Role of intracellular Ca\(^{2+}\) and pH in positive inotropic response of cardiomyocytes to diacylglycerol

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ACTIVATION OF protein kinase C (PKC) by diacylglycerol has been hypothesized to mediate the positive inotropic response of myocardium to the \(\alpha\)-adrenergic agonists angiotensin II and endothelin (28, 35). Phorbol esters have been useful in implicating PKC in the regulation of cardiac function (28, 35), but a clear picture of the role of PKC has not emerged, in part, because there are conflicting reports in the literature concerning the effects of phorbol esters on ventricular tissues. Positive (24), negative (5, 15, 21, 42), and both positive and negative inotropy (38) have been described, and these differences cannot be attributed to age or species differences. Moreover, a number of investigators have suggested that the negative inotropic effects of phorbol esters are independent of PKC (38, 39). In an attempt to clarify the role of PKC in ventricular muscle, we developed a method for controlled elevation of diacylglycerols within living cells using light activation of caged diacylglycerol (14). We observed for the first time that the widely used short-chain analog dioctanoylgllycerol (diC\(_8\)) is capable of initiating a strong positive inotropic effect in ventricular myocytes that is dose dependent, stereospecific, and blocked by PKC antagonists (26).

The magnitude of the response to photoreleased diC\(_8\) rivaled the magnitude of the myocyte response to the \(\beta\)-agonist isoproterenol (26). The intracellular mechanisms underlying the \(\beta\)-adrenergic response have been well characterized and involve stimulation of Ca\(^{2+}\) influx via phosphorylation of the L-type Ca\(^{2+}\) channel (10, 13), stimulation of the sarcoplasmic reticulum (SR) Ca\(^{2+}\) pump via phosphorylation of phospholamban (17, 40), and desensitization of the myofilaments to Ca\(^{2+}\) as a result of phosphorylation of troponin I (19). Thus regulation of excitation-contraction coupling, SR Ca\(^{2+}\) content, and myofilament properties all contribute to the cardiomyocyte response to \(\beta\)-stimulation. These changes can be mimicked by elevation of cAMP and stimulation of intracellular protein kinase A (PKA) (10, 13, 17, 40).

Diacylglycerol acting through PKC has been hypothesized to regulate some of these same processes such as L-type Ca\(^{2+}\) channel activity (8, 21), Ca\(^{2+}\) pumping (17, 31), and myofilament Ca\(^{2+}\) sensitivity (6, 12, 36). In contrast to the consensus that exists for the mechanisms of the positive inotropic and lusitropic action of PKA, regulation of contractile function by PKC is much less clearly defined. In the present study, we took advantage of the robust positive inotropic response produced by photogeneration of diacylglycerol to characterize PKC-dependent mechanisms with good signal-to-noise ratio in living cardiomyocytes. Reported here are results of measurements of intracellular Ca\(^{2+}\) and intracellular pH during this large enhancement of contractility. We found that the vast majority of the positive inotropic response to diacylglycerol is attributable to enhancement of systolic Ca\(^{2+}\) as a result of stimulation of the process of excitation-contraction coupling. Some of this work was presented in preliminary form to the Biophysical Society (27).

MATERIALS AND METHODS

All reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise noted. Endothelin-1, ryanodine, 2,3-butanediol monoxime (BDM), and \(\text{NH}_4\text{Cl}\) were prepared fresh in distilled water. Caffeine was prepared fresh in 1 mM Ca\(^{2+}\) Ringer buffer (see below). Caged diC\(_8\) was the \(\alpha\)-carboxy-2-nitrobenzyl form synthesized and purified as previously described (14). Fura 2, fluo 3, and 2',7'-bis(2-carboxylethyl)-5(6)-carboxyfluorescein (BCECF) were obtained in their acetoxymethyl ester (AM) forms from Molecular Probes (Eugene, OR). Ventricular myocytes were enzymatically dissociated from adult male rats as previously described (26). The yield was \(\sim\)1–3 \(\times\) 10\(^6\) cells per heart, and >80% were rod-shaped and Ca\(^{2+}\) tolerant. CaCl\(_2\) was added to Ca\(^{2+}\)-free Ringer to achieve the indicated Ca\(^{2+}\) concentration. Ca\(^{2+}\)-free Ringer buffer had the following composition (in mM): 125 NaCl, 2
NaH₂PO₄, 5 KCl, 1.2 MgSO₄, 25 HEPES, 5 pyruvate, 11 glucose, and 0.001 insulin, and pH was adjusted to 7.4 with NaOH at room temperature.

