the forward rate constant and  $\beta$  is the backward rate con-



**Fig. 8.** Effect of the PKA inhibitor  $R_p$ -cAMPS on the response of  $I_{Ca}$  and  $I_{Cl}$  to 10  $\mu$ M microcystin and 1  $\mu$ M Iso (A) or 10  $\mu$ M Fsk (B).  $\circ$ ,  $I_{Ca}$ ;  $\blacksquare$ ,  $I_{Cl}$ . Cells were first perfused internally with 2 mM  $R_p$ -cAMPS, which had no effect on basal  $I_{Ca}$ . A, Response to Iso and microcystin. B, Response to microcystin and Fsk.

stant. Because both the stimulation and washout of the Iso effect were faster for  $I_{Cl}$ , we conclude that the dephosphorylation reaction for  $I_{Cl}$  is faster than that for  $I_{Ca}$ . We do not currently have any insights into why dephosphorylation of  $I_{Cl}$  may be faster than  $I_{Ca}$ ; possibilities include different phosphatase isoforms responsible for dephosphorylation of the two channels or differences in the rate of dephosphorylation of the different substrates by the same phosphatase.

**$I_{Ca}$  and  $I_{Cl}$  are regulated by PKA and another protein kinase.** The observation that  $I_{Cl}$  was more rapidly dephosphorylated than  $I_{Ca}$  suggested that application of a protein phosphatase inhibitor might result in a larger basal stimulation of  $I_{Cl}$  than  $I_{Ca}$ . However, we found that  $I_{Cl}$  increased much less and more slowly than  $I_{Ca}$  in response to protein phosphatase inhibitors. This difference in the kinetics of response of the two currents to Iso and microcystin was explained by the fact that the increase in both basal currents produced by microcystin was not caused by PKA but by another, as yet unidentified, protein kinase. The fact that the



**Fig. 9.** Regulation of  $I_{Ca}$  and  $I_{Cl}$  by PKC and microcystin.  $\circ$ ,  $I_{Ca}$ ;  $\blacksquare$ ,  $I_{Cl}$ . A, Effects of the PKC activator TPA on  $I_{Ca}$  and  $I_{Cl}$ . At  $0.1 \mu\text{M}$ , TPA was applied to the bath solution during the time indicated (bar).  $I_{Ca}$  began to increase within 1 min after exposure of TPA.  $I_{Cl}$  began to increase slower than  $I_{Ca}$ . B, Effects of a PKC pseudosubstrate inhibitor on the response of  $I_{Ca}$  and  $I_{Cl}$  to microcystin;  $100 \mu\text{M}$  myr-PKC $_{(19-31)}$  was applied internally with  $2 \text{ mM}$  Rp-cAMPS. After a 6-min perfusion with myr-PKC $_{(19-31)}$  and Rp-cAMPS,  $0.1 \mu\text{M}$  TPA had no effect on basal  $I_{Ca}$  and  $I_{Cl}$ . Subsequent internal perfusion with  $10 \mu\text{M}$  microcystin increased both  $I_{Ca}$  and  $I_{Cl}$  to  $7.1$  and  $1.8 \text{ pA/pF}$ , respectively.

effects of microcystin are blocked by low concentrations of the nonselective protein kinase inhibitor staurosporine, however, argues that the effect of microcystin is mediated through a phosphorylation reaction. This protein kinase is clearly not PKA because it is not inhibited by PKA inhibitors, which in the same cell do inhibit the effect of Iso or Fsk. Likewise, the kinase is not PKC because the peptide inhibitors of PKC that inhibit the effects of TPA in the same cell do not inhibit the effects of microcystin. Finally, it is unlikely that the kinase is a  $\text{Ca}^{2+}$ -dependent kinase because the cells were perfused internally with  $10 \text{ mM}$  EGTA, which should inhibit the activation of  $\text{Ca}^{2+}$ -dependent protein kinases. This novel protein kinase is very similar to one we described in frog ventricular myocytes and have termed PKX (6, 11).

We believe that the microcystin-stimulated current at  $-50$

mV is the CFTR  $\text{Cl}^-$  current (16–18) for the following reasons. (1) The current at  $-50 \text{ mV}$  that is stimulated by microcystin is a  $\text{Cl}^-$  current because it has a reversal potential near  $E_{\text{Cl}}$  and the reversal potential changes as predicted with changes in  $[\text{Cl}^-]_o$ . Furthermore, the current-voltage relationship is the same as reported for the cAMP-activated  $\text{Cl}^-$  current. (2) The current does not resemble other  $\text{Cl}^-$  currents that have been described in cardiac muscle, including a stretch-activated  $\text{Cl}^-$  current (19, 20), a  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  current (21, 22), and a PKC-activated  $\text{Cl}^-$  current (23, 24). Collier and Hume (25) recently reported that this PKC-activated  $\text{Cl}^-$  current was the CFTR  $\text{Cl}^-$  current in guinea pig ventricular myocytes. It is unlikely that the current is a  $\text{Ca}^{2+}$ -activated  $\text{Cl}^-$  current because intracellular  $\text{Ca}^{2+}$  is highly buffered with EGTA. Furthermore, the fact the microcystin-stimulated current is time independent and requires phosphorylation to be activated suggests it is the CFTR  $\text{Cl}^-$  current.

