

Effects of Protein Phosphatase and Kinase Inhibitors on the Cardiac L-type Ca Current Suggest Two Sites Are Phosphorylated by Protein Kinase A and Another Protein Kinase

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ABSTRACT We previously showed (Frace, A. M. and H. C. Hartzell. 1993. *Journal of Physiology*. 472:305–326) that internal perfusion of frog atrial myocytes with the nonselective protein phosphatase inhibitors microcystin or okadaic acid produced an increase in the L-type Ca current (I_{Ca}) and a decrease in the delayed rectifier K current (I_K). We hypothesized that microcystin revealed the activity of a protein kinase (PKX) that was basally active in the cardiac myocyte that could phosphorylate the Ca and K channels or regulators of the channels. The present studies were aimed at determining the nature of PKX and its phosphorylation target. The effect of internal perfusion with microcystin on I_{Ca} or I_K was not attenuated by inhibitors of protein kinase A (PKA). However, the effect of microcystin on I_{Ca} was largely blocked by the nonselective protein kinase inhibitors staurosporine (10–30 nM), K252a (250 nM), and H-7 (10 μ M). Staurosporine and H-7 also decreased the stimulation of I_{Ca} by isoproterenol, but K252a was more selective and blocked the ability of microcystin to stimulate I_{Ca} without significantly reducing isoproterenol-stimulated current. Internal perfusion with selective inhibitors of protein kinase C (PKC), including the autoinhibitory pseudosubstrate PKC peptide (PKC₍₁₉₋₃₁₎) and a myristoylated derivative of this peptide had no effect. External application of several PKC inhibitors had negative side effects that prevented their use as selective PKC inhibitors. Nevertheless, we conclude that PKX is not PKC. PKA and PKX phosphorylate sites with different sensitivities to the phosphatase inhibitors calyculin A and microcystin. In contrast to the results with I_{Ca} , the effect of microcystin on I_K was not blocked by any of the kinase inhibitors tested, suggesting that the effect of microcystin on I_K may not be mediated by a protein kinase but may be due to a direct effect of microcystin on the I_K channel.

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INTRODUCTION

The L-type Ca current (I_{Ca}) plays a key role in excitation-contraction coupling in the heart and is one of the most important targets for the autonomic neurotransmitters that regulate the force of cardiac contraction (Hartzell and Duchatelle-Gourdon, 1992). Stimulation of β -adrenergic receptors increases I_{Ca} by stimulating cAMP-dependent phosphorylation via the G_s -adenylyl cyclase-cAMP-cAMP dependent protein kinase cascade (Hartzell, 1988; Trautwein and Hescheler, 1990). Muscarinic acetylcholine receptors antagonize the effects of β -adrenergic receptors by inhibiting cAMP synthesis in a G_i -dependent manner. The involvement of the cAMP-dependent phosphorylation pathway in effecting the action of β -adrenergic receptors has been well established by experiments showing that β -receptors stimulate adenylyl cyclase and cAMP-dependent protein kinase (PKA), that forskolin (a direct stimulator of adenylyl cyclase), exogenous cAMP, and exogenous catalytic subunit of cyclic AMP dependent protein kinase mimic the effects of β -adrenergic agonists, and that inhibitors of PKA completely inhibit the effects of β -adrenergic agonists on I_{Ca} .

Despite the considerable evidence for the involvement of cAMP-dependent phosphorylation in regulation of I_{Ca} , there remain a number of questions (Hartzell, 1993). First, it is unclear whether the α_1 subunit of the Ca channel is the final target for cAMP-dependent phosphorylation or whether there are other intermediates (possibly other subunits of the Ca channel) between the phosphorylated substrate and the Ca channel itself. Secondly, there is evidence to suggest that the Ca channel may be regulated by other mechanisms in addition to cAMP-dependent phosphorylation. For example, it has been proposed that the Ca channel may be regulated directly by $G_s\alpha$ (Brown and Birnbaumer, 1990), although this remains controversial (Hartzell and Fischmeister, 1992). In addition, the Ca channel is regulated by protein kinase C (Lacerda, Rampe, and Brown, 1988; Walsh, Begenisich, and Kass, 1989; Singer-Lahat, Gershon, Lotan, Hullin, Biel, Flockerzi, Hofmann, and Dascal, 1992) and this phosphorylation has been suggested to mediate the effects of angiotensin II (Dösemeçi, Dhallan, Cohen, Lederer, and Rogers, 1988) and possibly α_1 adrenergic agonists as well, although this is less certain (Dirksen and Sheu, 1990).

Recently, we showed that nonselective protein phosphatase inhibitors, such as okadaic acid and microcystin, in the absence of β -adrenergic stimulation, cause a large increase in I_{Ca} and a decrease in the delayed rectifier K current, I_K (Frace and Hartzell, 1993). The kinase(s) responsible for these effects were not identified, although we did show that the increase in I_{Ca} was likely to be caused by phosphorylation because the effect was dependent upon intracellular ATP. The purpose of the present experiments was to determine the nature of the protein kinase revealed by protein phosphatase inhibition and to determine whether this protein kinase has a physiological role in the regulation of the Ca or K currents.

METHODS

Cell isolation and electrophysiological techniques have been described in detail previously (Fischmeister and Hartzell, 1986; Duchatelle-Gourdon et al., 1989; Hartzell et al., 1991). Briefly, frog

(*Rana catesbiana*) ventricular and atrial cells were enzymatically isolated and patch clamped by the whole-cell patch clamp technique. Internal solution for recording I_{Ca} from ventricular cells was (in millimolar) 118 CsCl, 4 MgCl₂, 2.8 Na₂K₂ATP, 10 K₂PIPES, 5 Na-creatine phosphate, 5 K₂EGTA, pH to 7.15 with KOH. For recording I_K and I_{Ca} from atrial cells the internal solution contained 118 KCl, 4 MgCl₂, 2.8 Na₂K₂ATP, 10 K₂PIPES, 5 Na-creatine phosphate, 5 K₂EGTA, pH to 7.15 with KOH. Various test compounds were applied internally via pipet perfusion. External solution for ventricular cells was 103 NaCl, 20 CsCl, 10 HEPES, 1.8 CaCl₂, 1.8 MgCl₂, 5 pyruvic acid, 5 D-glucose, 0.3 μM tetrodotoxin, pH to 7.4 with NaOH. External solution for atrial cells replaced CsCl with NaCl and contained, in addition, 2.5 mM KCl. All experiments were conducted at room temperature. Cells were patch clamped with borosilicate patch pipets having resistances of 1–2 MΩ. Total series resistance was usually <3 MΩ. I_{Ca} and I_K were elicited at various frequencies by voltage pulses delivered by a programmable digital stimulator (Challenger DB, W. Goolsby, Kinetic Software and Emory University, Atlanta, GA). Routine pulses were from –80 mV holding potential to 0 or to +20 mV. In ventricular cells, the pulse was 200–400 ms in duration and I_{Ca} was measured as the peak inward current minus the current at the end of the pulse. In atrial cell experiments, I_{Ca} was measured by subtracting the current at 200 ms from the peak inward current and I_K was measured by subtracting the current at 200 ms from the outward current at the end of a 10–20-s duration pulse.

Reagents included cAMP-dependent protein kinase inhibitor peptide, PKI(5-22) [TTYADFIAS-GRTGRRNAIHD-NH₂] (Promega Corp., Madison, WI); microcystin-LR [cyclo-(D-alanine)-(L-leucine)-(D-erythro-β-methylaspartic acid)-(L-arginine)-(3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6-dienoic acid)-(D-glutamic acid)-(N-methyldehydroalanine)] (GIBCO BRL, Gaithersburg, MD); H-7 [(±)-1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride] (LC Laboratories, Woburn, MA); protein kinase C autoinhibitory peptide, PKC₍₁₉₋₃₁₎ [RFARKGALRQKNV] (LC Laboratories); myristoylated protein kinase C autoinhibitory peptide [myr-RFARKGALRQKNV] (Promega Corp.); adenosine cyclic 3',5'-(R_p)-phosphorothioate (provided by Dr. Ira Cohen, SUNY, Stony Brook, NY); K252a (Calbiochem Corp., LaJolla, CA); staurosporine (Calbiochem Corp.); calphostin C (LC Laboratories); forskolin (Calbiochem Corp.); calyculin A (LC Laboratories); acetylcholine (Sigma Chemical Co., St. Louis, MO). Stock solutions of staurosporine, K252a, and calphostin C were prepared at 1 mM in DMSO. Microcystin was made 0.5 mM in 10% methanol. Other peptides were dissolved in water at 1–5 mM. Forskolin was 10 mM in 100% ethanol. Acetylcholine was 1 M in 0.1 mM HCl. Stock solutions were stored at –20°C.