Fluorescence measurements. Cells were loaded with fluorescent indicators in their AM forms. Fura 2-AM, fluo 3-AM, or BCECF-AM were separately added to a suspension of 2–3 × 10⁶ cells/ml to a final concentration of 2 μM and then incubated for 1 h at room temperature. Myocytes were then pelleted, washed twice with 0.5 mM Ca²⁺ exchange activator nigericin to equilibrate intracellular and extracellular pH. At the end of each experiment, 1 mM Ca²⁺ Ringer solution was changed to calibration solution and signals were recorded for three pH standards. Calibration buffers contained 4 mM HEPES-KOH, 120 mM KCl, 0.5 mM EGTA, 5 mM pyruvate, 5.6 mM glucose, 10 mM K-ATP, and the ionophores nigericin (20 μM), ionomycin (4 μM), and carbonyl cyanide m-chlorophenylhydrazone (0.2 μM). pH was adjusted at room temperature with KOH or HCl to standard pH values 7.42, 6.72, and 7.23.

Twitch shortening. Electrical field stimulation was carried out in a custom-designed 200 μl Plexiglas chamber with a glass floor and two platinum electrodes. The standard stimulation protocol was 0.4 Hz, 1 ms duration, and 40 V with the use of a Grass SD9 stimulator (Quincy, MA) at 20–22°C. The chamber was mounted on a Nikon Diaphot inverted microscope. The myocyte image was created with transmitted light filtered to pass red light. A DM-600 dichroic mirror reflected the red light emerging from the output port up onto a Panasonic charge-coupled device video camera. Individual cell length was monitored with a model VED 104 video edge detector and plotted on an X-Y plotter.

Statistics. Data are expressed as means ± SE. Statistical significance was tested with a Student’s paired or unpaired t-test, and P < 0.05 was taken as significant.

RESULTS

Ca²⁺ transients. Figure 1 summarizes the effects of controlled release of diacylglycerol on electrically paced twitch amplitude and the corresponding intracellular Ca²⁺ transients measured with flou 3. Release of sufficient diC₈ to increase twitch amplitude by fourfold increased the Ca²⁺ transient amplitude by about twofold (Fig. 1A). The increase of systolic Ca²⁺ developed in parallel with the increase in twitch amplitude (Fig. 1B). Fluo 3 was chosen for these studies because it is excited with visible light, so its use eliminates potential cross talk between fluorophore excitation and photolysis of the caged compound. However, fluo 3 is not a ratiometric indicator, so it is susceptible to artifacts that alter the effective dye concentration, such as dye leakage or cell motion. To control for the effects of cell motion, myocytes were incubated with 20 mM BDM after photorelease of diC₈. This treatment greatly reduced the extent of twitch shortening (by inhibiting actomyosin interactions), but the twofold increase in systolic Ca²⁺ was still observed (Fig. 1C).

In other experiments the ratiometric indicator fura 2 was used. Results were similar to those obtained with flou 3 (Fig. 2), further ruling out a significant influence of dye leakage and cell shortening on the fluorescence records. The extent to which the fura 2 excitation source photolyzed the caged compound as well as the degree of bleaching of fura 2 by the photolysis beam was also evaluated (see MATERIALS AND METHODS). We found such cross talk to be minimal with the specific protocols used.

