Endothelin-1 and photoreleased diacylglycerol increase L-type Ca^{2+} current by activation of protein kinase C in rat ventricular myocytes

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1. The amphotericin B-perforated whole-cell patch clamp technique was used to determine the modulation of L-type Ca^{2+} channels by protein kinase C (PKC)-mediated pathways in adult rat ventricular myocytes.

2. Application of 10 nM endothelin-1 (ET-1) increased peak Ca^{2+} current (I_{Ca}) by 28·2 ± 2·5% ($n = 13$) and slowed current decay. These effects were prevented by the endothelin receptor antagonist PD145065 (10 μM) and by the PKC inhibitor chelerythrine (8 μM).

3. To establish if direct activation of PKC mimicked the ET-1 effect, the active and inactive phorbol esters (phorbol-12-myristate-13-acetate and 4α-phorbol-12,13-didecanoate) were tested. Both phorbol esters (100 nM) resulted in a small (~10%) increase in I_{Ca}, suggesting PKC-independent effects.

4. Bath application of dioctanoylglycerol (diC_8), a diacylglycerol (DAG) analogue which is capable of directly activating PKC, caused a gradual decline in peak I_{Ca} (50·4 ± 6·2%, $n = 5$) and increased the rate of current decay. These effects were unaffected by the PKC inhibitor chelerythrine (8 μM).

5. Intracellular photorelease of caged diC_8 with 3 or 10 s exposure to UV light produced a concentration-dependent increase in peak I_{Ca} (20·7 ± 8·5% ($n = 8$) for 3 s UV and 60·8 ± 11·4% ($n = 13$) for 10 s UV), which could be inhibited by chelerythrine.

6. Our results demonstrate that both ET-1 and intracellularly photoreleased diC_8 increase I_{Ca} by a PKC-mediated pathway, which is in direct contrast to the PKC-independent inhibition of I_{Ca} produced by bath-applied diC_8. We conclude that specific cellular pools of DAG are crucially important in the regulation of I_{Ca} by PKC.

Many extracellular hormones and neurotransmitters regulate the electrical and contractile properties of cardiac muscle. These neurohormones can bind to a variety of transmembrane receptors, initiating multiple signalling cascades leading to regulatory changes in the myocyte. A subset of G-protein-coupled receptors act via G_q to stimulate phospholipase C (PLC), which hydrolyses phosphatidyl-inositol 4,5-bisphosphate, generating inositol trisphosphate (IP_3) and DAG (Berridge, 1997). The liberated DAG can then activate PKC, which phosphorylates a wide spectrum of cardiac proteins responsible for myocardial excitability and contraction (Puceat & Vassort, 1996; Dorn & Brown, 1999). In cardiac muscle, multiple receptors, including endothelin (Endoh *et al.* 1998; Dorn & Brown, 1999), α_1-adrenergic (Stiles, 1996) and angiotensin II receptors (Van Heugten *et al.* 1996), have been demonstrated to couple with G_q and ultimately lead to activation of PKC.

The L-type Ca^{2+} channel plays a critical role in cardiac excitability and in excitation–contraction coupling, and thus it represents an important potential target for PKC modulation. However, the effect of activation of PKC on cardiac L-type Ca^{2+} channels remains unclear. For example, some studies of ET-1 have shown clear increases in I_{Ca} (Bkaily *et al.* 1995), while others have shown a decrease in I_{Ca} (Cheng *et al.* 1995) or no effect (Habuchi *et al.* 1992). Similar apparently conflicting findings have resulted from studies of direct activators of PKC, such as diC_8, 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) as well as phorbol esters (Dosemeci *et al.* 1988; Walsh & Kass, 1988; Lacerda *et al.* 1988; Tseng & Boyden, 1991; Schreur & Liu, 1996; Zhang *et al.* 1997). These contradictory data may be caused by the wide differences in experimental conditions, including different species, tissues, preparations and temperatures. In addition, the method employed to measure I_{Ca} could be of critical

importance in maintaining the physiological response to ET-1 and other neurohormones. Most of the initial studies were performed using the ruptured whole-cell patch clamp technique. In contrast, the perforated whole-cell technique allows voltage-clamp characterization of I_{Ca} without the dialysis of large molecules and proteins that occurs with the ruptured patch technique. This has recently been revealed to be of critical importance in characterizing the effect of α_1-adrenergic and arginine vasopressin regulation of L-type Ca^{2+} channels in isolated rat and guinea-pig ventricular myocytes (Liu & Kennedy, 1998; Kurata *et al.* 1999).

Walker and colleagues have developed a novel tool to investigate the role of intracellular DAG and related activation of PKC by synthesizing a caged DAG analogue, caged diC_8, which can be released in a controlled fashion by near-UV light (Huang *et al.* 1996). In isolated adult rat ventricular myocytes, intracellular photorelease of diC_8 induced a strong positive inotropic effect, demonstrated by enhanced cell shortening, and this effect was stereospecific, concentration-dependent, and blocked by a PKC inhibitor, chelerythrine (Pi *et al.* 1997). Further investigation demonstrated that the positive inotropic effect was due primarily to a large increase in the intracellular Ca^{2+} transient in response to photorelease of diC_8 (Pi & Walker, 1998). The increased Ca^{2+} transient did not reflect increased sarcoplasmic reticulum Ca^{2+} load as assessed by the caffeine releasable pool, nor did it reflect changes in sarcoplasmic reticulum Ca^{2+} uptake (Pi & Walker, 1998). Therefore, we hypothesized that influx of Ca^{2+} through L-type Ca^{2+} channels may increase following photorelease of caged diC_8 via activation of PKC pathways.

The purpose of the present study was to determine the modulation of rat ventricular L-type Ca^{2+} channels by PKC activation using the perforated patch technique. We used ET-1 as a representative agonist to activate G_q–PKC pathways and compared those results to putative direct activation of PKC using phorbol esters, bath-applied diC_8, and photoreleased diC_8. Our results demonstrate that ET-1 increases I_{Ca} in a PKC-dependent fashion that can be mimicked by intracellular photoreleased diC_8 but not by bath-applied diC_8 or phorbol esters.

Some of the preliminary results from this study have been presented to the Biophysical Society (He *et al.* 1999).

METHODS

Ventricular myocyte isolation

Single ventricular myocytes were enzymatically isolated from the hearts of adult male Sprague–Dawley rats (200–250 g) killed with metofane (inhalation for 3 min) following a protocol approved by the University of Wisconsin Animal Care and Use Committee as previously described (Pi *et al.* 1997). Briefly, hearts were rapidly excised, cannulated, and subjected to retrograde perfusion on a Langendorff apparatus at 37 °C via the aorta with oxygenated Ringer solution of the following composition (mM): 125 NaCl, 2 NaH_2PO_4, 5 KCl, 1·2 $MgSO_4$, 25 Hepes, 5 sodium pyruvate, 11 glucose, and 1 $CaCl_2$ (pH adjusted to 7·4 with NaOH). The hearts were briefly perfused with Ca^{2+}-free Ringer solution followed by Ca^{2+}-free Ringer solution containing 0·6 mg ml^{-1} collagenase and 0·36 mg ml^{-1} hyaluronidase. The left ventricle was cut away from the rest of the tissue and further incubated in the enzyme solution. Isolated myocytes were washed, pelleted in a tabletop centrifuge, and resuspended in 0·5 mM Ca^{2+} Ringer solution at room temperature at a density of $\sim 10^5$ cells ml^{-1}. The yield was 1×10^6 to $1{\cdot}5 \times 10^6$ cells per heart, of which typically 85% were viable rod-shaped cells. Myocytes which displayed clear striations were used for experiments within 8 h of isolation.

Caged diC_8 loading in myocytes and photorelease

Myocytes at a density of 3×10^4 to 6×10^4 cells ml^{-1} were incubated in the dark with 800 μM α-carboxyl caged diC_8 dissolved in dimethyl sulfoxide (DMSO; final concentration of DMSO 0·05%) in a siliconized Eppendorf tube for 45 min at room temperature. The cell pellet was gently suspended twice in order to provide adequate oxygenation and then washed twice with fresh Ringer solution containing 0·5 mM Ca^{2+} without caged diC_8. Ventricular myocytes were then placed in an experimental chamber and perfused at $\sim$0·5 ml min^{-1} with a normal Ringer solution with 0·5 mM Ca^{2+}(see above) throughout the experiment.

An inverted Nikon Diaphot 200 microscope (Tokyo, Japan) with Nikon epi-fluorescence attachments was used for the experiments. The UV beam from a HBO 100 W/2 mercury lamp was passed through sequential neutral density filters (ND2 and ND4) and reflected onto the cell via a DM 400 dichroic mirror and Nikon 0.55 LWD × 40 objective lens. After the perforated whole-cell configuration was obtained, illumination was initiated and exposure time (1–30 s as desired) was controlled by hand-switching the light path off or on. Control experiments showed that exposure for up to 3 min to the UV light alone was without effect on I_{Ca} (data not shown).