RESULTS

PKA Inhibition Has No Effect on Responses to Microcystin

Fig. 1 A is a control experiment showing the effect of internal perfusion with microcystin on I_{Ca} and I_K in a frog atrial myocyte. In this experiment, the cell was first exposed to 10 μM forskolin, which increased I_{Ca} ~16-fold and I_K ~2.5-fold. After washing out forskolin, the cell was perfused internally with 10 μM microcystin-LR (Rinehart, Harada, Namikoshi, Chen, and Harvis, 1988; Honkanen, Zwiller, Moore, Daily, Khatra, Dukelow, and Boynton, 1990; MacKintosh, Beattie, Klumpp, Cohen, and Codd, 1990). This resulted in a ~10-fold increase in I_{Ca} and a ~50% decrease in I_K . As we have published previously (Frace and Hartzell, 1993), microcystin does not change the shape of the *I-V* curve for I_{Ca} or the steady state activation curve for I_K . The effect of microcystin is to increase I_{Ca} and decrease I_K at all potentials where these currents are activated. Although microcystin affects I_{Ca} and I_K with similar time courses in Fig. 1 A, the effect of microcystin on I_K was frequently more rapid

than the effect on I_{Ca} . This is illustrated for an extreme example in Fig. 1 *B*. This cell was perfused with 20 μ M microcystin, and the subsequent change in I_K and I_{Ca} were fitted to single exponentials. The decrease in I_K occurred with a $\tau = 1.06$ min, whereas the effect on I_{Ca} occurred with a $\tau = 10.4$ min. In four cells where we fitted the change in current amplitude to exponentials, I_K decreased with $\tau = 2.5 \pm 0.6$ min and I_{Ca} increased with a $\tau = 7.5 \pm 1.9$ min.

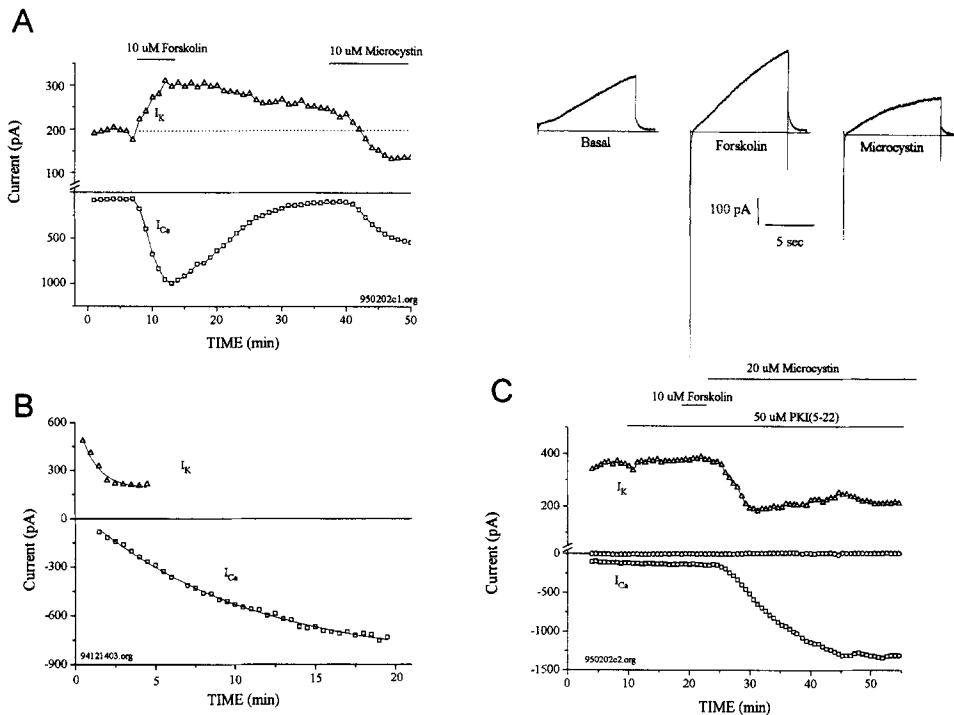


FIGURE 1. Effect of a PKA inhibitor on response to microcystin in frog atrial myocytes. (*A*) Control effect of microcystin on I_{Ca} and I_K . A patch-clamped atrial myocyte was stimulated once every 30 s with 10-s duration pulses to 0 mV from a holding potential of -80 mV. I_{Ca} (open squares) was measured as the difference between peak inward current and current at 200 ms. I_K (open triangles) was measured as outward current at the end of the pulse minus the current at 200 ms. Holding current (open circles) was measured at -80 mV. 10 μ M forskolin was applied to the superfusion during the bar. After forskolin was washed out, 10 μ M microcystin-LR was applied by internal perfusion. Internal perfusion was begun at the time indicated, but measurements with fluorescent dyes showed that 1–5 min was required for the new solution to reach the tip of the pipette depending on the flow rate of the internal perfusion system and the proximity of the internal capillary to the pipette tip. Representative current traces are shown for control, forskolin, and microcystin. Although it appears that the activation kinetics of I_K are accelerated by microcystin, this is not a consistent observation. Often, such acceleration is observed spontaneously during the course of an experiment without any drug exposure. (*B*) Time course of the effect of microcystin on I_{Ca} and I_K . A cell was perfused internally with 20 μ M microcystin as in *A*. The record shown begins with the first current that is different from basal after adding microcystin to the internal perfusion. The changes in current amplitudes were fitted to single exponentials. (*C*) Effect of 50 μ M PKI₍₅₋₂₂₎ on the response to 20 μ M microcystin and 10 μ M forskolin. The effect of forskolin is inhibited, whereas the response to microcystin is unaffected.

Fig. 1 C shows that neither the effect on I_{Ca} nor the effect on I_K was mediated by phosphorylation catalyzed by PKA, because internal perfusion with 50 μ M PKI₍₅₋₂₂₎ (Cheng, Kemp, Pearson, Smith, Misconi, VanPatten, and Walsh, 1986) did not affect the response to microcystin. As a positive control to show that PKI₍₅₋₂₂₎ actually inhibited PKA, we show in Fig. 1 C that the response to 10 μ M forskolin is blocked by this inhibitor. These results confirm our previous preliminary conclusions (Frace and Hartzell, 1993) and show clearly that the changes in I_{Ca} and I_K produced by microcystin are not due to inhibition of dephosphorylation of a site phosphorylated by PKA. For convenience, the presumptive protein kinase responsible for the microcystin effect is termed PKX.

Staurosporine Blocks the Effect of Microcystin on I_{Ca}

We screened several protein kinase inhibitors to determine their ability to block the effects of microcystin. Initially, we examined the effect of these inhibitors only on I_{Ca} in ventricular myocytes. Both staurosporine (Tamaoki, Nomoto, Takahashi, Kato, Morimoto, and Tomita, 1986; Rüegg and Burgess, 1989) and K252a (Kase, Iwahashi, Nakanishi, Matsuda, Yamada, Takahashi, Murakata, Sato, and Kaneko, 1987; Rüegg and Burgess, 1989) were able to block the effect of microcystin on I_{Ca} . Fig. 2 illustrates the effects of staurosporine. Staurosporine was applied extracellularly at concentrations between 3 and 100 nM before internal perfusion with microcystin was begun. At concentrations ≥ 30 nM, staurosporine almost completely attenuated the increase in I_{Ca} produced by microcystin (Fig. 2 A). On average, in the presence of 30 nM staurosporine, microcystin increased I_{Ca} only 0.97 ± 0.4 -fold ($n = 3$) compared to a normal 10.9 ± 1.0 -fold ($n = 6$) increase in the absence of staurosporine. Even though 30 nM staurosporine nearly completely blocked the response to microcystin, subsequent exposure to Iso (with 30 nM staurosporine outside and 10 μ M microcystin inside) stimulated I_{Ca} 10.0 ± 3.3 -fold ($n = 3$). This level of stimulation was somewhat less than the 19.4 ± 2.2 -fold ($n = 4$) increase in current produced by Iso and microcystin together in the absence of staurosporine. It should be noted that fold changes cited in the text are derived from measuring changes in current amplitudes in response to an intervention in individual cells and then computing the average. This "average change" (cited in the text) usually differs somewhat from the difference between "average current densities" (plotted in the figures) under different conditions. Fig. 2 A also shows that I_{Ca} is irreversibly stimulated by Iso in the presence of microcystin. When Iso is washed out, I_{Ca} remains elevated for at least 5 min. A similar example is shown in Fig. 5 B, although in this case, I_{Ca} did decrease a small amount over a 10-min time period.