In contrast to the consistent enhancement of systolic Ca²⁺ by diC₈, it was more difficult to characterize changes in diastolic Ca²⁺ because there was considerable variability from cell to cell. Approximately one-half of the cells examined showed a significant increase in diastolic Ca²⁺ and a corresponding decrease in resting cell length (e.g., Figs. 1A and 4C), whereas the other
one-half showed no change in either parameter (e.g., Fig. 1C). This variability appears to be intrinsic to the cell population because it was observed on each experimental day and did not correlate with temperature, dye loading conditions, or time after myocyte isolation. It was also independent of the Ca\(^{2+}\) indicator used. Cells derived from the left versus right ventricle or from different transmural regions of the heart may show a different diastolic response to diC\(_8\).

One possible explanation for dramatic changes in systolic Ca\(^{2+}\) after diC\(_8\) might be that the SR Ca\(^{2+}\) content increases (2). This occurs, for example, during \(\beta\)-adrenergic stimulation as a result of a dramatic enhancement of the rate of Ca\(^{2+}\) pumping (17). Figure 1A, inset, shows the time course of Ca\(^{2+}\) transients measured in the same cell before and after diC\(_8\). There was no significant change in the overall duration of the transients and no change in the rate of decay to baseline, suggesting that Ca\(^{2+}\) pumping is unaltered by diC\(_8\) under these experimental conditions. Figure 2 shows the results of an experiment in which the caffeine-releasable Ca\(^{2+}\) content of the SR was compared before and after diC\(_8\). There was no significant difference in the total fura 2 fluorescence signal produced by rapid caffeine application between control cells and cells exposed to a level of diC\(_8\) that increased the Ca\(^{2+}\) transient by more than twofold (Fig. 2).

Further information about SR Ca\(^{2+}\) content and Ca\(^{2+}\)-handling kinetics was obtained by investigating the phenomenon of postrest potentiation. Figure 3 shows a typical negative staircase in the twitch and Ca\(^{2+}\) transient responses of a rat myocyte after a 2-min rest period. The first twitch is very large, and then subsequent twitches decrease in amplitude to a steady-
state level that depends on the stimulation frequency. Again, changes in systolic Ca\(^{2+}\) paralleled the twitch changes. This phenomenon is thought to be due to a balance of Ca\(^{2+}\) leak and Ca\(^{2+}\) pump fluxes that causes the SR to be loaded during periods of rest. Photorelease of diC\(_8\) did not alter the maximum Ca\(^{2+}\) release or twitch response after the rest period, and diC\(_8\) did not alter the nature or kinetics of the negative staircase phenomenon (Fig. 3B). Thus there is no indication that Ca\(^{2+}\) pumping or SR Ca\(^{2+}\) content was changed significantly by this inotropic stimulus.

The principle change then appears to occur at the level of coupling electrical excitation to contraction. To test whether the enhancement of systolic Ca\(^{2+}\) after diC\(_8\) was due to stimulation of the normal excitation-contraction mechanism or to mobilization of Ca\(^{2+}\) via a distinct pathway, we treated myocytes with the Ca\(^{2+}\) release channel blocker ryanodine. At 2 µM, ryanodine inhibited both basal systolic Ca\(^{2+}\) and the diC\(_8\)-stimulated systolic Ca\(^{2+}\) by 50%. At 6 µM, ryanodine inhibited both types of Ca\(^{2+}\) transients by 80%. Thus diC\(_8\) appears to stimulate a ryanodine-inhibitable process consistent with diC\(_8\) regulating the traditional excitation-contraction coupling mechanism.

