Protein Kinase C Inhibits Ca\(^{2+}\) Accumulation in Cardiac Sarcoplasmic Reticulum*

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It is now recognized that phorbol esters are negative inotropic agents in mammalian heart which presumably act via stimulation of Ca\(^{2+}\)-activated phospholipid-dependent protein kinase (PKC). The goal in the present study was to identify the underlying cellular processes. Digitonin-permeabilized cultured neonatal rat ventricular myocytes were used to study biochemical and functional effects of phorbol esters on cardiac sarcoplasmic reticulum (SR). These cells contracted spontaneously at 3 \(\mu\)M Ca\(^{2+}\). Beating was inhibited by 10 \(\mu\)M ryanodine and was insensitive to 1 \(\mu\)M nifedipine. Thus, beating behavior results from the phasic oscillation of Ca\(^{2+}\) transport by SR in this preparation. Phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA), decreased frequency by 30%, such that Ca\(^{2+}\) transport by SR had been reduced. Whereas cAMP stimulated the rate of oxalate-supported \(\text{Ca}^{2+}\) uptake 2-fold, phorbol esters, TPA, and phorbol 12,13-dibutyrate inhibited this process by about 45%. The effects of phorbol esters were specific: (a) the \(\alpha\)-analogs of TPA and phorbol 12,13-dibutyrate were inactive; and (b) the phorbol esters had no effect on Ca\(^{2+}\) transport in cells that had been depleted of PKC. TPA decreased oxalate-stimulated Ca\(^{2+}\) uptake over the entire range of Ca\(^{2+}\) concentrations, from 0.1 to 10 \(\mu\)M, by at least 70% without shifting the half-maximal effective Ca\(^{2+}\) concentration. Taken together these results indicate that the effects of phorbol ester on cardiac contraction are due to decreased Ca\(^{2+}\) transport by the SR and that these responses are mediated by PKC.

These studies support the interpretation that the negative inotropic effects of phorbol esters are due, in part, to decreased SR function.

The Ca\(^{2+}\)-activated phospholipid-dependent protein kinase, protein kinase C (PKC), occurs in various forms and is widely distributed in many tissues (1-3). This family of enzymes is an important mediator of signal transduction in tissues in which receptors increase the accumulation of 1,2-diacylglycerol, the endogenous activator, as the result of increased hydrolysis of phospholipids by phospholipase C (4). Recently, there has been interest in understanding the role of PKC in the regulation of heart function. In cardiac tissue there are a number of receptor systems, including the muscarinic cholinergic, \(\alpha\)-adrenergic, adenosine, and angiotensin II, which stimulate the hydrolysis of phosphoinositides and presumably activate PKC (5-7). Since the functional responses of heart to these various agents are markedly different, the effects of PKC activity on cardiac contraction are poorly understood. Synthetic activators of PKC, phorbol esters, have been used as probes to examine this question. The accumulating evidence reveals that TPA evokes a negative inotropic effect in a variety of cardiac preparations, including chick heart, rat heart, and cultured rat heart myocytes (8-10). The molecular events underlying this response are not known at this time.

Recently we have utilized a system of cultured neonatal rat myocytes to explore the role of PKC in heart. Activation of PKC in these cells by either angiotensin II or TPA results in increases in inward calcium current via dihydropyridine-sensitive L-type calcium channels (5, 10). This finding has been extended and verified at the single-channel level (11). Although these results are consistent with the increases in beating frequency which were also observed, they are inconsistent with the decreases in contraction force evoked by either the hormone or the phorbol ester.

The simplest explanation of these results is that PKC has other sites of action beyond those that regulate Ca\(^{2+}\) channels. There are a number of possible sites of action in the cardiac cell, including the myofilaments and the SR. In regard to the latter organelle, it has been shown that phospholamban is a substrate for PKC in these cells by either angiotensin II or TPA results in extended and verified at the single-channel level (11). Al-though these results are consistent with the increases in beating frequency which were also observed, they are inconsistent with the decreases in contraction force evoked by either the hormone or the phorbol ester.

The abbreviations used are: PKC, Ca\(^{2+}\)/phospholipid-dependent protein kinase (protein kinase C); SR, sarcoplasmic reticulum; pCa, \(-\log [\text{Ca}^{2+}];\) EGTA, (ethylenbis(oxyethylenenitrilo)tetraacetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; TPA, 12-O-tetradecanoylphorbol-13-acetate; \(\alpha\)-TPA, 4-\(\alpha\)-12-O-tetradecanooylphorbol-13-acetate; PDBu, phorbol 12,13-dibutyrate; \(\alpha\)-PDBu, 4-\(\alpha\)-phorbol 12,13-dibutyrate.
Experimental Procedures

Materials—All of the phorbol esters were obtained either from Sigma or LC Services Corp. (Woburn, MA). Digitonin, histone type III, Dubelcco’s modified Eagle’s medium, and bovine serum albumin were obtained from Sigma. Ryanodine was purchased from Agri-Systems International (Wind Gap, PA). All other reagents were purchased as reagent grade.