The experiment of Fig. 2 A suggested that staurosporine inhibits the protein kinase that is responsible for stimulation of I_{Ca} by microcystin but has only a small effect on PKA. This conclusion is surprising considering that staurosporine has been shown to inhibit PKA at these concentrations. However, this interpretation requires circumspection for the following reason. If PKA activity is not completely inhibited by staurosporine, the fraction that remains active could be sufficient to increase I_{Ca} , because every phosphorylated substrate molecule will be "trapped" in the phosphorylated state as a result of inhibition of the protein phosphatase. For this reason, to compare the selectivity of staurosporine for PKA and PKX it was nec-

essary to examine the effect of staurosporine on the Iso response in the absence of phosphatase inhibitors. Fig. 2 *B* shows that in the absence of microcystin, staurosporine produced a dose-dependent inhibition of the Iso response. The IC_{50} for the effect of staurosporine on Iso-stimulated I_{Ca} was 12 nM (shown qualitatively in

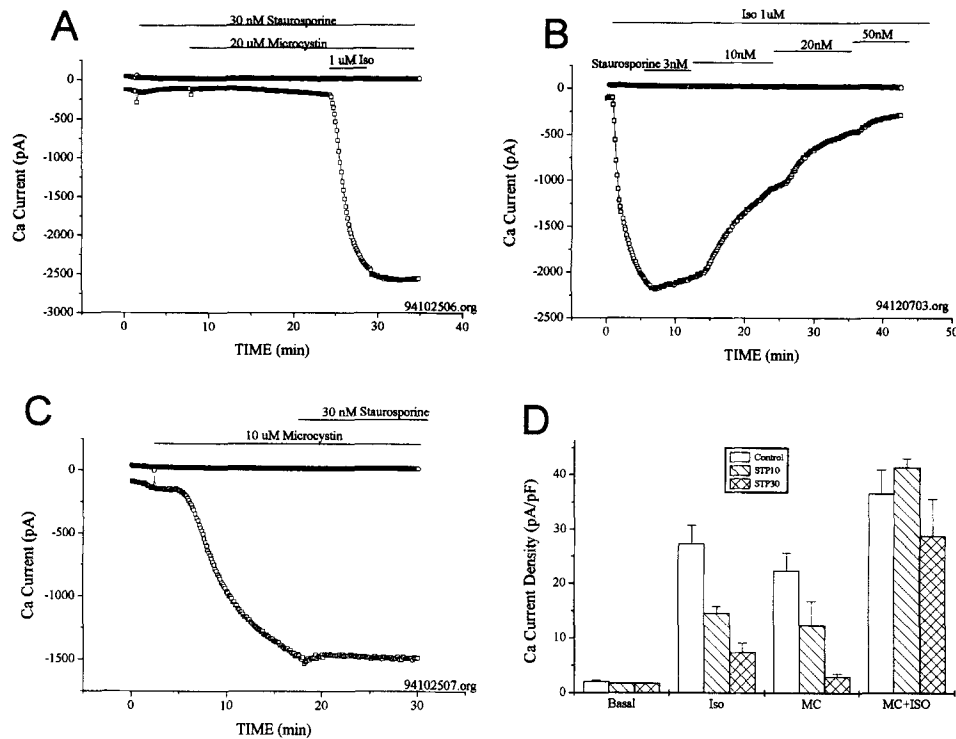


FIGURE 2. Effect of staurosporine on response to microcystin in frog ventricular myocyte. (A–C) Patch clamp records obtained as described in Fig. 1, legend. Holding current: *open circles*. I_{Ca} : *open squares*. 30 nM staurosporine and 1 μ M isoproterenol were applied by superfusion and microcystin was applied by internal perfusion during the times indicated. (A) Effect of microcystin and isoproterenol on I_{Ca} after exposure to staurosporine. (B) Effect of staurosporine on I_{Ca} previously stimulated by isoproterenol. (C) Effect of staurosporine on I_{Ca} previously stimulated by microcystin. (D) Summary of the effect of staurosporine on I_{Ca} . Current densities are plotted for control conditions (*open bars*) and after exposure to 10 nM staurosporine (*hatched bars*) or 30 nM staurosporine (*cross-hatched bars*). Basal current was measured before exposure to any other drug. The Iso columns were obtained by first stimulating I_{Ca} with Iso and then sequentially testing the responses to different concentrations of staurosporine as in Fig. 2 *B*. The microcystin (MC) and microcystin + Iso (MC+ISO) columns were obtained by first exposing the cell to staurosporine and then testing the effect of microcystin and microcystin plus Iso as in Fig. 2 *A*. Means are averages of 4–18 cells.

Fig. 2 *D*, but quantitative data not shown). Quantitative analysis of dose-response curves such as the one in Fig. 2 *B* were slightly compromised by the slow effect of staurosporine and failure to reach a true steady state, even after 10 min staurosporine exposure. Nevertheless, we estimate that the error introduced by failure to

reach true steady state is <10%. These data show clearly that the potency of staurosporine to inhibit stimulation of I_{Ca} by either Iso alone or microcystin is similar. In contrast, staurosporine has little effect on the ability of Iso to stimulate the current in the presence of microcystin.

Our interpretation of the experiments of Fig. 2, *A* and *B*, were based on the assumption that staurosporine was inhibiting microcystin- and Iso-stimulated I_{Ca} by inhibiting a protein kinase. To test whether staurosporine might have other, possibly direct effects on the Ca channel, we examined the effect of staurosporine on I_{Ca} previously elevated by microcystin. We reasoned that after microcystin had completely inhibited phosphatase activity and all the substrate was phosphorylated, inhibition of the protein kinase would have no effect. In Fig. 2 *C*, staurosporine was applied somewhat before the effect of microcystin was complete. This application of staurosporine arrested the continuing increase in I_{Ca} , but did not reduce I_{Ca} below the level that had already been stimulated by microcystin. This result shows that staurosporine does not inhibit microcystin stimulation of I_{Ca} by acting directly on the Ca channel, for example.

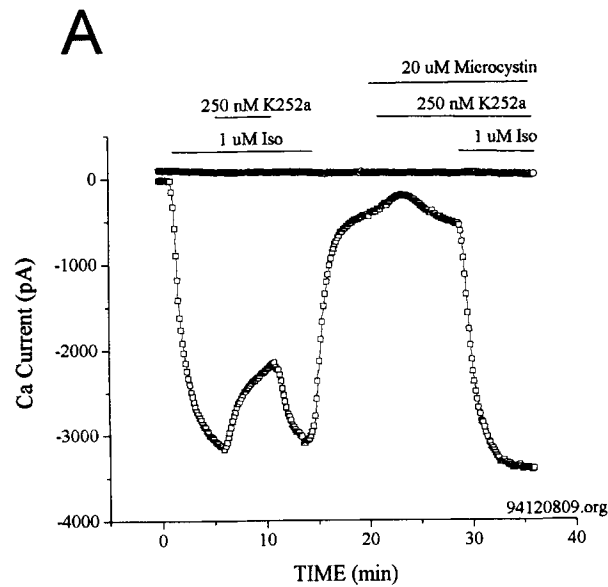
The effects of staurosporine are summarized in Fig. 2 *D*. Staurosporine at both 10 and 30 nM significantly reduced the stimulation of I_{Ca} by microcystin and decreased I_{Ca} previously elevated by Iso. These data show that staurosporine does not selectively inhibit PKX over PKA. Staurosporine also reduced basal I_{Ca} . On average, 30 nM staurosporine reduced basal I_{Ca} $44 \pm 10\%$ ($n = 3$). This decrease was not due to rundown, because in most cells I_{Ca} increased during the first 20 min of the experiment.

K252a Selectively Blocks the Effect of Microcystin on I_{Ca}

The protein kinase inhibitor K252a, which is closely related to staurosporine (Rüegg and Burgess, 1989), was more selective in inhibiting microcystin stimulation of I_{Ca} than was staurosporine. In Fig. 3 *A*, 250 nM K252a inhibited Iso stimulated I_{Ca} only ~30%, but strongly inhibited the stimulation produced by microcystin. On average, Iso-stimulated I_{Ca} was reduced only $18 \pm 11\%$ ($n = 4$) by 250 nM K252a, whereas microcystin-stimulation of I_{Ca} was reduced 70%. Like staurosporine, K252a had much less effect on the response to Iso in the presence of microcystin (Fig. 3, *A* and *B*) and caused a $37 \pm 16\%$ ($n = 4$) decrease in basal I_{Ca} (Figs. 3 *B* and 4 *A*).