Ca\(^{2+}\) sensitivity of myofilaments. Another possible contributor to the positive inotropic action of diC\(_8\) is an increase in the Ca\(^{2+}\) responsiveness of the myofilaments. The fact that the twitch amplitude increased by three- to fourfold, whereas the Ca\(^{2+}\) transient only increased by twofold, opens the possibility that the Ca\(^{2+}\) regulatory system is also more sensitive to Ca\(^{2+}\) after diC\(_8\). However, it is also possible that changes in twitch shortening and free Ca\(^{2+}\) are not strictly proportional. To investigate Ca\(^{2+}\) sensitivity in living myo-

Fig. 2. Effect of diC\(_8\) on Ca\(^{2+}\) content of sarcoplasmic reticulum (SR). Measurements were performed on myocytes loaded with fura 2 and caged diC\(_8\). Top: 2 basal Ca\(^{2+}\) transients were measured, and then electrical stimulation was halted for 20 s. A rapid brief pulse of 20 mM caffeine was applied onto cell surface to induce SR Ca\(^{2+}\) release. Bottom: same protocol was repeated after a 20-s pulse of diC\(_8\) from caged diC\(_8\). Top inset: superimposed caffeine transients before and after diC\(_8\). Bottom inset: summary of SR Ca\(^{2+}\) content before and after diC\(_8\) in 12 myocytes. Mean area under caffeine Ca\(^{2+}\)-response curve was similarly unchanged by diC\(_8\). Data are means ± SE.

Fig. 3. Simultaneous measurements of twitch contractions and fluo 3 fluorescence during postrest potentiation. A: original records of twitch contractions (top) and fluo 3 fluorescence (bottom) showing 2 steady-state twitches followed by a 2-min rest period (break), and then stimulation was resumed until steady state was reestablished. Left: control records. Right: records 5 min after a 20-s diC\(_8\) pulse. B: time courses of recovery of twitch amplitude (top) or Ca\(^{2+}\) transient amplitude (bottom) during postrest potentiation before (control) and after diC\(_8\). Each data point represents mean ± SE for at least 8 cells. Curves show fits to a monoeponential function as previously described (41), which gave apparent rate constants of 2.2 s\(^{-1}\) (control twitch amplitude), 1.8 s\(^{-1}\) (diC\(_8\) twitch amplitude), 1.9 s\(^{-1}\) (control Ca\(^{2+}\) amplitude), and 2.0 s\(^{-1}\) (diC\(_8\) Ca\(^{2+}\) amplitude). Errors in these values are estimated to be ±15%.
cytes, we first examined the relationship between free Ca\(^{2+}\) and twitch shortening during the large swings in these two parameters observed during postrest potentiation. A comparison was then made between control and diC\(_8\) treated cells using this relationship. In most myocytes (13 of 22), there was no detectable difference in the ratio of cell shortening to Ca\(^{2+}\) transient amplitude before and after diC\(_8\) (Fig. 4A). In ~40% of myocytes (9 of 22), there was a small leftward shift in this relationship after diC\(_8\) treatment (not shown). The direction of this shift was the same as that observed after treatment with endothelin (Fig. 4B). However, unlike the diC\(_8\) response, the endothelin response was observed in all eight cells tested. The magnitude of this effect was also at least twofold greater in response to endothelin than to diC\(_8\).

It has been argued that a plot of peak shortening versus peak Ca\(^{2+}\) transient is an imperfect measure of myofilament Ca\(^{2+}\) dynamics because Ca\(^{2+}\) binding does not reach an equilibrium at this point in the twitch (34). A better assessment of myofilament Ca\(^{2+}\) responsiveness may be obtained by examining the relengthening limb of cell length versus free Ca\(^{2+}\) phase in a plane diagram. It is during the relengthening (relaxation) phase of the twitch that Ca\(^{2+}\) binding to the myofilaments may achieve a quasi-equilibrium (34). A plot of cell length versus intracellular free Ca\(^{2+}\) shows characteristic phase-plane loops in which the relengthening limb of the loops occurred along a common trajectory. The position of this relengthening trajectory is a measure of responsiveness of the myofilaments to Ca\(^{2+}\) (34), and this trajectory did not change in response to diC\(_8\) (Fig. 4C). For the cell shown in Fig. 4C, a significant increase in diastolic Ca\(^{2+}\) was observed after exposure to diC\(_8\). Interestingly, the new starting position at the lower left of each loop (representing diastolic Ca\(^{2+}\) and diastolic cell length) also moved along this common trajectory. Thus there was little evidence for a consistent increase or decrease of the myofilament responsiveness to Ca\(^{2+}\) after exposure of these intact, electrically stimulated myocytes to diC\(_8\).