Electrophysiological measurements

The amphotericin B-perforated whole-cell technique was employed to record I_{Ca} (Rae *et al.* 1991). Ventricular myocytes were placed in the experimental chamber mounted on the stage of an inverted microscope (Nikon Diaphot 200). The cells were perfused at 0·5 ml min^{-1} with 0·5 mM Ca^{2+} Ringer solution (see above). The pipette solution consisted of (mM): 100 caesium glutamate, 40 CsCl, 10 Hepes, 0·5 $CaCl_2$ (pH adjusted to 7·2 with CsOH). The amphotericin B was prepared as a stock solution (0·1 mg μl^{-1} in DMSO) and frozen in small aliquots for up to 5 days. The diluted amphotericin B was prepared hourly from the stock solution by diluting in pipette solution to a final concentration of 300 μg ml^{-1}. The pipette solution with amphotericin B was sonicated for 2–5 s. The final solution was used within 1 h after preparation. The tip of the patch pipette was first filled with amphotericin B-free solution by dipping the tip into the solution for 2–5 s. The rest of the pipette was backfilled with amphotericin B-containing solution. Patch electrodes were fabricated from borosilicate glass (TW150F-4, World Precision Instruments, Inc.) with a Flaming/Brown Micropipette Puller Model 87 (Sutter Instruments). The electrode resistance was 1–2 MΩ when filled with the pipette solution. The potential of the electrode was adjusted to zero current between the pipette solution and the bath solution immediately before seal formation. After a giga-seal between the pipette and myocyte had formed, the pipette potential was stepped from −80 mV to −90 mV for 10 ms at 1 Hz. The development of electrical access could be monitored by the appearance of a capacitative current evoked by the −10 mV hyperpolarization test pulse. The uncompensated access resistance of the cell typically dropped to a stable level of $11{\cdot}8 \pm 0{\cdot}4$ MΩ ($n = 117$) in 10–40 min after seal formation. Series

Table 1. Decay of I_{Ca} at +10 mV after 40 and 200 ms in response to ET-1, photoreleased diC8 and bath-applied diC_8

	Experimental groups	n	I_{Ca} decayed relative to peak I_{Ca} (%) At 40 ms	At 200 ms
ET-1	Control	13	47·7 ± 4·3	96·1 ± 0·6
	10 nM ET-1	13	42·6 ± 3·8**	94·2 ± 0·4**
Photoreleased diC_8	Control	11	49·3 ± 3·1	96·6 ± 0·9
	10 s UV	11	42·7 ± 3·2**	89·4 ± 1·3**
Bath-applied diC_8	Control	7	48·5 ± 6·2	95·6 ± 1·3
	7·5 μM diC_8	7	59·5 ± 4·0**	98·2 ± 0·8**

** $P < 0·01$, compared to control.

resistance and whole-cell capacitance were analog compensated using the Axopatch 200B circuitry. The series resistance was compensated 70–85%. Access resistance was periodically monitored during the course of an experiment and the level of compensation was adjusted as needed. Once the perforated whole-cell configuration was formed, the bath solution was switched to a solution containing (mM): 130 NaCl, 10 TEA-Cl, 1 $MgCl_2$, 10 Hepes, 10 glucose, 1·8 $CaCl_2$ (pH adjusted to 7·4 with 20% TEA-OH), with 1 μM saxitoxin (STX). Currents were recorded at 25 kHz and filtered at 5 kHz using an Axopatch 200B amplifier (Axon Instruments) with pCLAMP 6.04 as acquisition software at room temperature. Under the perforated whole-cell recording conditions, cell contraction was clearly observed during the depolarizing voltage steps. Inclusion of 0·5 mM Ca^{2+} in the pipette solution ensured that only cells in the perforated whole-cell configuration were studied. The holding potential for these experiments was −80 mV.

Current through L-type Ca^{2+} channels (I_{Ca}) was evoked using 200 ms test pulses over a range of potentials from −30 to +60 mV following a 300 ms prepulse to −40 mV. The prepulse was used to inactivate the Na^+ current (I_{Na}), the T-type Ca^+ current, and the transient outward K^+current ($I_{to,K}$) (Dukes & Morad, 1991). In addition, 10 mM TEA-Cl and 1 μM STX in the bath solution and 140 mM Cs in the pipette solution were used to inhibit voltage-dependent I_K and I_{Na} during the measurements of L-type I_{Ca}. Only cells which showed no detectable change in I_{Ca} during the initial 5 min of observation were used for these experiments, and control experiments revealed that I_{Ca} was stable in these cells, with less than 5% run-down or run-up in 30 min of recording.

The voltage-dependent inactivation relationships were determined using a gapped double-pulse protocol, i.e. a conditioning prepulse for 1000 ms to potentials between −50 and +40 mV, followed by a 10 ms step returning to −50 mV, and then a 200 ms test pulse to +10 mV. These data were fitted to a Boltzmann distribution using the following equation:

$$I/I_{max} = 1/[1 + \exp((V - V_{0·5})/k)],$$

where $V_{0·5}$ is the half-maximum inactivation potential, and k is the slope factor.

Percentage decay of I_{Ca} at 40 and 200 ms was compared between control and experimental groups. The depolarization protocol was the same as above, i.e. a prepulse to −40 mV for 300 ms followed by a test pulse to +10 mV for 200 ms. The percentage decay of I_{Ca} was established by measuring I_{Ca} at 40 and 200 ms as a percentage of the peak current.

Each myocyte response was recorded from a single cell obtained from a fresh aliquot of cells. Myocytes from one heart were used each experimental day, with data collected from one to four myocytes per day. The mean capacitance of cells used in the present study was 105·5 ± 3·3 pF ($n = 117$).

Chemicals

All reagents were purchased from Sigma Chemical Co. unless otherwise stated. Chelerythrine (chloride), free diC_8, phorbol-12-myristate-13-acetate (PMA), and 4α-phorbol-12,13-didecanoate (4αPDD) stock solutions were prepared in DMSO. ET-1 stock solutions were prepared in 5% acetic acid. PD145065 and STX (Calbiochem, CA) were dissolved in distilled water. All stock solutions were stored at −20 °C. The stock solutions were freshly diluted into bath solution immediately prior to experimental recording. The final concentrations of DMSO and acetic acid in the bath were < 0·1% and < 0·0005%, respectively, which had no discernible effect on I_{Ca} (data not shown). α-Carboxyl caged diC_8 was synthesized and purified as described previously (Sreekumar *et al.* 1997).

Statistics

All values are presented as means ± S.E.M., with n values representing the number of myocytes in the data set. Statistical significance was evaluated using Student's paired or unpaired t test (two-tailed). ANOVA was used for multiple comparisons. Differences with $P < 0·05$ were considered statistically significant.

RESULTS

ET-1 increases I_{Ca}

ET-1 has been shown to have positive inotropic and chronotropic actions in a variety of cardiac preparations. To resolve the role of L-type Ca^{2+} channels in these responses to ET-1, we used the perforated whole-cell technique to assess the effect of ET-1 on I_{Ca} in adult rat ventricular myocytes with or without the specific PKC blocker, chelerythrine. I_{Ca} was elicited from a holding potential of −80 mV with a 300 ms prepulse to −40 mV followed by 25 or 200 ms depolarizing pulses to +10 mV every 15 s. The peak I_{Ca} was plotted in response to test pulses to +10 mV as a function of time for a representative cell in Fig. 1*A*. The measured peak I_{Ca} slowly increased with a time to half-maximal effect ($t_{½}$) of 7·9 ± 1·2 min ($n = 13$) when 10 nM ET-1 was perfused. The inset shows the superimposed

control (○) and ET-1-treated (□) current traces. Figure 1*B* shows the voltage dependence of the effect of 10 nM ET-1 on I_{Ca}. The stimulus protocol employed a 300 ms prepulse to −40 mV followed by a family of 200 ms test pulses from −40 mV to +60 mV in 10 mV steps. Representative original current traces in response to these test pulses are shown (○, control; □, during 10 nM ET-1). Peak I_{Ca} densities measured in six different myocytes from four rat hearts are plotted as a function of membrane potential. ET-1 (10 nM) increased I_{Ca} by 28·2 ± 2·5% at +10 mV ($n = 13$, $P < 0·001$), and a comparable increase was observed across a wide range of membrane potentials from −10 to +60 mV. To confirm the specificity of the ET-1 effect on I_{Ca}, a non-selective antagonist of ET receptors, PD145065, was tested (Kelso *et al.* 1998). Pretreatment with 10 μM PD145065 completely blocked the ET-1 (10 nM)-mediated stimulation of I_{Ca} (1·8 ± 2·5%, $n = 4$, data not shown). ET-1 also significantly reduced the decay of I_{Ca} measured at 40 ms (47·7 ± 4·3% in control *vs.* 42·6 ± 3·8% in ET-1, $n = 13$, $P < 0·01$, see Table 1) and at 200 ms (96·1 ± 0·6% in control *vs.* 94·2 ± 0·4% in ET-1, $n = 13$, $P < 0·01$, see Table 1).