Staurosporine and K252a Do Not Block Effect of Microcystin on I_K

The next question was whether the parallel decrease in I_K caused by microcystin was also due to the same protein kinase that increased I_{Ca} . Fig. 4 *A* shows that 250 nM K252a did not inhibit the effect of internal perfusion with microcystin on I_K in an atrial cell. Internal perfusion with microcystin in the presence of K252a caused a ~60% decrease in I_K , even though stimulation of I_{Ca} was largely inhibited. On average, 20 μ M microcystin produced a somewhat greater decrease in I_K in the presence of K252a than under control conditions (Fig. 4 *F*). Thus, the effect of microcystin on I_K could be due to the action of a protein kinase that is not inhibited by K252a or might be due to another (possibly direct) effect of microcystin on I_K . We prefer the hypothesis that the decrease in I_K produced by microcystin is not medi-



B

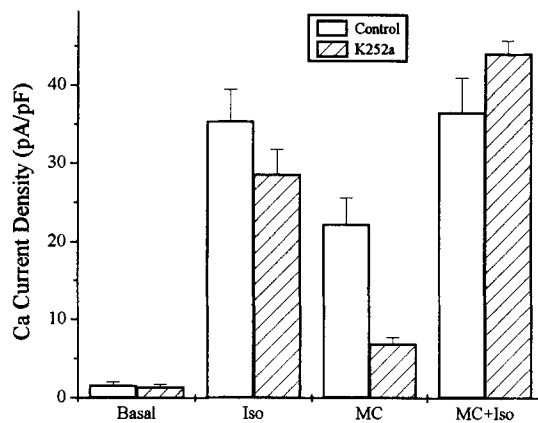


FIGURE 3. Effect of K252a on response of I_{Ca} to microcystin in frog ventricular myocytes. (A) Patch clamp record. 250 nM K252a and 1 μ M Iso were applied by superfusion and 20 μ M microcystin was applied by internal perfusion during the periods indicated. (Open circles) Holding current. (Open squares) I_{Ca} . (B) Summary of effects of K252a on I_{Ca} in ventricular myocytes. See Fig. 2 D for details. (Open bars) Control. (Hatched bars) 250 nM K252a. Means are averages of 4–8 cells.

ated by the action of a protein kinase, because we have not been unable to identify any protein kinase inhibitor that reduces the effect of microcystin on I_K . For example, microcystin also reduced I_K at least 40% in the presence of 100 nM staurosporine (Fig. 4 B), which did inhibit the response of I_{Ca} to Iso or microcystin (Fig. 2, A and B). The effects of K252a in atrial cells are summarized for I_{Ca} in Fig. 4, C and D and for I_K in Fig. 4, E and F. The effects of K252a on I_{Ca} are very similar in atrial and ventricular cells (compare Fig. 3 B with Fig. 4, C and D). However, K252a has little or no effect on the ability of microcystin to inhibit I_K (Fig. 4, E and F).

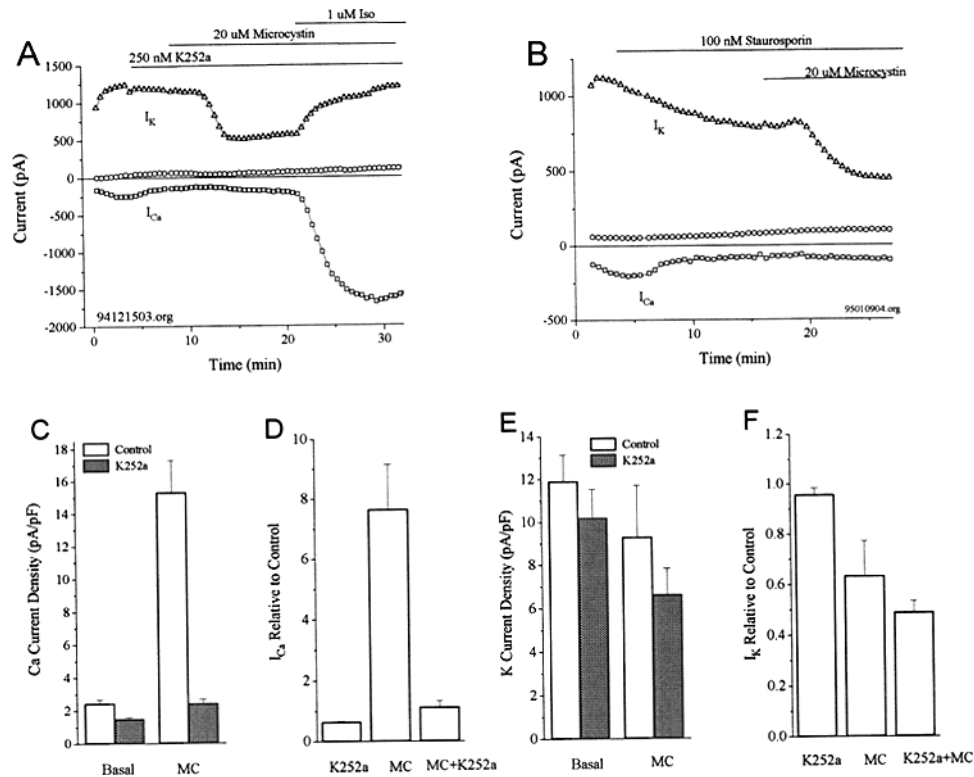


FIGURE 4. Effect of K252a and staurosporine on response of I_{Ca} and I_K to microcystin in frog atrial myocytes. (A) Patch clamp record showing inhibition of I_K by microcystin in the presence of K252a. 250 nM K252a and 1 μ M Iso were applied via superfusion and 20 μ M microcystin was applied by internal perfusion during the periods indicated. (Open squares) I_{Ca} ; (Open triangles) I_K ; (Open circles) Holding current. (B) Patch clamp record as in A showing inhibition of I_K by microcystin in the presence of 100 nM staurosporine. (C) Summary of effects of K252a on I_{Ca} current density in atrial myocytes. (D) Data in C expressed as relative Ca current. For each individual cell analyzed in C, relative I_{Ca} was calculated by dividing I_{Ca} in the presence of 250 nM K252a (K252a), 20 μ M microcystin (MC), and 20 μ M microcystin plus 250 nM K252a (MC+K252a) by the control basal I_{Ca} . Thus, these values reflect the real changes occurring in I_{Ca} amplitude with different treatments in individual cells, whereas the data in C plots only Ca current density. Similar conclusions are obtained from the two methods, but the numbers differ slightly. (E) Summary of effects of K252a on I_K density in atrial myocytes. (F) Data in E expressed as relative I_K as described in D. Means are averages of 4–14 cells.

Does Microcystin Stimulate I_{Ca} via Protein Kinase C?

Staurosporine and K252a are both relatively nonselective protein kinase inhibitors, but they both inhibit protein kinase C *in vitro* at submicromolar concentrations (Rüegg and Burgess, 1989). To determine whether the stimulation of I_{Ca} by microcystin was due to protein kinase C, we examined the effects of several selective protein kinase C inhibitors.