Differences in the rates of phosphorylation/dephosphorylation by PKA and PKX could be due to differences in the association of kinases and phosphatases with  $\text{Cl}^-$  and  $\text{Ca}^{2+}$  channels. For example, PKX might be more closely associated with  $\text{Ca}^{2+}$  channels than  $\text{Cl}^-$  channels such that  $\text{Ca}^{2+}$  channels are phosphorylated preferentially. This possibility is made more attractive by recent studies that have identified several “scaffold” proteins that bind and target a variety of signaling enzymes to appropriate locations in the cell (26, 27). Recently, it was shown that voltage-dependent potentiation of L-type  $\text{Ca}^{2+}$  channels requires a cAMP-anchoring protein (28) and that L-type  $\text{Ca}^{2+}$  channels are associated with a 15-kDa cAMP-anchoring protein (29). Thus, differential regulation of different ion channels by protein kinases and phosphatases may be affected by differential localization of the kinases and phosphatases to the immediate neighborhood of the channel.

**Physiological role of PKX.** We hypothesize that the role of PKX in ventricular myocytes may be to regulate the basal level of  $I_{Ca}$ . Our reasons for suggesting this hypothesis are discussed below. The basal level of  $I_{Ca}$  is not determined by PKA-dependent phosphorylation because we find that internal perfusion of guinea pig myocytes with the PKA inhibitor Rp-cAMPS did not have a significant effect on basal  $I_{Ca}$  (see, for example, Fig. 8A). Ono and Fozzard (30) reported that the phosphatase inhibitor okadaic acid slowed rundown of  $\text{Ca}^{2+}$  channel activity in excised inside-out patches. They suggested that dephosphorylation was an important component of rundown and that phosphorylation was needed for channel-opening activity under basal conditions. They speculated that the intact cell may have protein kinases other than PKA that could maintain some level of phosphorylation. This is consistent with our results, and we conclude PKX may be important for maintaining basal phosphorylation of  $I_{Ca}$  in guinea pig ventricular myocytes. In support of this suggestion is the observation that  $300 \text{ nM}$  staurosporine reduced basal  $I_{Ca}$ , which may reflect basal activation of  $I_{Ca}$  by PKX.

Hescheler *et al.* (31, 32) have also reported that cell dialysis with protein phosphatase 1 inhibitor or okadaic acid stimulated basal  $I_{Ca}$  and suggested that there was a high activity of intracellular protein kinase even in the absence of agonist that was normally counteracted by a high level of protein phosphatase activity.

The role of PKX in regulating  $I_{Cl}$  is less clear.  $I_{Cl}$  is stim-

ulated much more slowly and to a much lesser extent than  $I_{Ca}$ . Thus, it is not clear that this phosphorylation is physiologically relevant. The observation that  $I_{Cl}$  is activated slowly by phosphatase inhibitors suggests that the  $Cl^-$  channel is also a substrate for PKX but that either the  $Cl^-$  channel is a relatively poor substrate for PKX or protein phosphatases that are insensitive to microcystin keep the accumulation of the phosphorylated state low. Cheng *et al.* (33) detected basal levels of phosphorylation in CFTR  $Cl^-$  channel and suggest that basal phosphorylation sites do not represent PKA sites in CFTR  $Cl^-$  channel. In contrast, Nakashima and Ono (34) observed that the phosphatase inhibitor okadaic acid increased  $I_{Cl}$ , but they speculated that this was due to basal activity of PKA.

**Inhibitory effects of PKA stimulation in the presence of protein phosphatase inhibitors.** Another novel finding of these studies is that Fsk or Iso in the presence of microcystin or calyculin A seemed to have a dual effect on  $I_{Ca}$ ; initially, Fsk or Iso stimulated  $I_{Ca}$ , but then it initiated a run-down of  $I_{Ca}$ . This decrease in  $I_{Ca}$  was not paralleled by any change in  $I_{Cl}$ , suggesting that run-down of  $I_{Ca}$  was not due to deterioration of the cell or to activation of a nonselective microcystin-insensitive phosphatase. The effect was observed with both Fsk and Iso, suggesting that the effect was not due to a pharmacological effect of Fsk but rather to activation of the cAMP cascade. This run-down phenomenon was much more pronounced at 36°. The decrease in the amplitude was accompanied by increased inactivation of the current, supporting the idea that the effect was specific.

In conclusion, we found that  $I_{Ca}$  and  $I_{Cl}$  are stimulated by phosphatase inhibitors in the absence of  $\beta$ -adrenergic stimulation. The conclusion that this stimulatory effect is mediated by a basally active kinase is supported by the observation that this effect is inhibited by a protein kinase inhibitor. The kinase is not PKA nor PKC because it is not inhibited by PKA or PKC inhibitors. We speculate that this kinase, which we term PKX, contributes to set the basal level of  $I_{Ca}$  but plays a lesser role in regulation of the CFTR  $Cl^-$  channel.

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