To decide if PKC was involved in the modulation of I_{Ca} by ET-1, a specific inhibitor of PKC, chelerythrine, was examined. At the concentrations tested, chelerythrine is a specific inhibitor for PKC compared to other protein kinases (Herbert *et al.* 1990), and is insensitive to near-UV light (Pi *et al.* 1997). In control experiments, we did not find any

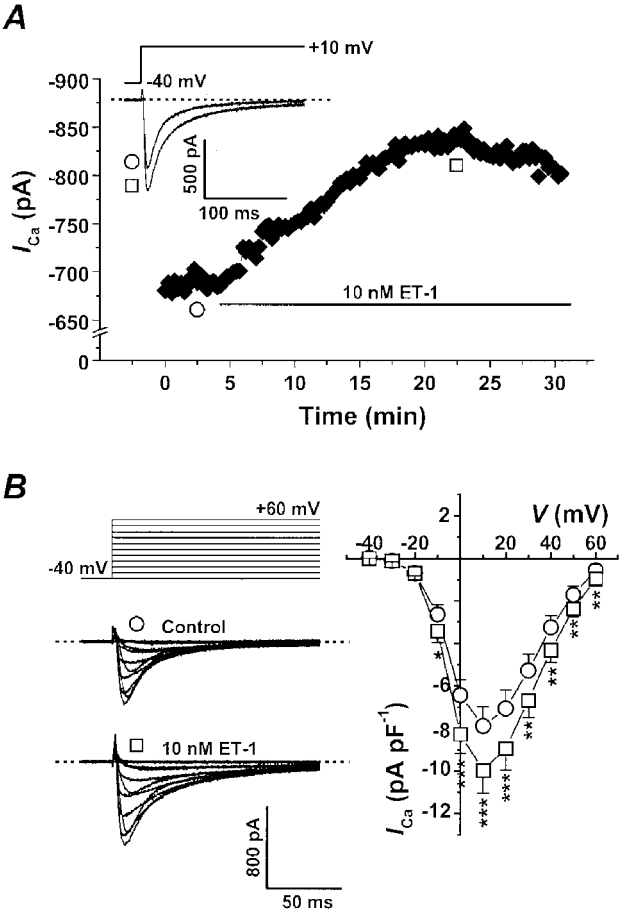

Figure 1. ET-1 increases I_{Ca}

A, the time course of I_{Ca} stimulation by 10 nM ET-1 in a representative myocyte. Peak I_{Ca} was plotted in response to test pulses to +10 mV every 15 s. The horizontal bar indicates application of 10 nM ET-1. The inset shows the stimulus protocol and the superimposed original current traces in the absence (○) and presence of ET-1 (□). The dashed line indicates the zero current level. The membrane capacitance (C_m) of this cell was 78 pF. *B*, the stimulus protocol, representative original current traces ($C_m = 79$ pF), and the mean I_{Ca}–V relationship before (○) and during (□) ET-1 exposure. Peak I_{Ca} densities (pA pF^{-1}) were measured in 6 different myocytes from 4 rat hearts. Vertical bars in *I–V* plots indicate the standard errors (* $P < 0·05$; ** $P < 0·01$; *** $P < 0·001$).

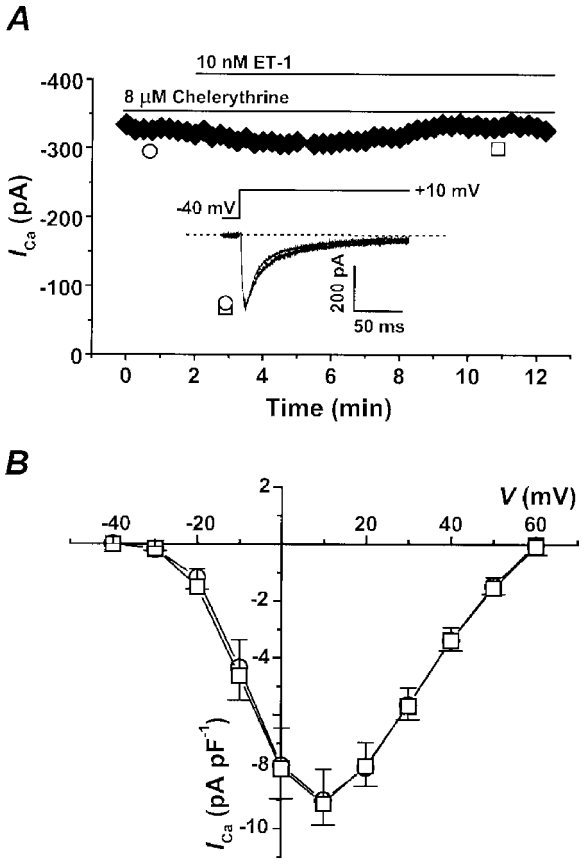

Figure 2. Chelerythrine, a specific PKC inhibitor, blocks the effect of ET-1 on I_{Ca}

A, the time course of I_{Ca} during perfusion of 10 nM ET-1 in a representative myocyte preincubated with 8 μM chelerythrine. Peak I_{Ca} is plotted in response to test pulses to +10 mV every 15 s. The inset shows the stimulus protocol and the superimposed original current traces before (○) and during 10 nM ET-1 (□) in a representative myocyte. The dashed line indicates the zero current level. $C_m = 39$ pF. *B*, the mean *I–V* relationship before (○) and during (□) ET-1 exposure in myocytes pre-incubated with 8 μM chelerythrine measured in 4 different myocytes from 4 rat hearts. Upward vertical bars in the *I–V* plot indicate the standard errors of the control data and the downward bars indicate standard errors of the ET-1 data.

discernible effects of 8 μM chelerythrine on I_{Ca}. After 10 min pretreatment with chelerythrine, application of 10 nM ET-1 (□) had no significant effect on I_{Ca}, as shown in the time course from a representative cell in Fig. 2*A*. Figure 2*B* shows the mean *I–V* relationship before (○) and during (□) ET-1 exposure. The stimulus protocol was the same as in Fig. 1. Peak I_{Ca} densities measured in four different myocytes from four rat hearts were plotted *vs.* membrane potential. Chelerythrine eliminated the response of myocytes to ET-1 at all potentials examined. These data suggest that the stimulation of I_{Ca} by ET-1 is PKC dependent.

Effect of phorbol esters on I_{Ca}

To confirm that the increase in I_{Ca} in response to ET-1 was mediated by PKC, two phorbol esters, PMA and 4αPDD, were tested. PMA should be capable of directly activating PKC, bypassing receptor stimulation, while 4αPDD is inactive with regard to PKC stimulation. Perfusion of 100 nM PMA resulted in a small increase in peak I_{Ca}, as shown in the time course of I_{Ca} from a representative cell in Fig. 3*A*. The effect was readily reversible with washout of PMA. The mean data from 11 cells treated with 100 nM PMA showed an 11·7 ± 2·2% increase in I_{Ca} at +10 mV ($P < 0·001$). Figure 3*B* shows that extracellular perfusion of 100 nM 4αPDD also increased I_{Ca}. The mean I_{Ca} from four cells treated with 100 nM 4αPDD showed an increase of 10·3 ± 2·1% ($P < 0·05$), which was also reversible upon washout. No significant difference was found between the effects of PMA and 4αPDD. Control experiments exposing the cells to 0·1% DMSO revealed no effect on I_{Ca}. Therefore, the small effect of both phorbol esters on I_{Ca} suggests PKC-independent modulation of I_{Ca}. The rapid reversibility of the effect of the phorbol esters following washout is also consistent with a PKC-independent effect.

Extracellular application of diC_8 inhibits I_{Ca}

As phorbol esters did not produce a clear PKC-dependent effect on I_{Ca}, we investigated another putative direct activator of PKC, diC_8. Figure 4*A* shows the time course of I_{Ca} during bath application of 7·5 μM free diC_8 in a representative cell. During perfusion of free diC_8, I_{Ca} was significantly inhibited, with a $t_{½} = 6·7 \pm 0·7$ min ($n = 7$). Peak I_{Ca} densities were measured in five different myocytes from five rat hearts and are plotted as a function of membrane potential (Fig. 4*B*). With test pulses to +10 mV, 7·5 μM free diC_8 decreased mean I_{Ca} by 50·4 ± 6·2% ($n = 5$, $P < 0·01$) without changing the voltage dependence of current activation, as shown by the *I–V* relations. Bath application of diC_8 resulted in a significantly greater decay