In our previous paper (Frace and Hartzell, 1993), we reported, but did not show, that a pseudosubstrate PKC peptide fragment ($\text{PKC}_{(19-36)}$) had no effect on the stimulation of I_{Ca} by microcystin. In the present series of experiments, we found that another pseudosubstrate PKC inhibitor ($\text{PKC}_{(19-31)}$) (House and Kemp, 1990) applied by internal perfusion at concentrations of $20 \mu\text{M}$ had no effect on the response to $20 \mu\text{M}$ microcystin. A myristoylated derivative of this inhibitor ($\text{myr-PKC}_{(19-31)}$), which has been reported to be a more effective inhibitor of PKC because it is targeted to the membrane (Eichholtz, deBont, deWidt, Liskamp, and Ploegh, 1993), also had no effect when applied internally at concentrations of $100 \mu\text{M}$ (Fig. 5 A). This myristoylated PKC inhibitor has also been reported to be an effective PKC in-

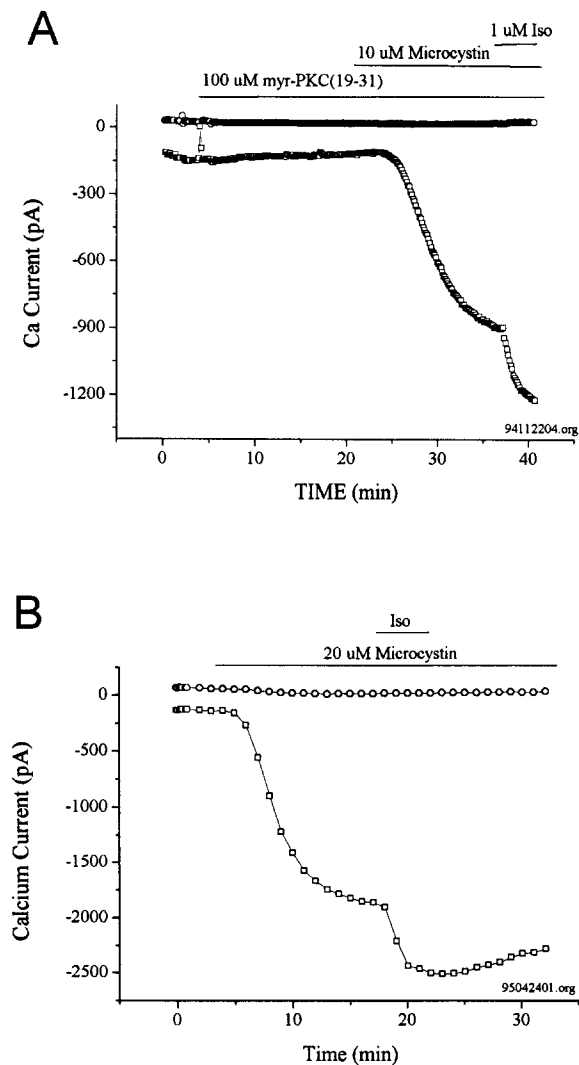


FIGURE 5. Effects of a PKC inhibitor and BAPTA on the effect of microcystin on I_{Ca} in ventricular myocytes. (Open squares) I_{Ca} . (Open circles) Holding current. (A) Patch clamp record showing lack of effect of a myristoylated derivative of $\text{PKC}_{(19-31)}$ on the response of I_{Ca} to microcystin. $100 \mu\text{M}$ $\text{myr-PKC}_{(19-31)}$ and $10 \mu\text{M}$ microcystin were applied by internal perfusion and Iso was applied by superfusion during periods indicated. (B). Patch clamp record showing effect of microcystin in the presence of an internal solution containing BAPTA. 5 mM BAPTA was added to the standard internal solution (Methods) from the onset of the experiment. The cell was depolarized by 200-ms pulses to 0 mV once every minute. $20 \mu\text{M}$ microcystin was applied by internal perfusion and Iso was applied by superfusion during periods indicated.

hibitor when applied externally. Applied externally, this inhibitor completely and rapidly blocked the basal Ca current at concentrations $>1 \mu\text{M}$ (not shown). We believe that this block was not associated with protein kinase inhibition for the following reasons. (a) The block was usually associated with a significant increase in holding current. (b) The block was very rapid in onset and was also rapidly reversible (complete within several seconds). This seems too fast to be mediated by kinase-phosphatase mechanisms. (c) Externally-applied $1 \mu\text{M}$ myr-PKC₍₁₉₋₃₁₎ rapidly and reversibly reduced the Ca current stimulated by Iso in the presence of $20 \mu\text{M}$ microcystin. If we assume that the phosphatase was completely inhibited by microcystin, this PKC inhibitor could not reduce I_{Ca} by inhibiting a kinase. (d) In the presence of low concentrations of myr-PKC₍₁₉₋₃₁₎ (0.1 – $0.5 \mu\text{M}$) that did not completely block the basal Ca current, microcystin continued to elevate I_{Ca} normally.

We also tested the effect of calphostin C, which has been reported to selectively inhibit PKC in the presence of light (Kobayashi, Nakano, Morimoto, and Tamaoki, 1989; Bruns, Miller, Merriman, Howbert, Heath, Kobayashi, Takahashi, Tamaoki, and Nakano, 1991). External application of 0.3 – $1 \mu\text{M}$ calphostin C in the dark had no effect on basal I_{Ca} or on stimulation of I_{Ca} by microcystin. However, if the cell was illuminated by the microscope lamp at moderate intensity in the presence of $0.3 \mu\text{M}$ calphostin C, I_{Ca} stimulated by microcystin and Iso was irreversibly and completely blocked within 1 min. Because inhibition of the kinase should have no effect on the stimulated current after the protein phosphatase is inhibited with microcystin, we conclude that calphostin C blocks I_{Ca} by a mechanism that does not involve protein kinase inhibition (Hartzell and Rinderknecht, manuscript in preparation).

It seems unlikely that PKX is regulated by Ca, because our internal solution is designed to buffer Ca to $<10 \text{ nM}$. However, the experiments above utilized 10 mM EGTA as the internal Ca buffer, but it is clear that EGTA does not control internal Ca concentration on a fast time scale in these cells (cf. Argibay et al., 1988). Consequently, several experiments were done to test whether PKX might be Ca-regulated by buffering Ca with the faster chelator, BAPTA. In Fig. 5 B, 5 mM BAPTA was added to our standard internal solution with no added Ca, and the cell was depolarized only once per minute, rather than once per 8 s as in the other figures, to reduce Ca influx. Under these conditions, internal perfusion with $20 \mu\text{M}$ microcystin increased I_{Ca} with the same time course and to the same extent as under our standard conditions. This result, coupled with the finding that the pseudo-substrate peptide inhibitor of CaM-Kinase II had no effect (data not shown), suggests that PKX is not regulated by Ca.

In summary, interpretation of experiments with PKC inhibitors was complicated by what we interpret as direct effects of PKC inhibitors on Ca channels or nonspecific effects on the membrane. In these cells, we found no evidence for an effect of PKC on I_{Ca} . Further evidence to support this contention is the observation that the PKC activators TPA ($0.1 \mu\text{M}$) and indolactam-V ($0.1 \mu\text{M}$) had no effect on I_{Ca} (data not shown).

PKX and PKA Phosphorylate Different Sites

The data above show that there are two different protein kinases that can elevate I_{Ca} . Next we wanted to know whether these two kinases phosphorylated the same or

different sites. At maximal concentrations, the effects of Iso and microcystin are not additive, but the two together do produce a larger response than either alone (Fig. 3 B). To examine further whether the two kinases phosphorylate different sites, we compared the effects of protein phosphatase inhibitors on basal I_{Ca} and Iso-stimulated I_{Ca} . We found that low concentrations of protein phosphatase inhibitors that did not stimulate basal I_{Ca} nevertheless could partially subdue the ability of ACh to inhibit Iso stimulation of I_{Ca} . This result is shown for microcystin in Fig. 6 A. In this experiment, the cell was first exposed to Iso, which stimulated I_{Ca} ~14-fold, and then Iso + ACh, which reduced I_{Ca} to basal levels. Afterwards, 0.5 μ M microcystin was perfused internally. In this cell, this concentration of microcystin had no effect on basal I_{Ca} after ~10 min. Subsequent Iso application stimulated I_{Ca} to the same level as before. Nevertheless, when ACh was added, I_{Ca} was reduced only ~50%. In the presence of Iso and ACh, I_{Ca} amplitude was 1 nA with microcystin, compared to 200 pA without microcystin. Thus, the inhibition of the effect of Iso by ACh was more sensitive to microcystin than was basal I_{Ca} . Fig. 6 B shows a control experiment where the cell was exposed to Iso and Iso+ACh twice separated by 15 min as in Fig. 6 A. In both cases, ACh completely inhibited the Iso response. The purpose of this experiment was to show that the partial inhibition of Iso-stimulated I_{Ca} by the second ACh exposure was not due to desensitization of muscarinic receptors or some time-dependent rundown of the muscarinic signal transduction pathways. The time course of the effect of ACh is analyzed for another cell in Fig. 6 C. The decrease in I_{Ca} produced by ACh was fitted to a single exponential. In the absence of microcystin I_{Ca} declined with $\tau = 3.15$ min, whereas in the presence of microcystin the decline was somewhat faster, $\tau = 1.75$ min. For comparison, the time course of the increase in I_{Ca} produced by Iso is also shown ($\tau = 0.83$ min).