The enhancement of contractility by diC\(_8\) was also blocked by the actinomyosin ATPase inhibitor BDM (Fig. 1C). The concentration for 50% inhibition was ~8 mM BDM in both control and diC\(_8\)-stimulated myocytes. This observation indicates that BDM inhibits control and diC\(_8\)-stimulated twitches proportionately and that diC\(_8\) did not alter how BDM interacts with the filaments. That myofilament properties were altered in at least subtle ways is suggested by the observation that the time course of the twitch was abbreviated after diC\(_8\) treatment (Fig. 1B, inset).

Intracellular pH. The positive inotropic actions of a number of agonists in the heart appear to be correlated with an intracellular alkalization (11, 16, 18, 29). To assess the contribution of pH changes to the mechanism of diC\(_8\) action, we measured intracellular pH with the fluorescent indicator BCECF. Intracellular pH was typically 7.1–7.2 before photorelease of diC\(_8\), and it did not change significantly under conditions in which diC\(_8\) caused a fourfold increase in twitch amplitude (Fig. 5A). The primary mechanism underlying alkalization is thought to be an increase in activity of a sarcolemmal Na\(^+\)/H\(^+\) exchanger (11, 16, 18, 24, 29, 32). We also measured this activity directly by determining the rate of recovery of pH from an acid load (24). First, it was confirmed in these experiments that the pH recovery process was virtually completely inhibited by the amiloride analog N-ethylisopropyl amiloride (EIPA) (not shown), consistent with this intracellular pH change being mediated by the Na\(^+\)/H\(^+\) exchanger. Figure 6A shows that diC\(_8\) did not accelerate the recovery from an acid load, indicating that diC\(_8\) did not stimulate Na\(^+\)/H\(^+\) exchange activity. The change in Na\(^+\)/H\(^+\) exchange activity measured in this way averaged −12 ± 13% (n = 6) after diC\(_8\). Further control experiments showed that regulation of the exchanger was normal in these cells because treatment with endothelin-1 resulted in a measurable alkalization (Fig. 5B) that was blocked by EIPA (Fig. 5C). Endothelin-1 also

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**Fig. 4. Effects of diC\(_8\) on myofilament responsiveness to Ca\(^{2+}\).**

A: relationship between cell twitch amplitude and intracellular Ca\(^{2+}\) amplitude in a control cell derived by plotting values obtained during postrest potentiation experiments as in Fig. 3. Twitch and Ca\(^{2+}\) transient amplitudes were normalized to their respective maxima. The cell was then exposed to a 20-s pulse of diC\(_8\), and the new relationship between twitch amplitude and Ca\(^{2+}\) transient amplitude was derived in the same manner. B: relationships between twitch amplitudes and intracellular Ca\(^{2+}\) amplitude before (control) and after 10 nM endothelin (ET). Lines in A and B represent linear regressions. C: plane loops for cell length versus free Ca\(^{2+}\) phase in a myocyte before diC\(_8\) (control) and at various times after exposure to diC\(_8\). Dotted line is shown to help visualize relengthening trajectory.
stimulated exchange activity by 125 ± 42% (n = 4) (Fig. 6B), consistent with previous reports of stimulation of the Na⁺/H⁺ exchanger by agonists (11, 16, 18, 29).

To rule out the possibility that the effects of diC₈ on Na⁺/H⁺ exchange and intracellular alkalinization escaped detection by BCECF because changes were either too small or too localized, we carried out further experiments with the Na⁺/H⁺ antiport inhibitor EIPA. Blockade of Na⁺/H⁺ antiporter activity with EIPA was without effect on the positive inotropic response to diC₈ (Fig. 7A). Therefore, the positive inotropic response did not require Na⁺/H⁺ antiport activity. In contrast to the effects of EIPA on diC₈ responses, the response to endothelin-1 was reduced by ~50% in the presence of EIPA (Fig. 7B). Overall, both the exchanger and its inhibitor behaved as expected in control experiments. We conclude that under conditions in which diC₈ gave rise to a large positive inotropic effect, diC₈ did not activate the Na⁺/H⁺ exchange mechanism and therefore did not cause intracellular alkalinization that could influence myofilament function.