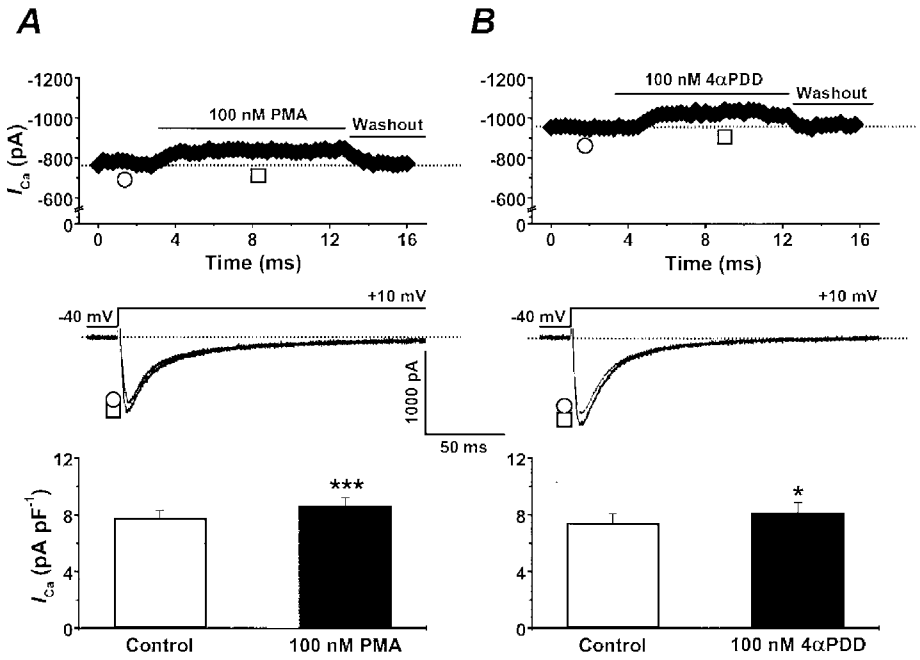

Figure 3. Effect of phorbol esters on I_{Ca}

A, the effect of 100 nM PMA on peak I_{Ca}. The top panel in *A* plots peak I_{Ca} obtained by a depolarization to +10 mV every 15 s during exposure to 100 nM PMA in a representative myocyte. The middle panel in *A* displays the superimposed original current traces before (○) and during PMA (□) exposure. The dashed line indicates the zero current level. $C_m = 86$ pF. The bottom panel in *A* charts the mean I_{Ca} densities (pA pF^{-1}) measured in 11 different myocytes from 4 rat hearts before and after 100 nM PMA. *B*, the same experiment as *A*, but examining the effects of an inactive form of phorbol ester, 4αPDD. The time course of I_{Ca} and current traces during 100 nM 4αPDD in a representative myocyte are shown in the upper and middle panels, respectively ($C_m = 141$ pF). The mean I_{Ca} densities measured in 11 cells from 4 rat hearts are summarized in the bar chart comparing I_{Ca} before and after 100 nM 4αPDD. Vertical bars indicate the standard errors (* $P < 0·05$; *** $P < 0·001$, compared to control).

of I_{Ca} measured at 40 ms (48·5 ± 6·2% in control *vs.* 59·5 ± 4·0% in diC_8, $n = 7$, $P < 0·01$, see Table 1) and at 200 ms (95·6 ± 1·3% in control *vs.* 98·2 ± 0·8% in diC_8, $n = 7$, $P < 0·01$, see Table 1).

To resolve if the effect of free diC_8 on I_{Ca} is PKC dependent, chelerythrine (8 μM) was pre-incubated with the cells prior to bath application of 7·5 μM diC_8. In six cells, I_{Ca} was inhibited by diC_8 by 65·2 ± 10·5% at +10 mV ($P < 0·01$, data not shown), which was not significantly different from the 50·4 ± 6·2% decrease seen in the absence of chelerythrine. The results show that the inhibitory effect of extracellular perfusion of free diC_8 on I_{Ca} is likely to be PKC independent.

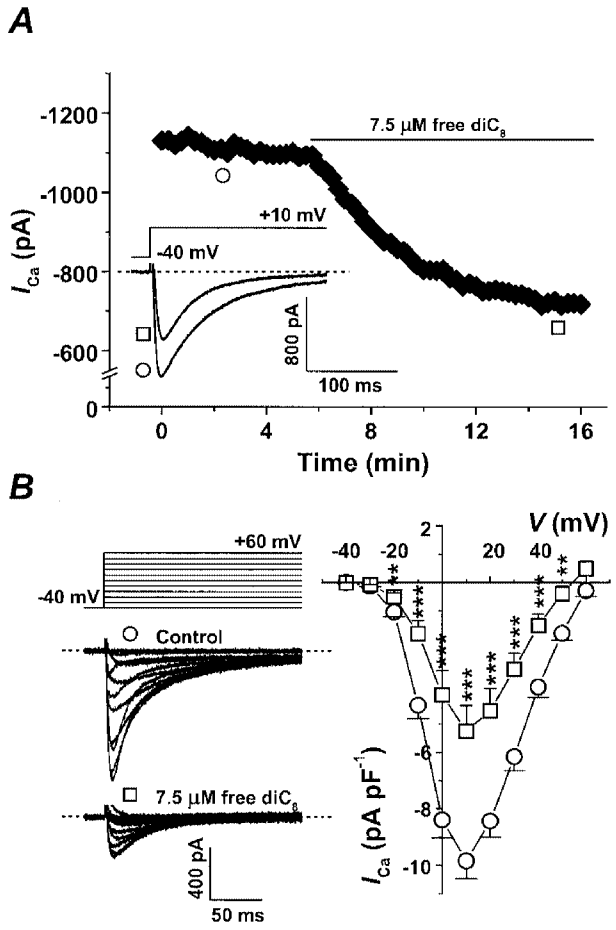

Figure 4. Bath application of diC_8 inhibits I_{Ca}
A, the time course of I_{Ca} inhibited by bath-applied diC_8 in a representative myocyte. Peak I_{Ca} is plotted in response to test pulses to +10 mV every 15 s before and during application of 7·5 μM diC_8. The inset shows the stimulus protocol and the superimposed original current traces of I_{Ca} before (○) and after (□) perfusion of free diC_8. The dashed line indicates the zero current level. $C_m = 117$ pF. *B*, the stimulus protocol, representative original current traces, and mean *I–V* relationship before (○) and during (□) bath application of diC_8. Peak I_{Ca} densities (pA pF^{-1}) are measured in 5 different myocytes from 5 rat hearts. Vertical bars in *I–V* plots indicate the standard errors (** $P < 0·01$; *** $P < 0·001$).

Intracellular photorelease of caged diC_8 increases I_{Ca}

As our initial attempts to directly activate PKC failed to produce clear PKC-dependent effects on I_{Ca}, we next tested intracellular photorelease of caged diC_8, which has recently been demonstrated to stimulate a PKC-dependent positive inotropic response in rat cardiac myocytes (Pi *et al.* 1997). Isolated adult rat ventricular myocytes were first loaded with caged diC_8 (see Methods) and then continuous perfusion was carried out for at least 10 min to remove the extracellular unincorporated caged diC_8 prior to studying a given cell. Figure 5*A* shows the time course of the effect of photoreleased caged diC_8 on I_{Ca}. The arrow indicates the time point of exposure to 10 s UV light. Photorelease of diC_8 produced a gradual increase in I_{Ca} with $t_{½} = 5·6 ± 1·0$ min ($n = 13$), which plateaued and then

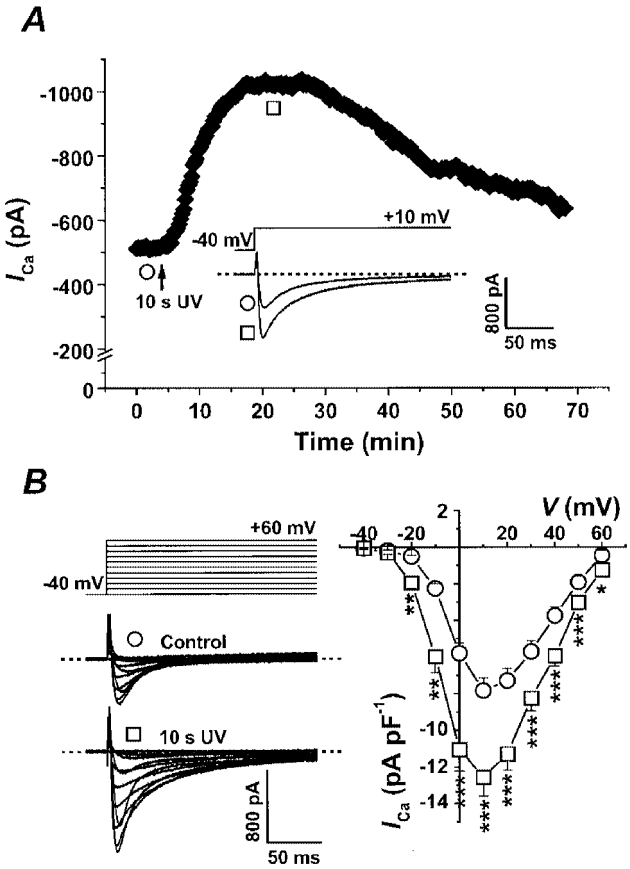

Figure 5. Photoreleased diC_8 increases I_{Ca}
A, the time course of I_{Ca} stimulated by photoreleased diC_8 in a representative myocyte. Peak I_{Ca} is plotted in response to test pulses to +10 mV every 15 s. The arrow indicates the time point of 10 s UV light exposure. The inset displays the stimulus protocol and the superimposed original current traces of I_{Ca} before (○) and after (□) UV light exposure. The dashed line indicates the zero current level. $C_m = 72$ pF. *B* shows the stimulus protocol, representative original current traces, and mean *I–V* relationship before (○) and after (□) photorelease of diC_8. Peak I_{Ca} densities (pA pF^{-1}) were measured in 13 different myocytes from 10 rat hearts. Vertical bars in *I–V* plots indicate the standard errors (* $P < 0·05$; ** $P < 0·01$; *** $P < 0·001$).

gradually declined. The inset displays the stimulus protocol and the superimposed representative current traces illustrated before (○) and after (□) photorelease of diC_8. Peak I_{Ca} densities measured in 13 different myocytes from 10 rat hearts are plotted as a function of membrane potential in Fig. 5*B*. Photorelease of diC_8 increased I_{Ca} $60{\cdot}8 \pm 11{\cdot}4\%$ at +10 mV following a 10 s UV light exposure ($n = 13$, $P < 0{\cdot}01$), and a comparable increase was seen throughout the voltage range studied.