The effects of protein phosphatase inhibitors were analyzed more quantitatively using calyculin A (Ishihara, Martin, Brautigam, Karaki, Ozaki, Kato, Fusetani, Watabe, Hashimoto, Uemura, and Hartshorne, 1988). Because calyculin A, unlike microcystin, can be applied extracellularly, dose-response curves are easier to obtain. Fig. 7 A shows that 30 nM calyculin A had no effect on basal I_{Ca} but rendered the stimulation of I_{Ca} by Iso partly irreversible. A second application of Iso increased the current to the same level as the initial stimulation, and the second washout caused the current to decline to the same level as the first application. Concentrations of calyculin A >30 nM alone often stimulated basal I_{Ca} . In Fig. 7 B, 100 nM stimulated I_{Ca} approximately eightfold over a period of 15 min. Subsequent application of Iso stimulated I_{Ca} in this cell, but often the combination of high concentrations of calyculin and Iso together appeared to be toxic to the cell as the cell blebbed and I_{Ca} decreased. In Fig. 7 C we plotted the amplitude of I_{Ca} stimulated by calyculin A alone (as indicated by the arrow in Fig. 7 B) (*solid line, circles*), the total current stimulated by calyculin A and Iso together (*solid line, squares*), and the total current after Iso had been washed out 10 min (*dashed line, triangles*). Comparing these curves shows that concentrations of calyculin <100 nM do not significantly increase basal I_{Ca} , whereas they do inhibit the washout of the Iso response. By fitting the data to a rectangular hyperbola, the EC_{50} for calyculin inhibition of Iso washout was 24.6 nM, whereas the EC_{50} for stimulation of basal I_{Ca} was estimated to be 149 nM.

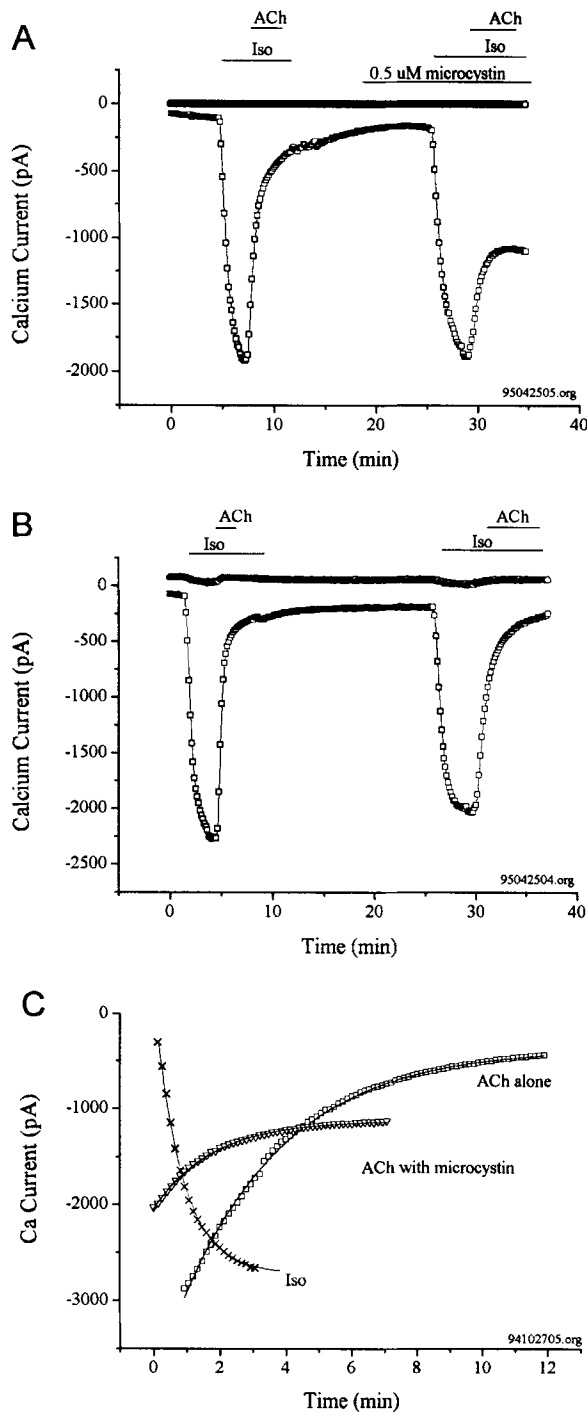


FIGURE 6. Effects of a low concentration of microcystin on I_{Ca} in a ventricular myocyte. (A) (Open squares) I_{Ca} , (Open circles) Holding current. Iso and ACh were applied by superfusion and microcystin was applied by internal perfusion for the periods indicated. (B) Control cell not perfused with microcystin. This experiment shows that repeated exposure to Iso and ACh produce similar effects during a 40-min experiment. (C) Fits of changes in I_{Ca} in response to Iso and ACh. The changes in I_{Ca} produced by Iso (crosses), ACh in the presence of Iso (squares), and ACh in the presence of Iso and microcystin (triangles) were fitted to single exponentials.

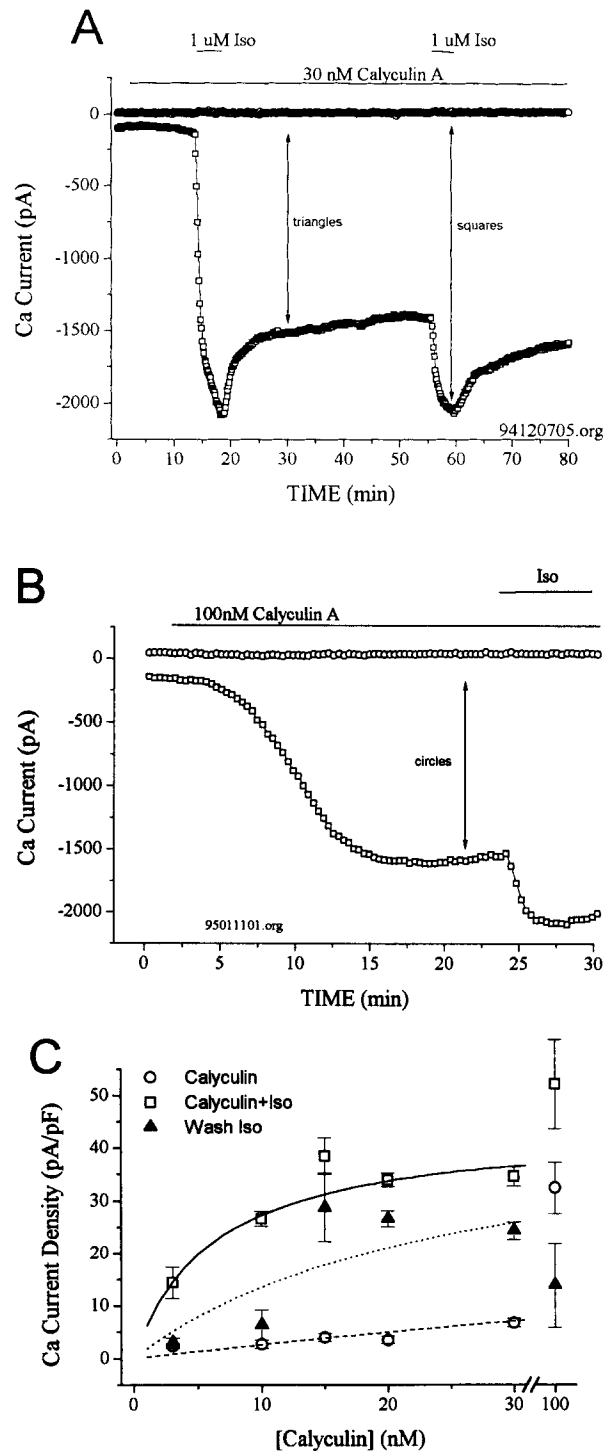


FIGURE 7. Effects of the protein phosphatase inhibitor calyculin A on I_{Ca} in a ventricular myocyte. (A) Patch clamp record showing effect of a low concentration of calyculin A. (Open squares) I_{Ca} . (Open circles) Holding current. Both Iso and calyculin A were applied by superfusion as indicated. (B) Patch clamp record showing effect of a high concentration of calyculin A. (C) Dose response curve for the effect of calyculin A on I_{Ca} . (Circles) I_{Ca} density stimulated by calyculin A alone (measured as shown by double-headed arrow in B). (Squares) I_{Ca} density in the presence of calyculin and Iso. (Triangles) I_{Ca} density in the presence of calyculin A after washing out 1 μ M Iso (as shown in A). Data were fitted to rectangular hyperbolas (lines). The best-fit parameters were: calyculin + Iso: $K_{1/2} = 6.0$ nM, $I_{max} = 43$ pA/pF; wash Iso: $K_{1/2} = 24.6$ nM, $I_{max} = 46$ pA/pF; calyculin alone: $K_{1/2} = 149$ nM, $I_{max} = 45$ pA/pF. Data points are means of 3–13 cells.