**DISCUSSION**

Two well-established mechanisms for increasing the contractility of cardiac muscle are an increase in systolic Ca²⁺ and an increase in responsiveness of the myofilaments to Ca²⁺ (9). Under the conditions of our experiments, the positive inotropic response to diacylglycerol can be accounted for by an increase in the magnitude of the systolic Ca²⁺ transient. Diacylglycerol did not consistently alter the myofilament responsiveness to Ca²⁺. The Ca²⁺ transient can be influenced by the degree of Ca²⁺ loading in the SR (2), but we found no evidence that the SR was “superloaded” with Ca²⁺ as a result of diacylglycerol treatment. Another possibility is that diacylglycerol/PKC stimulated L-type Ca²⁺ channel activity. This channel is a major player in cardiac excitation-contraction coupling, and its activity has been shown to be stimulated by agonists that elevate diacylglycerol (8). Moreover, both the α- and β₂-subunits of the cardiac L-type channel have been...
shown to be good substrates for PKC in vitro (30). The effects of PKC phosphorylation on this channel, however, have been difficult to pin down. Most investigators report inhibitory effects of phorbol esters and diacylglycerol analogs on cardiac L-type channel activity, but these may be PKC independent (7, 33). Other investigators have reported stimulatory or biphasic effects of PKC activators on voltage-gated Ca\(^{2+}\) channel function in ventricular tissue (8, 21).

Another candidate protein that could be influenced by diC\(_8\) is the SR Ca\(^{2+}\) release channel/ryanodine receptor. This channel has been shown to be regulated by phosphorylation (23), but evidence for regulation by PKC phosphorylation is sparse. Other possibilities include cardiac K channels, which have been shown to be regulated by PKC activators under various conditions (1, 25). A likely candidate is the transient outward K channel, whose inhibition by PKC activators prolongs the action potential (1), which in turn increases the duration of transsarcolemmal Ca\(^{2+}\) influx. This is typically associated with an increase in duration of the twitch and the Ca\(^{2+}\) transient, but we did not observe such increases after photo-release of diC\(_8\). At present we cannot distinguish among these three molecular targets (L-type Ca\(^{2+}\) channels, ryanodine receptors, or K channels) with any degree of certainty, so we lump them together as changes in excitation-contraction coupling.

Another major control mechanism shown to occur under both physiological and pathological conditions is alteration of myofilament sensitivity to intracellular Ca\(^{2+}\) (9, 11, 12, 18, 29). We employed methods developed by Lakatta and co-workers (34) to assess dynamic interactions between intracellular Ca\(^{2+}\) and shortening myofilaments, and we found that diC\(_8\) caused no change in this interaction. A similar lack of an effect of diC\(_8\) on myofilament Ca\(^{2+}\) sensitivity was reported in a study using force-pCa measurements in skinned myocytes that had been treated with diC\(_8\) before skinning (22). However, using similar approaches, other groups have detected an increase (36) or a decrease (12) in Ca\(^{2+}\) sensitivity after treatment with PKC activators. In none of these experiments was the inotropic response to the activator measured in parallel. In skinned cells or reconstituted myofilaments, treatment with exogenous PKC has consistently failed to enhance the myofilament Ca\(^{2+}\) sensitivity (37) unless myosin light chain kinase was also present (6). In experiments analogous to ours in intact myocytes, Capogrossi et al. (5) reported a negative inotropic response and either no change or an increase in myofilament Ca\(^{2+}\) responsiveness after diC\(_8\). The cause of the variability was not identified. Therefore, although we cannot completely rule out the possibility that activation of PKC under some conditions increases myofilament Ca\(^{2+}\) sensitivity, in many cells we observed a very large positive inotropic response without such a change in Ca\(^{2+}\) responsiveness.