To confirm that the measured currents before and after UV photorelease of diC_8 were through L-type Ca^{2+} channels, we investigated the sensitivity of these currents to Ca^{2+} channel blockers. First, we examined the effect of the inorganic Ca^{2+} channel blocker, Cd^{2+}, which potently blocks L-type Ca^{2+} channels. Figure 6*A* displays that, in cells loaded with caged diC_8, 0·2 mM Cd^{2+} can completely inhibit the inward current at +10 mV before and after UV photorelease of diC_8. The inhibitory effect of the Cd^{2+} is also largely reversible. We next examined the effect of the dihydropyridine nifedipine, which is a selective and voltage-dependent blocker of L-type Ca^{2+} channels. Figure 6*B* shows the effect of nifedipine on peak inward currents at +10 mV, which have been enhanced by photorelease of diC_8. Application of 1·5 μM nifedipine at the holding potential of −80 mV resulted in about a 40% decrease in the inward current. Changing the holding potential to −40 mV from −80 mV produced no significant change in I_{Ca} measured under control conditions (data not shown); however, in the presence of 1·5 μM nifedipine, there was a large enhancement of block. Application of 10 μM nifedipine further blocked the currents, again in a voltage-dependent fashion. From a holding potential of −40 mV, 10 μM nifedipine produced near complete block of the measured inward current. These data confirm that under the present ionic and experimental conditions, the measured currents are through L-type Ca^{2+} channels with minimal contamination by other currents.

As the photorelease of diC_8 is proportional to UV exposure time (Huang *et al.* 1996), we next examined whether there was a concentration-dependent effect of diC_8 on I_{Ca}. Concentration-dependent effects of photoreleased diC_8 were previously shown for the positive inotropic effect of this compound on isolated rat ventricular myocytes (Pi *et al.* 1997). The composite time courses for the response of I_{Ca} to 3 s and 10 s UV exposure are shown in Fig. 7*A*. The peak I_{Ca} gradually increased in response to diC_8 and reached a plateau. The kinetics of current increase were comparable for both 3 s and 10 s UV light exposure as the $t_{½}$ values were not significantly different, $4{\cdot}2 \pm 0{\cdot}7$ min ($n = 8$) and $5{\cdot}6 \pm 1{\cdot}0$ min ($n = 13$), respectively. However, 10 s UV exposure did cause a significantly greater increase in I_{Ca} compared to 3 s UV exposure, $60{\cdot}8 \pm 11{\cdot}4\%$ *vs.* $20{\cdot}7 \pm 8{\cdot}5\%$, respectively ($P < 0{\cdot}01$). These data suggest that there is a concentration-dependent effect of intracellular diC_8 on I_{Ca}, and that the rate-limiting steps in this effect are downstream from diC_8 production.

We also investigated whether photorelease of diC_8 altered the kinetics of I_{Ca} decay. The percentage of I_{Ca} decay at 40 and 200 ms was measured at a test potential of +10 mV. The decay of I_{Ca} has previously been demonstrated to be due to both Ca^{2+}- and voltage-dependent inactivation. We anticipated an acceleration of current decay due to the larger currents after photorelease of diC_8 and greater Ca^{2+}-dependent inactivation. However, we found that the percentage of I_{Ca} decay at 40 ms was reduced from $49{\cdot}3 \pm 3{\cdot}1\%$ in control to $42{\cdot}7 \pm 3{\cdot}2\%$ after 10 s UV photorelease of diC_8 ($n = 11$, $P < 0{\cdot}01$, see Table 1). Likewise, the percentage of I_{Ca} decay measured at 200 ms

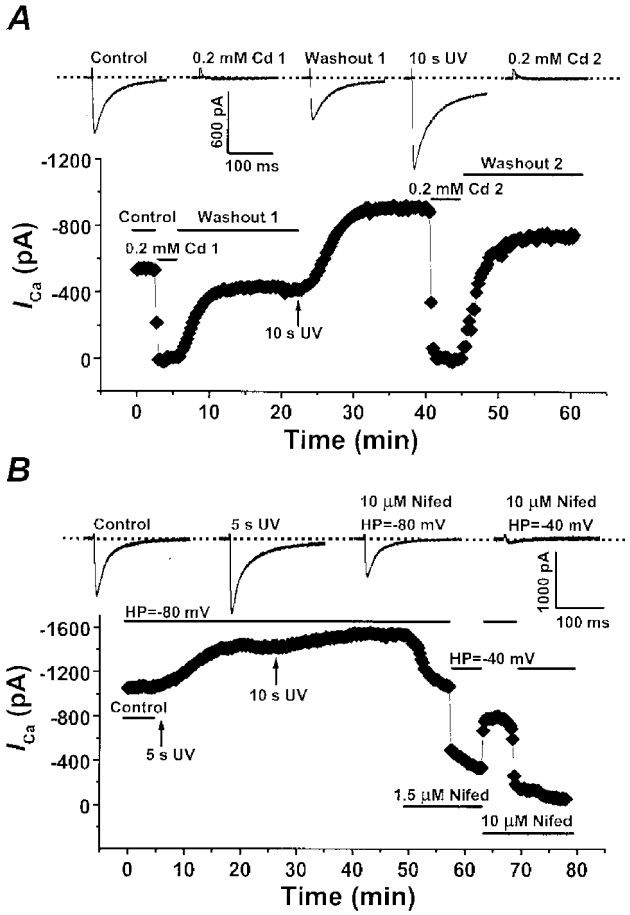

Figure 6. Cadmium and nifedipine block I_{Ca} stimulated by photoreleased diC_8

A, the inhibition of I_{Ca} by 0·2 mM cadmium (Cd^{2+}) before and after photorelease of diC_8. Peak I_{Ca} is plotted in response to test pulses to +10 mV every 15 s. The vertical arrow indicates the time point of 10 s UV light exposure and the horizontal bars describe the experimental conditions. The inset displays the original current traces in response to the each treatment during experiment. The dashed line indicates the zero current level. $C_m = 90$ pF. *B*, the voltage-dependent inhibition of I_{Ca} by nifedipine (Nifed). The vertical arrows indicate the time of 5 s and 10 s UV light exposure. The horizontal bars indicate the experimental conditions. The inset displays the original current traces in response to each treatment. The dashed line indicates the zero current level. HP, holding potential. $C_m = 140$ pF.

was reduced from 96·6 ± 0·9 % in control to 89·4 ± 1·3 % after photorelease ($n = 11$, $P < 0·01$, see Table 1). The slowing of the current decay suggests alterations in Ca^{2+}- or voltage-dependent gating of the channels following modulation by diC_8.

The initial experiments revealed that photorelease of diC_8 caused a concentration-dependent increase in I_{Ca}, similar in characteristics to that stimulated by ET-1. The following experiment examined whether this increase in I_{Ca} was sensitive to the PKC inhibitor chelerythrine, like the response to ET-1. Figure 8 plots the time course of the effect of photoreleased diC_8 on I_{Ca} without (left) or with a pre-incubation with 4 μM (middle) and 8 μM (right) chelerythrine. Photorelease of diC_8 for 10 s only resulted in a 22·2 ± 12·9 % ($n = 6$) increase in I_{Ca} of myocytes pretreated with 4 μM chelerythrine compared to a 60·8 ± 11·4 % ($n = 13$) increase in control. However, following pre-incubation with 8 μM chelerythrine, 10 s of UV exposure led to a 14·0 ± 16 % decrease in I_{Ca} relative to the baseline ($n = 8$, $P < 0·001$). Control experiments with chelerythrine alone or chelerythrine plus 10 s UV light exposure did not show any significant effect on I_{Ca} over the period of time studied. We conclude that the concentration-dependent inhibition of the effect of photoreleased diC_8 on I_{Ca} produced by chelerythrine is consistent with this effect being mediated by a PKC-dependent pathway. The inhibition of I_{Ca} in the presence of 8 μM chelerythrine following photorelease of diC_8 may reflect an unmasking of the PKC-independent blocking effect demonstrated by bath application of diC_8 (Fig. 4).

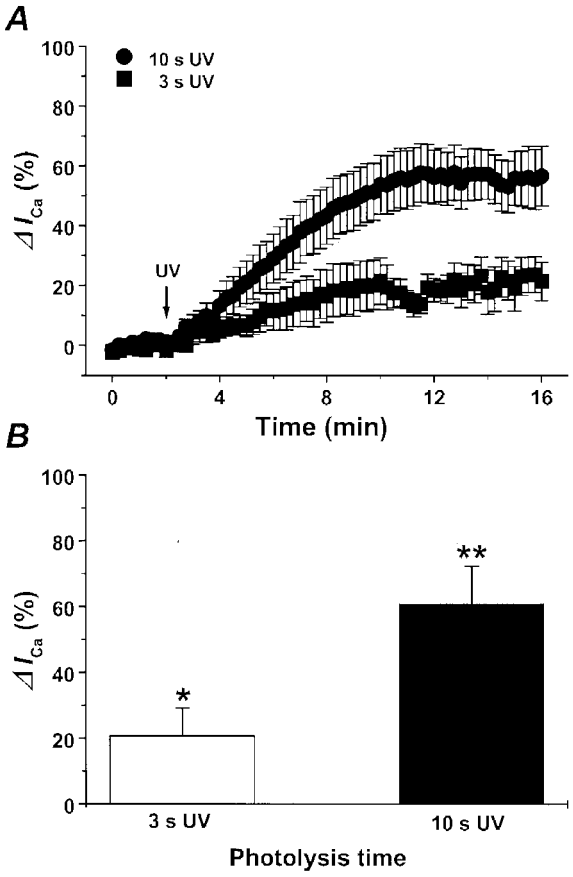

Figure 7. Average time course of I_{Ca} in response to UV photorelease of diC_8

A, the mean percentage increase of I_{Ca} induced by 3 s (■) and 10 s (●) of UV light. Peak I_{Ca} is plotted in response to test pulses to +10 mV every 15 s. The vertical arrow indicates the time point of UV light exposure. *B*, the mean maximum responses to 3 s (□) and 10 s (■) of UV light exposure. Vertical bars in both *A* and *B* indicate the standard errors (* $P < 0·05$; ** $P < 0·01$, compared to control).