DISCUSSION

Conclusions

(a) The stimulation of I_{Ca} by high concentrations of microcystin is due to inhibition of a protein phosphatase that dephosphorylates site(s) phosphorylated by a basally active protein kinase. This protein kinase is not protein kinase A, because it is not blocked by PKA inhibitor peptides, and is probably not PKC, because it is not blocked by PKC inhibitor peptides. In the case of PKA inhibitors, we had the positive control that these inhibitors blocked the increase in I_{Ca} produced by Iso or forskolin. In the case of the PKC inhibitors, although we had no positive controls, we do not think PKC is involved because three different PKC inhibitor peptides had no effect and we were unable to stimulate I_{Ca} with PKC activators. The kinase is also unlikely to be calmodulin-kinase II because free Ca^{2+} is buffered to $<10^{-9}$ M. Thus, it appears that the increase in I_{Ca} produced by microcystin involves phosphorylation by a novel protein kinase (PKX). We are confident that the effect of microcystin on I_{Ca} is, indeed, due to a protein kinase and not to some other effect of microcystin (as described below for I_k) because the effect of microcystin requires ATP (Frace and Hartzell, 1993) and is blocked by two protein kinase inhibitors, staurosporine and K252a.

(b) The regulation of the Ca channel involves phosphorylation of two different sites. The observation that low concentrations of microcystin or calyculin A only partially block the washout of Iso (Figs. 6 and 7) suggests that the effect of Iso involves phosphorylation of two different sites or involves two different mechanisms. We prefer the conclusion that Iso stimulates cAMP-dependent phosphorylation of two different sites because the effect of Iso is completely blocked by inhibitors of PKA (Hartzell et al., 1991). Furthermore, it is unlikely that the fraction of I_{Ca} stimulation that is reversible in the presence of low concentrations of microcystin is due to a direct G protein effect (Brown and Birnbaumer, 1990) because high concentrations of microcystin completely block the washout of the Iso effect. From Fig. 7 C, it appears that $\sim 50\%$ of the sites are sensitive to low concentrations of calyculin, although scatter in the data make a precise quantification difficult. The existence of two sites is also supported by the observation that high concentrations of microcystin stimulate basal I_{Ca} . The site that is phosphorylated by PKX and responsible for this increase in basal I_{Ca} may be the same site phosphorylated by PKA with low sensitivity to microcystin or calyculin A. The idea of two different phosphorylation sites regulating Ca channel availability and gating was first proposed by Tsien, Bean, Hess, Lansman, Nilius, and Nowycky (1986). More recently, single-channel analysis by Herzig, Patil, Neumann, Staschen, and Yue (1993) and by Ono and Fozzard (1993) have provided more direct experimental support for this idea (see below).

(c) The effect of microcystin on the delayed rectifier current I_k does not seem to be mediated by a protein kinase that is sensitive to either staurosporine or K252a. Although the possibility still exists that the inhibition of I_k by microcystin is due to inhibition of a protein phosphatase, this seems unlikely for several reasons. First, the effect of microcystin on I_k is considerably faster than the effect on I_{Ca} (Fig. 1 B). We think that the rate of decrease in I_k is related to the time it takes for microcystin

to equilibrate with the cell contents, whereas the increase in I_{Ca} reflects the rate of phosphorylation. Also, in our previous paper (Frace and Hartzell, 1993) we showed that the effect of microcystin on I_{Ca} required ATP (which suggested the role of a protein kinase), but we were unable to test this for I_K because I_K disappeared when the cell was perfused internally with ATP-free solutions. Thus, we have no reason to believe that a protein kinase is involved and hypothesize that the effect of microcystin may be due to a direct effect on the delayed rectifier channel. Because a chemically unrelated protein phosphatase inhibitor, okadaic acid, produces the same effect (Frace and Hartzell, 1993), the hypothetical binding site on the K channel may share some similarity with the protein phosphatases.

Kinetic model of phosphorylation and dephosphorylation. If we assume that the amplitude of I_{Ca} is proportional to the concentration of a phosphorylated substrate (either the Ca channel itself or a regulator of the channel), then our data is consistent with the model shown in Fig. 8. The substrate can be phosphorylated by PKA

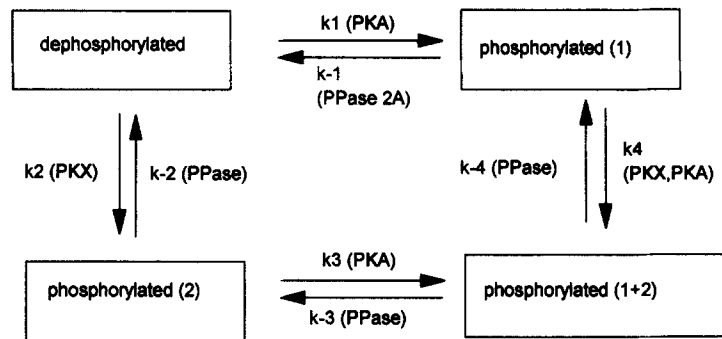


FIGURE 8. Model of regulation of I_{Ca} by phosphorylation. It is assumed that a single substrate is the target of PKA and PKX. This substrate may be the Ca channel or another regulatory protein. The substrate can be phosphorylated on two different sites independently by PKA and PKX. Each site is dephosphorylated by a protein phosphatase (PPase).

(at rate k_1) or PKX (at rate k_2) to give singly phosphorylated states or can be phosphorylated by both kinases (via k_2 and k_3 or via k_1 and k_4). We believe that in the absence of β -receptor stimulation, that PKA activity is very low (k_1 and k_3 very small), because inhibition of PKA has no effect on basal I_{Ca} (Fig. 1 C, see also Hartzell et al., 1991). In contrast PKX does exhibit basal activity because inhibition of dephosphorylation (k_{-2}) with microcystin causes I_{Ca} to increase and because inhibition of PKX with K252a or staurosporine causes a 40–50% decrease in basal I_{Ca} . Thus, under basal conditions, we hypothesize that most of the substrate exists in the dephosphorylated state but a small fraction is in the phosphorylated (2) state. Upon application of microcystin most of the substrate is converted to the phosphorylated (2) state, but very little exists in the doubly phosphorylated state [phosphorylated (1 + 2)] because PKA inhibitors have little or no effect on the increase in I_{Ca} produced by microcystin and because Iso usually increases I_{Ca} further beyond that produced

by microcystin alone. Application of Iso alone converts the substrate to the phosphorylated (1) state and the phosphorylated (1 + 2) state. We do not know whether phosphorylation of site 2 in the presence of Iso is actually catalyzed by PKA or PKX. PKA might phosphorylate both sites 1 and 2, but because PKX is basally active, one might also expect a certain proportion of the substrate to be phosphorylated both by PKA and PKX in the presence of Iso. It is also possible that the rate constants k_2 and k_4 are different, such that a substrate phosphorylated by PKA is phosphorylated more rapidly by PKX or that PKX activity itself is regulated by PKA phosphorylation. The results of Figs. 6 and 7 show, however, that in the presence of Iso, two sites with different sensitivities to phosphatase inhibitors are phosphorylated. Our finding that K252a reduces Iso stimulated current could be interpreted in two ways: either K252a is nonselective for PKX and PKA or K252a reduces Iso-stimulated I_{Ca} because PKX-mediated phosphorylation comprises 25% of the Iso-stimulated increase in I_{Ca} . Although this second alternative is a possibility, K252a is known to inhibit PKA in vitro with an IC_{50} of ~ 20 nM (Kase et al., 1987).