To further investigate this important issue, we examined the involvement of another possible target of PKC, namely the Na\(^{+}/H^+\) exchanger. PKC regulation of Na\(^{+}/H^+\) exchange is controversial. It has been demonstrated in many cell types that the growth factor-regulated Na\(^{+}/H^+\) exchanger (NHE-1) is activated by growth factors and phorbol esters in a PKC-dependent manner (32). Sequence analysis of the NHE1 gene, however, has revealed no phosphorylation sites for PKC, although sites are present for other kinases. This has led to the proposal that PKC activates the exchanger indirectly for example by activating a kinase cascade. In ventricular tissue, several groups have provided evidence of activation of Na\(^{+}/H^+\) antiport by phorbol esters (24, 28). Growth factor-stimulated alkalization in the heart has also been blocked by PKC inhibitors. However, these findings have not been confirmed in all cases (29). Our results do not resolve this controversy, but they do show that, under conditions in which diacylglycerol produces a dramatic inotropic response, in some ways mimicking agonist responses (26), the Na\(^{+}/H^+\) exchanger is not activated. It is possible that diC\(_8\) is compartmentalized under our experimental conditions so that certain natural PKC targets are not accessible to the signal. We consider this unlikely because the levels of caged compound in the cell are substantial, the levels of diC\(_8\) produced are at the high end of the physiological range (26), and diC\(_8\) is known to readily diffuse in aqueous and membrane environments (14). Our results suggest that diC\(_8\) alone is not sufficient to stimulate the Na\(^{+}/H^+\) exchanger in ventricular tissue.

Cardiac cross-bridge kinetics is another factor that has been reported to be regulated by PKC phosphorylation. These conclusions were derived from measurements of myosin ATPase activity (37) or myocyte shortening velocity (22), two parameters that were not evaluated here. We did not detect a significant change in the time course of the cardiac twitch, which may be
sensitive to changes in cross-bridge kinetics, although this latter point is still under debate. It is also not clear how changes in cross-bridge kinetics might contribute to inotropic changes in cardiac muscle, although it has been suggested that PKC-mediated inhibition of cross-bridge kinetics may underlie negative inotropic effects (37).

In view of the relatively large number of cardiac proteins suggested to be substrates for, or under the control of, PKC (28, 35), our observation that diacylglycerol predominantly influences one measurable parameter in intact myocytes is surprising. Reasons for the unusual selectivity afforded by this experimental approach are currently unknown. The ability to precisely control diacylglycerol concentration may be one factor. This selectivity may also be due to the species of diacylglycerol used, although there is no indication from in vitro studies that diC3 is selective for one PKC isoform or directs PKC to a particular substrate or sequence motif. The observed selectivity does indicate that certain PKC substrates are preferred over others; in this case, the preferred substrates appear to be proteins involved in excitation-contraction coupling. The selective action of diacylglycerol on myocyte function contrasts with the pleiotropic effects of agonists that activate phospholipases C and/or D (8, 9, 16, 28, 35, 36). This is an indication that diacylglycerol/PKC signaling in cardiac tissue represents only a part of the complex signaling that occurs downstream of receptor occupation by agonists.

In summary, many cellular processes have been hypothesized to be regulated by PKC in cardiac muscle, including Ca2+ channels, K channels, the SR Ca2+ pump, the Na+/H+ exchanger, and the myofilament Ca2+ regulatory system. Under conditions in which the diacylglycerol analog diC8 produced a large positive inotropic effect in isolated rat ventricular myocytes, the only significant and consistent change we detected was an increase in systolic Ca2+. Because the SR Ca2+ load did not change, these observations suggest that cardiac excitation-contraction coupling is a major locus of regulation by PKC. Details of the molecular mechanism such as the isoform of PKC responsible and the target cardiac proteins involved remain to be determined.

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