If ET-1 and photorelease of diC_8 act by the same PKC-dependent pathway to modulate I_{Ca}, then we predicted that following a saturating response to photoreleased diC_8, application of ET-1 would not further upregulate I_{Ca}. Figure 9 displays the response of I_{Ca} measured in a myocyte following five successive exposures to UV light. I_{Ca} was increased by 38, 66, 83 and 100 % in response to successive exposures of 1, 5, 10 and 20 s of UV light, respectively. An additional exposure of 30 s UV light failed to further increase I_{Ca}, suggesting that the response was saturated. Addition of 10 nM ET-1 to the cell after saturating the photoreleased diC_8 effect not only failed to further increase I_{Ca}, but actually resulted in a decrease in I_{Ca}. This result suggests that the regulation of I_{Ca} by diC_8 and ET-1 is complex, but is consistent with a PKC-dependent upregulation of I_{Ca} sharing a final common pathway for photoreleased diC_8 and ET-1. The observed inhibition by ET-1 may represent the unmasking of an inhibitory influence of ET-1 on I_{Ca}, perhaps acting through a distinct ET-1 receptor subtype. The effect of ET-1 was reversible upon washout and actually resulted in an overshoot of I_{Ca} compared to the pre-ET-1 levels.

To investigate the mechanism for the proposed PKC-dependent modulation of I_{Ca} by photoreleased diC_8, we examined possible changes in the inactivation of L-type Ca^{2+} channels. For example, a shift in the voltage dependence of Ca^{2+} channel inactivation to more positive potentials by photoreleased diC_8 could underlie the observed increase in I_{Ca}. A gapped double-pulse protocol (see Fig. 10*A*) was employed to examine the voltage dependence of current inactivation following a 1000 ms prepulse over a range of potentials from −50 to +40 mV. Figure 10*B* displays the mean data from four cells studied before and after photorelease of diC_8. The peak current from the test pulse normalized to the maximal test pulse current was plotted as a function of membrane potential and then fitted to a Boltzmann distribution. The mean $V_{0·5}$ and k were unchanged (−22 ± 0·77 mV and 4·1 ± 0·15 mV in control and −22·6 ± 0·57 mV and 4·4 ± 0·12 mV after photorelease of diC_8). These data argue that photoreleased diC_8 does not result in a significant voltage shift in the inactivation of I_{Ca}.

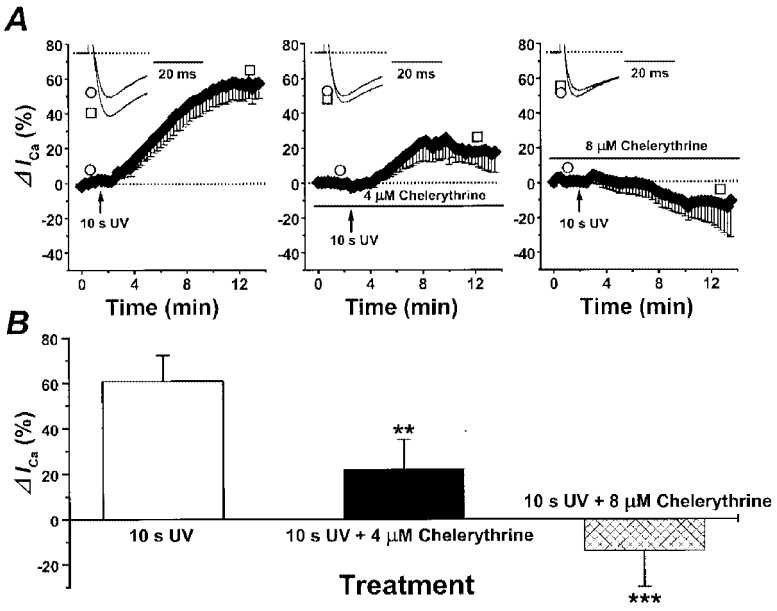

Figure 8. Chelerythrine blocks the effect of photoreleased diC_8 on I_{Ca}

A, the mean percentage increase in I_{Ca} induced by 10 s UV light exposure in the absence (left) and presence of 4 μM (middle) or 8 μM chelerythrine (right). Peak I_{Ca} is plotted in response to test pulses to +10 mV every 15 s. The insets display the normalized superimposed original current traces of control I_{Ca} (○) and photoreleased diC_8 (□). The vertical arrows indicate the time point of UV light exposure. The dashed line indicates the zero current level. *B*, the sum of the maximal effect of photoreleased diC_8 on I_{Ca} in the absence (□) and presence of 4 μM (■) or 8 μM (▨) chelerythrine. Vertical bars in *A* and *B* indicate the standard errors (** $P < 0{\cdot}01$; *** $P < 0{\cdot}001$, compared to 10 s UV exposure alone).

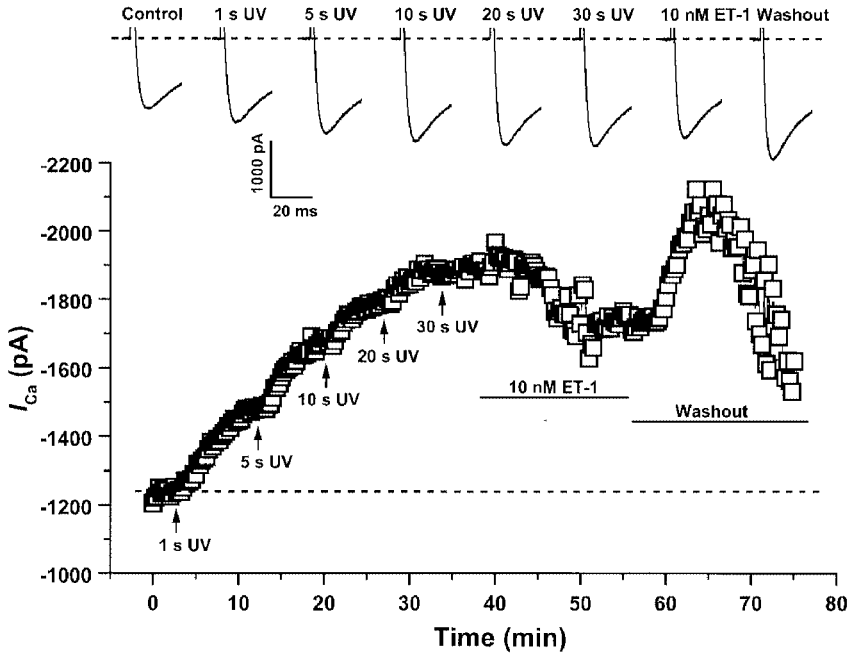

Figure 9. Effect of successive UV photorelease of diC_8 and ET-1 on I_{Ca}

Peak I_{Ca} is plotted in response to test pulses to +10 mV every 15 s. The vertical arrows indicate the times of each UV light exposure. Exposure of 1, 5, 10 and 20 s UV light, in the presence of caged diC_8, gradually increased I_{Ca} by 38, 66, 83 and 100%, respectively. A final 30 s UV light exposure failed to increase I_{Ca} further, and ET-1 (10 nM) was then applied where indicated. The original traces corresponding to each UV light exposure are shown above. The dashed lines indicates the zero current level. $C_m = 188$ pF.

DISCUSSION

ET-1 regulation of I_{Ca}

ET-1 was initially described as a potent vasoconstrictor, but further investigations showed that ET-1 also produces a positive inotropic effect when applied to many isolated cardiac muscle preparations (Ishikawa *et al.* 1988; Takanashi & Endoh, 1991; Pi *et al.* 1997). Multiple studies have also demonstrated that an increase in the intracellular Ca^{2+} transient contributes to this positive inotropic effect (Qiu *et al.* 1992; Damron *et al.* 1993; Ebihara *et al.* 1996). It was originally postulated that the basis for the increased Ca^{2+} transient was a stimulation of I_{Ca} (Ishikawa *et al.* 1988), and the results of the present study are consistent with this hypothesis, as 10 nM ET-1 caused a reproducible increase in I_{Ca} in adult rat ventricular myocytes. However, previous investigations examining the effect of ET-1 on L-type Ca^{2+} channels have produced conflicting results with decreases (Ono *et al.* 1994; Xie *et al.* 1996), increases (Lauer *et al.* 1992; Bkaily *et al.* 1995), and no effect (Thomas *et al.* 1997) on basal I_{Ca} being reported. Comparison of these different studies reveals differences in species, differences in concentrations of ET-1 tested, and differences in experimental techniques.