Relative rates of phosphorylation by PKA and PKX. We would like to know how the rates of phosphorylation by PKX and PKA compare, because if the rate of PKX-mediated phosphorylation is similar to PKA activity, this might suggest that PKX has a significant physiological role on basal I_{Ca} . Conversely, if the activity is very small, it might suggest that PKX is not physiologically important unless it is regulated. We can estimate k_1 and k_2 in Fig. 8 if we make some simplifying assumptions. First, we assume that Iso increases only k_1 . This is justified by the finding that PKA inhibitors completely block the response to Iso (Hartzell et al., 1991). We also assume that k_1 is the rate-limiting step in the increase in I_{Ca} produced by Iso. This assumption is less secure. We have previously shown that the rate-limiting step is subsequent to cAMP production, although we have not shown that the rate-limiting step is the phosphorylation itself (Frace, Méry, Fischmeister, and Hartzell, 1993). For example, if PKA phosphorylates a regulator of the Ca channel rather than the Ca channel itself, association of the regulator with the channel could be rate-limiting. Nevertheless, under the assumption that k_1 is the only rate constant affected by Iso and is limiting, k_1 can be estimated by measuring the rate of I_{Ca} increase in response to Iso in the presence of microcystin to reduce k_{-1} to zero. The phosphorylation rate due to PKA was estimated in this manner to be $1.20 \pm 0.03 \text{ min}^{-1}$ (Fig. 6 B). It should be noted that the increase in I_{Ca} produced by Iso in the presence of microcystin and kinase inhibitors was not greatly different (0.7 min^{-1} for Fig. 2 A), which confirms that even if Iso does stimulate phosphorylation by PKX, PKX phosphorylation is not rate-limiting. To estimate k_2 , we assume that PKA is inactive in the absence of Iso (as shown by the insensitivity of the increase in I_{Ca} caused by microcystin to PKA inhibitors, Fig. 1 C) and that the increase in I_{Ca} in response to microcystin is due solely to phosphorylation via k_2 . If microcystin reduces k_{-2} to zero, then the rate of increase in I_{Ca} in response to microcystin will be a lower estimate of k_2 . The average was $0.13 \pm 0.03 \text{ min}^{-1}$, but in some cells the rate was as high as 0.43 min^{-1} . Thus, the rate of phosphorylation by PKX may be between 10 and 35% of the rate of PKA phosphorylation in the presence of a maximally stimulating dose of Iso. These calculations support the view that PKX activity is sufficiently high to be physiologically relevant.

Which protein phosphatases are involved? In cell-free systems, microcystin inhibits protein phosphatase 1 and 2A with a K_i of 0.1 nM, protein phosphatase 2B with a K_i of 0.2 μ M, and protein phosphatase 2C with a $K_i >10 \mu$ M (Honkanen et al., 1990; MacKintosh et al., 1990). Calyculin A inhibits phosphatase 1 and 2A with a K_i of 1–2 nM (Ishihara et al., 1988). In our experiments, the washout of Iso was partially inhibited by 0.1–1 μ M microcystin and 5–30 nM calyculin A. This is in a range consistent with the involvement of either type 1 or 2A phosphatases. Because internal perfusion with the regulatory subunit of protein phosphatase 1 (Inhibitor-2, Cohen, 1989) had no effect on Iso washout (not shown) and because protein phosphatase 2B was inhibited by buffering internal Ca^{2+} to <1 nM, we suggest that protein phosphatase 2A may be the major contributor to dephosphorylation of site 1.

Stimulation of basal I_{Ca} occurred at concentrations of microcystin $>1 \mu$ M and calyculin >30 nM. These concentrations are quite high relative to the K_i for inhibition of either type 1 or type 2A phosphatase. Other candidates for the protein phosphatase are phosphatase 2B (calcineurin), phosphatase 2C, and other uncharacterized protein phosphatases. Calcineurin does not seem to be a likely candidate, because it should not be active under conditions where intracellular Ca is buffered by BAPTA (Fig. 5 B) and because calcineurin inhibitor peptides had little effect on basal I_{Ca} (Frace and Hartzell, 1993). Protein phosphatase 2C exhibits a dependence on Mg^{2+} in the 1–10 mM range (Cohen, 1989). We have previously shown that changes in internal free Mg^{2+} in the range of 0.3 to 3 mM modulate the response of I_{Ca} to Iso by a mechanism which could involve protein phosphatase 2C (White and Hartzell, 1988; Hartzell and White, 1989). Unfortunately, protein phosphatase 2C does not appear to be inhibited by microcystin at concentrations as high as 4 μ M (MacKintosh et al., 1990). Thus, it appears that the phosphatase inhibited by high concentrations of microcystin in our studies may be a novel protein phosphatase.

Another issue relevant to the effect of microcystin is the observation that in this series of experiments, conducted from September, 1994, until May, 1995, the apparent potency and efficacy of microcystin were less than we observed in earlier experiments done between 1989 and 1993 (Frace and Hartzell, 1993). For example, in the present experiments 0.5 μ M microcystin had no effect on basal I_{Ca} , whereas previously this concentration produced a severalfold increase in I_{Ca} . 10 μ M microcystin in the present experiments produced a 10-fold increase in I_{Ca} ; previously it produced a 19-fold increase. This lower efficacy was found in five different lots of microcystin from two manufacturers, but unfortunately we have no samples of the microcystin from 1993 to compare.

Comparison to other studies. Some limited comparisons can be made to other studies. In their studies on the mammalian cardiac I_{Ca} , Hescheler, Kameyama, Trautwein, Mieskes, and Söling (1987) and Hescheler, Mieskes, Ruegg, Takai, and Trautwein (1988) have shown that 50 μ M okadaic stimulated basal I_{Ca} approximately twofold. Although there were no data presented as to the nature of the kinase responsible for this increase, it seems possible that the stimulation of basal I_{Ca} was due to a kinase analogous to our PKX. However, their results differ from ours in that the washout of Iso stimulation was only slowed two- to threefold but was not prevented by okadaic acid. In addition, inhibitor-2 enhanced the stimulation by Iso

and slowed washout of the Iso effect twofold, suggesting a role for protein phosphatase-1 in this cell type. However, phosphatase 2A is probably also involved because okadaic acid, which inhibits both phosphatase 1 and 2A at the concentrations used had a larger effect than inhibitor-2 which inhibits only phosphatase-1.

Recently, Ono and Fozzard (1993) showed that in cell-attached patches of rabbit ventricular myocytes, concentrations of okadaic acid $<1 \mu\text{M}$ increased channel availability, whereas high concentrations ($>10 \mu\text{M}$) of okadaic acid increased channel open time. These authors conclude that there are two phosphorylation sites that regulate the Ca channel. One of these sites controls channel availability and the other controls channel open time. From the analysis by Herzig et al. (1993), channel availability probably corresponds to channel gating mode 0, 1, and 2.

Somewhat analogous results have been obtained by Hwang, Horie, and Gadsby (1993) in their analysis of the regulation of the PKA-regulated Cl current in mammalian heart. They have found that the Cl channel is phosphorylated by PKA at two sites that have differing sensitivities to protein phosphatase inhibitors. Thus, multiple phosphorylation by protein kinases may be a common feature of ion channels. However, in contrast to the stimulation of basal I_{Ca} by microcystin, the basal Cl current is not stimulated by okadaic acid or microcystin alone which suggests that the Cl channel may not be regulated by PKX-like kinases.

Identity of PKX. We have not yet identified PKX, except to conclusively rule out PKA. The next most attractive candidate, PKC, has been difficult to rule out because the inhibitors that are available for PKC have been problematic. The most selective inhibitors, the pseudosubstrate peptides, have no effect when applied by internal perfusion in our system. Inhibitors applied extracellularly, such as the myristoylated pseudosubstrate peptide, sphingosine, and calphostin C had nonspecific effects that were clearly not related to their ability to block protein kinase C. These studies, which we will publish in detail elsewhere, underscore the problems with the selectivity of many protein kinase C inhibitors. Nevertheless, it is unlikely that PKC is not PKX because of the absence of effect of the pseudosubstrate peptide inhibitors and the lack of effect of protein kinase C stimulators.

Physiological significance. The physiological significance of phosphorylation by PKX remains to be determined. However, the fact that basal I_{Ca} is reduced 40–50% by K252a suggests that PKX may play a role in setting the amplitude of basal I_{Ca} . We have previously reported (Argibay, Fischmeister, and Hartzell, 1988) that basal I_{Ca} density in frog ventricular myocytes is extremely variable (from 1.3 pA/pF to 28 pA/pF). It is possible that this variability reflects the basal level of phosphorylation by PKX. If this is the case, one would predict that the degree of inhibition of basal I_{Ca} by K252a would correlate with basal I_{Ca} density, but we do not yet have sufficient data to answer this question. However, in some rare dissociations, I_{Ca} in nearly every cell increased spontaneously after patch break severalfold and sometimes approached 1 to 2 nA in amplitude. What stimulated this increase is unknown. Nevertheless, in two cells with a spontaneously-elevated current of $>1 \text{ nA}$, application of 250 nM K252a rapidly returned I_{Ca} to $\sim 100 \text{ pA}$, suggesting that the increase was caused by activation of PKX. Subsequent application of Iso produced a normal increase. This observation raises two questions: is protein kinase X regulated by neu-

rotransmitters or hormones? Can disorders of this PKX produce pathological states?

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