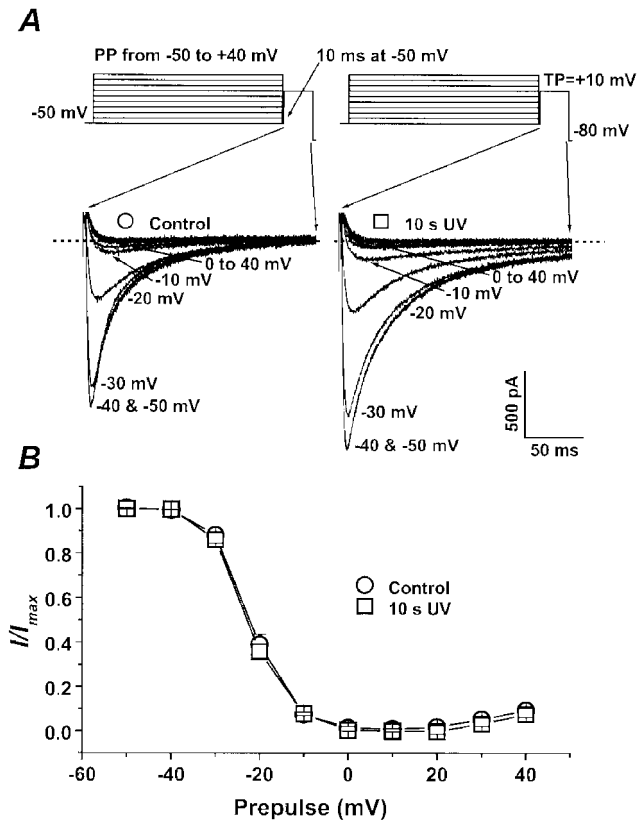

Figure 10. Photorelease of diC_8 does not change steady-state inactivation of I_{Ca}

A, the gapped double-pulse voltage protocol with 1000 ms prepulses (PP) over a range of potentials followed by a 200 ms test pulse (TP) to +10 mV. Representative current traces during the test pulses before (○) and after (□) photorelease of diC_8 are displayed, and the dashed lines indicate the zero current level. $C_m = 142$ pF. *B*, the mean normalized current (I/I_{max}) during the test pulse to +10 mV as a function of the 1000 ms prepulse potential before (○) and after photoreleased diC_8 (□). This inactivation relationship was examined in 4 different cells from 3 rat hearts. The data are fitted to a Boltzmann distribution using a non-linear least-squares regression. Vertical bars indicate standard errors of the data.

Recent pharmacological studies have suggested that multiple subtypes of ET receptors, ET_A and ET_B, exist, which may have opposing effects on I_{Ca} (Kelso *et al.* 1998). For example, ET_B receptors have been implicated in the stimulation of I_{Ca} in rabbit ventricular myocytes based on the greater potency of ET-3 as an agonist and on the sensitivity to specific ET receptor antagonists (Kelso *et al.* 1998). Based on our results, we suggest that such an upregulation of I_{Ca} may be mediated by ET_B receptor coupling to the G_q–PLC–DAG–PKC pathway. In contrast, the majority of studies characterizing the role of ET-1 in regulation of I_{Ca} have exhibited either no effect on basal currents or an inhibition of I_{Ca} (Ono *et al.* 1994; Xie *et al.* 1996; Thomas *et al.* 1997). In addition, several studies have demonstrated a clear inhibition of β-adrenergic (isoproterenol (isoprenaline))-stimulated I_{Ca} (Cheng *et al.* 1995; Xie *et al.* 1996; Thomas *et al.* 1997). Based on pharmacological studies (Ono *et al.* 1994; Thomas *et al.* 1997), the inhibition of basal currents and isoproterenol-stimulated currents by endothelin has been suggested to be mediated by ET_A receptors. For example, BQ123, a specific ET_A receptor antagonist, blocks the observed inhibition in several different preparations. Our results also reveal that ET-1 can inhibit I_{Ca} when it is maximally upregulated by photoreleased diC_8 (Fig. 9). Therefore, it is possible that multiple ET receptor subtypes can be present in ventricular muscle, which may exert opposing actions on L-type Ca^{2+} channels, and, furthermore, the receptor subtypes present may be dependent on the species studied. The system is probably even more complex, as subtypes of ET_A and ET_B receptors have also been proposed.

Differences in experimental techniques may also contribute to the diversity of results observed. Perhaps the most important variable is related to the type of patch clamp technique used. When the ruptured patch clamp technique is used, dialysis of large molecules from the intracellular compartment occurs, while use of the perforated patch clamp technique, as in the present study, allows electrical access to the cell and dialysis of monovalent cations and anions with no exchange of larger molecules, which may be involved in regulating the channels. A previous study using isolated rabbit ventricular myocytes showed that 1 nM ET-1 caused a 25% increase in I_{Ca} when studied using the nystatin-perforated patch clamp technique, but had no effect when the ruptured patch clamp technique was used (Kelso *et al.* 1996). One potential regulatory molecule which may be affected by cellular dialysis is GTP. Using the

ruptured patch technique, Lauer and colleagues reported an increase in I_{Ca} in response to 10 nM ET-1 only if GTP was included in the pipette; otherwise ET-1 caused a decrease in I_{Ca} (Lauer *et al.* 1992). Others have not confirmed that GTP is the critical intracellular constituent required for regulation (Cheng *et al.* 1995), but use of the amphotericin-perforated patch technique in the present study should preserve intracellular GTP as well as other potential regulatory molecules.

Many other neurohormone receptors in the heart are coupled via G_q to PLC and the resulting liberation of IP_3 and DAG. The regulation of I_{Ca} by these agents has also been investigated by others, with apparently conflicting results present in the literature. The best studied is the effect of α_1-adrenergic receptor stimulation on I_{Ca}. An initial study using bovine trabeculae and the sucrose-gap voltage clamp demonstrated an increase in I_{Ca} in response to α_1-adrenergic stimulation (Bruckner & Scholz, 1984); however, many subsequent studies using the ruptured whole-cell patch clamp technique failed to show any clear modulation of cardiac I_{Ca} in several species including the rat (Boutjdir *et al.* 1992; Fedida & Bouchard, 1992). More recent evidence has revealed, in rat ventricular myocytes, that if the perforated patch technique is employed, α_1-adrenergic stimulation causes an increase in I_{Ca} (Liu & Kennedy, 1998). Cell-attached single channel studies have confirmed that phenylephrine can increase the open probability of single L-type Ca^{2+} channels and enhance the ensemble Ca^{2+} channel currents, and these effects are blocked by the PKC inhibitor chelerythrine (Zhang *et al.* 1998). In aggregate, these findings suggest that regulation of I_{Ca} in rat ventricular myocytes by the G_q–DAG–PKC pathway is altered by the intracellular dialysis that occurs using the ruptured patch clamp technique, whereas the upregulation of I_{Ca} in response to PKC activation is clearly evident when the intracellular environment is preserved using the cell-attached or perforated patch clamp technique.

Phorbol ester modulation of I_{Ca}

Phorbol esters can directly activate PKC, bypassing surface membrane receptors, and therefore these agents can be useful tools for determining the role of PKC in regulatory pathways. In the present experiments, we found that both the active form of phorbol ester, PMA, and the inactive form, 4αPDD, produced comparable, small increases in I_{Ca} in rat ventricular myocytes. The similar effects of PMA and 4αPDD argue against a PKC-specific effect of these compounds on I_{Ca}. In a previous study using guinea-pig ventricular myocytes a small inhibition by both PMA and 4αPDD was found (Asai *et al.* 1996), which also suggested PKC-independent effects on I_{Ca}. However, our results are in direct contrast to the results of Zhang *et al.* (1997) which demonstrated that 100 nM PMA produced a 40% inhibition of basal I_{Ca} in adult rat ventricular myocytes while 4 αPDD was without effect. In addition, the specificity of the inhibition of PKC in their study was further confirmed by the use of peptide inhibitors of PKC. The most obvious difference between the studies is the use of the ruptured patch clamp technique by Zhang *et al.*, but the use of the perforated patch clamp in the present study. In addition, the complexity of the response of I_{Ca} to phorbol esters has been demonstrated in studies of neonatal rat ventricular myocytes and adult canine ventricular myocytes which show a biphasic effect on I_{Ca}, with an initial stimulation followed by an inhibition (Lacerda *et al.* 1988; Tseng & Boyden, 1991). Studies in neurons and endocrine cells have also revealed both stimulatory and inhibitory effects of phorbol esters on L-type Ca^{2+} channels, depending on the preparation (Di Virgilio *et al.* 1986; Yang & Tsien, 1993). It is possible that PMA is capable of activating different isoforms of PKC which may have opposing effects on L-type Ca^{2+} channels. The effect of phorbol esters on the chronotropic state of neonatal rat ventricular myocytes has previously been attributed to opposing effects of different PKC isoforms (Johnson & Mochly-Rosen, 1995). The net result of stimulation by phorbol esters probably depends on the PKC isoforms which are present in a given preparation and their ability to regulate I_{Ca}, which may be in part altered by intracellular dialysis.

diC_8 modulation of I_{Ca}

Several synthetic DAG analogs can directly activate PKC, and these agents have been used as tools to examine the role of PKC in the regulation of myocardial contraction and excitability. Previous studies by Walker and colleagues have shown that bath application of diC_8 to isolated adult rat ventricular myocytes engendered a marked negative inotropic effect, as measured by cell shortening which was not blocked by chelerythrine (Pi *et al.* 1997). In contrast, the intracellular release of caged diC_8 led to a large increase in cell shortening which was mainly due to an increase in the intracellular Ca^{2+} transient, and this response was abolished by chelerythrine suggesting a PKC-dependent positive inotropic effect (Pi *et al.* 1997; Pi & Walker, 1998). In the present study, a similar modulation of I_{Ca} by diC_8 has been revealed, as bath application of 7·5 μM diC_8 resulted in a large decrease in I_{Ca} while photorelease of intracellular diC_8 greatly upregulated I_{Ca}. Furthermore, chelerythrine did not affect the inhibition produced by bath-applied diC_8, but did block the stimulation of I_{Ca} produced by photorelease of diC_8. These parallel results suggest that the inotropic effects mediated by diC_8 are in large part due to modulation of L-type Ca^{2+} channels. A PKC-independent inhibition of I_{Ca} by bath-applied diC_8 is consistent with the results of others obtained using the same rat ventricular myocyte preparation (Schreur & Liu, 1996), embryonic chick cardiomyocytes (Conforti *et al.* 1995), rat myometrial cells (Kusaka & Sperelakis, 1995), and chick dorsal root ganglion neurons (Hockberger *et al.* 1989). The potential mechanism for this modulation of I_{Ca} remains unknown. However, it has become increasingly evident that multiple proteins besides PKC contain a DAG binding motif (Newton, 1997). Does DAG interact directly with a Ca^{2+} channel subunit or associated protein? Interestingly, previous studies have

provided evidence that another synthetic DAG analogue, OAG, does not inhibit I_{Ca} (Conforti *et al.* 1995; Schreur & Liu, 1996). Future studies will be needed to clarify this mechanism of modulation and its physiological relevance.

Photorelease of diC_8, in direct contrast to the effect of bath-applied diC_8, stimulates an increase in I_{Ca} which is inhibited by the PKC inhibitor chelerythrine. This effect is specific for photolysis of caged diC_8, as control experiments exposing cells loaded with vehicle (DMSO) to prolonged UV light failed to show any effect on I_{Ca}. Previous work by Walker and colleagues has demonstrated that the effect of photoreleased diC_8 on cell shortening is stereospecific, as only the enantiomer *S*-diC_8 stimulated cell shortening, arguing that the observed effects are due to the liberated diC_8 and not other by-products of photolysis (Pi *et al.* 1997). The present study used racemic caged diC_8, as did the majority of the previous experiments (Huang *et al.* 1996; Pi *et al.* 1997; Pi & Walker, 1998), and we assume the effect that we observed is primarily due to release of *S*-diC_8. The effect of photoreleased diC_8 was also shown to be concentration dependent, since increased photolysis time (3 s *vs.* 10 s) produced a greater stimulation of I_{Ca} (~20% *vs.* ~60%). Furthermore, the nearly identical kinetics for the increase in I_{Ca} by photorelease of diC_8 for either 3 s or 10 s suggest that the rate-limiting step in this regulation is downstream from diC_8 binding to its effectors. We propose that a major effector which binds photoreleased diC_8 is PKC and this activated kinase is then responsible for upregulation of I_{Ca}. A role for PKC in this process is supported by the ability of chelerythrine to concentration dependently inhibit the upregulation of I_{Ca}.

The finding that the same molecule, diC_8, can produce opposite effects on I_{Ca} when applied extracellularly or photoreleased intracellularly is a central paradox of these studies. The specific membrane pools of DAG liberated may be critically important in determining the action of this second messenger. Does intracellularly liberated DAG have preferential access to the critical PKC binding sites required for enhancement of I_{Ca}? Does diC_8 applied extracellularly directly interact with the Ca^{2+} channel or associated proteins, by virtue of entering via the surface membrane of the cell, to inhibit the channel prior to any potential effects on PKC? Can diC_8 produce both types of modulation on the channel which are additive, or are the effects exclusive in nature? In fact, we observed that 8 μM chelerythrine not only blocked the stimulation of I_{Ca} by 10 s UV photorelease of diC_8, but also unmasked a small but significant inhibition of I_{Ca}. This suggests that if the PKC-mediated enhancement of I_{Ca} is blocked, photoreleased diC_8 is capable of inhibiting the channel just as bath-applied diC_8 does. Our working hypothesis is that the two methods of applying diC_8 result in binding of diC_8 to different sites involved in two distinct mechanisms of modulation of I_{Ca}, causing opposite effects on I_{Ca}. How these two forms of modulation by diC_8 interact at the level of the Ca^{2+} channel will require future evaluation.

ET-1 and photoreleased diC_8 stimulate I_{Ca} through a common pathway

Both ET-1 and photorelease of diC_8 caused an increase in I_{Ca} in the present study, and we conclude that both of these agents lead to modulation of I_{Ca} by a common final pathway requiring activation of PKC. There are many similarities between the modulation of I_{Ca} by ET-1 and photoreleased diC_8 which lead to this conclusion. First, the increases in I_{Ca} in response to both ET-1 and photorelease of diC_8 are blocked by the specific PKC inhibitor chelerythrine. Second, the currents stimulated by ET-1 or photoreleased diC_8 show similar changes in the kinetics of current decay, with both treatments resulting in a statistically significant slowing of current decay. Third, if the effect of photoreleased diC_8 is maximal, ET-1 causes no further stimulation of I_{Ca}, suggesting a common final pathway which mediates upregulation of I_{Ca} (Fig. 9). Fourth, the slower time course for upregulation of I_{Ca} mediated by ET-1 relative to that mediated by photoreleased diC_8 is consistent with ET-1 action requiring additional steps (i.e. receptor binding, G_q stimulation, PLC activation) prior to PKC stimulation. The major difference between the effects of ET-1 and photoreleased diC_8 that we observed was the relative magnitude of the effects. Photorelease of diC_8 with 10 s UV light exposure produced about a two-fold greater increase in I_{Ca} than did 10 nM ET-1. It is possible that ET-1 at an optimal concentration may exert comparable effects, but 10 nM was tested as our previous study had demonstrated that this concentration produced the maximal positive inotropic effect in this preparation (Pi *et al.* 1997). The present observations imply that 10 nM ET-1 does not fully activate the DAG–PKC pathway or that competing inhibitory influences are also activated on by this agonist.

Molecular mechanisms of PKC regulation of L-type Ca^{2+} channels

The present study and previous studies suggest that PKC-activating pathways can upregulate the L-type Ca^{2+} channel in cardiac muscle; however, the substrate(s) for PKC and the underlying molecular mechanisms of this regulation remain unknown. The subunits of the L-type Ca^{2+} channel represent potential targets for PKC phosphorylation, and *in vitro* biochemical studies have demonstrated that both the α_{1C} and β_{2a} subunits of the L-type Ca^{2+} channel can be substrates for PKC (Puri *et al.* 1997). Heterologous expression studies of the cardiac α_{1C} subunit in *Xenopus* oocytes have revealed that the amino terminus of α_{1C} may be critical for upregulation of α_{1C} by PKC (Bouron *et al.* 1995; Shistik *et al.* 1998). Bouron *et al.* (1995) proposed that the cloned human α_{1C} subunit is not upregulated by PKC because it lacks the initial 41 amino acids present in the cloned rabbit α_{1C} subunit which is significantly stimulated by PKC in heterologous systems (Bouron *et al.* 1995). However, no studies have produced direct evidence for PKC-dependent phosphorylation of the α_{1C} subunit or other subunits in intact cells. It remains possible that the substrate for PKC modulation may be associated regulatory proteins rather than channel subunits.

A second important issue regarding the molecular mechanisms of the upregulation of cardiac L-type Ca^{2+} channels is what isoform(s) of PKC are responsible for the regulation. Our experiments using chelerythrine supported a role for PKC, but did not provide any evidence for the isoform of PKC responsible. Adult rat ventricular myocytes are known to express several PKC isoforms, which probably have distinct cellular targets (Puceat & Vassort, 1996). ET-1 has been suggested to cause the translocation of PKCε (Jiang *et al.* 1996), and therefore it is a potential candidate for regulation of I_{Ca}. Another possibility is that different PKC isoforms may exert opposing effects on L-type Ca^{2+} channels. This may explain some of the conflicting data in the literature. For example, Zhang *et al.* (1997) reported that inhibitory peptides for conventional PKC isoforms blunted the downregulation of I_{Ca} by PKC. Future studies using isoform-specific antagonists and agonists will be necessary to establish which isoform(s) of PKC are responsible for the upregulation of I_{Ca}.

In summary, the present work suggests that activation of PKC by either receptor stimulation using ET-1 or intracellular photorelease of diC_8 significantly upregulates I_{Ca} studied using the perforated patch technique in rat ventricular myocytes. In contrast, a strong inhibition of I_{Ca} by bath-applied diC_8 occurs independently of PKC activation and represents a distinct mechanism by which DAG can modulate L-type Ca^{2+} channels. Therefore, DAG and PKC can exert strong regulatory influences on I_{Ca}, but the nature of the modulation is critically dependent on the pools of DAG liberated and the techniques used to study I_{Ca}.

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