Tissue Culture—Spontaneously beating primary cultures of neonatal rat cardiac ventricular myocytes were prepared from 1-day-old Sprague-Dawley rats as described previously (10). The cells were plated in four-well 1.7-cm plastic tissue culture dishes with growth medium (Dubelcco’s modified Eagle’s medium, 10% fetal calf serum, 1% penicillin, and 1% streptomycin). The cells were maintained up to 6 days in a humidified atmosphere of 92.5% air, 7.5% CO₂.

Measurement of Contractile Properties—Contractions of single cardiocytes, attached to the culture dishes, were measured using an optical video dimension analysis system as described previously (5). In the experiments in which intact cells were studied, the cells were superfused at 1.4 ml/min with Dubelcco’s modified Eagle’s medium, 25 mm HEPES, pH 7.4. The cells were then superfused with medium containing various compounds as indicated under “Results.” In the functional studies with permeabilized cells, permeabilization was accomplished by incubating the cultures in Krebs-Ringer buffer, pCa 6.5 (16 mM NaH₂PO₄, 1.3 mM KH₂PO₄, 190 mM NaCl, 6 mM KCl, 10 mM HEPES, 0.1 mM EDTA, 10 mM NaCl, 0.1 mM MgCl₂, 5 mM CaCl₂, 0.2% bovine serum albumin, pH 7.2) supplemented with 10 μM digitonin. The permeabilization medium was removed, and the uptake reaction was initiated by the addition of 0.3 ml of medium containing 5 mM potassium oxalate and 4Ca²⁺ (about 2 μCi/ml). At the end of the reaction, the 4Ca²⁺-containing medium was removed, and the cells were rapidly rinsed twice with 0.5 ml of medium J. The cells were solubilized in 200 μl of 1% sodium dodecyl sulfate and counted in a scintillation counter.

Ca²⁺ Transport in Permeabilized Cells—Oxalate-stimulated 4Ca²⁺ uptake in detergent-permeabilized 3-5-day-old cultures was measured in the following manner. Culture dishes were washed with Dubelcco’s modified Eagle’s medium-HEPES, 0.25% bovine serum albumin, and incubated for 10 min at room temperature. The cells were permeabilized by incubating them for 10 min at room temperature in medium J, pCa 6.5 (40 mM HEPES, 20 mM NaCl, 5 mM MgCl₂, 5 mM CaCl₂, 0.2% bovine serum albumin, pH 7.1) supplemented with 10 μM digitonin. The permeabilization medium was removed, and the reaction was initiated by the addition of 0.3 ml of medium J containing 5 mM pCa 6.5 potassium oxalate and 4Ca²⁺ (about 2 μCi/ml). At the end of the reaction, the 4Ca²⁺-containing medium was removed, and the cells were rapidly rinsed twice with 0.5 ml of medium J. The cells were solubilized in 200 μl of 1% sodium dodecyl sulfate and counted in a scintillation counter.

Evaluation of Permeabilization—The Rb⁺ release measurements were performed to assess the extent of permeabilization of detergent-treated cells. Cells were incubated for 2 h with 0.4 ml of a medium containing 15 mM HEPES, 1.2 mM NaH₂PO₄, 35 mM NaHCO₃, 155 mM NaCl, 1.2 mM MgCl₂, 10 mM KCl, 0.2% bovine serum albumin, pH 7.2, with 1.7 μCi of [³²P]Rb⁺. The cells were washed twice with 0.5 ml of incubation medium to remove excess radioactivity. The cells were then incubated with the permeabilization buffer described above, containing a range of digitonin concentrations. The supernatant was removed and counted by scintillation methods. The remaining activity was extracted from the cells with 0.4 ml of 1% sodium dodecyl sulfate and was also counted. The results were expressed as percent of total radioactivity released. Permeability was also assessed by the release of lactate dehydrogenase under similar conditions. Lactate dehydrogenase activity released into the medium and remaining in the cells was quantitated as described previously (14).

Other Methods—Protein was measured according to Bradford (15). Free Ca²⁺ concentrations in the various media were calculated by the use of a computer program that accounts for the binding of ECTA and ATP and competition with other cations (16).

Results

There is physiological and biochemical evidence that cardiac SR may be expressed at extremely low levels and may play a relatively insignificant role in contraction at early stages of development in many species including rat (17, 18). To assess the contribution of SR to spontaneous contractions in cultured neonatal ventricular myocytes, it would be valuable to examine the effects of the inhibition of SR function on contractile behavior. Ryanodine has been shown to inhibit SR function selectively in a variety of cardiac preparations, although its exact mechanism of action appears complex (19-23). As shown in twitch records in Fig. 1, application of 10 μM ryanodine reduced contraction frequency and markedly decreased the relaxation rate. These characteristic changes in contractile behavior indicate that SR does play a prominent role in the regulation of contraction in these cultures.

One valuable approach to examine directly the biochemical properties of the SR in these cells would be to develop a preparation of myocytes in which the myolemma was permeabilized. Accordingly, the effects of detergents on cell permeability were examined. Cultures were incubated with increasing concentrations of digitonin, and the effects of this detergent on cell permeability were quantitated by two methods. As shown in Fig. 2, there was a dose-dependent release of two cytosolic markers, ³²P and lactate dehydrogenase. At an intermediate dose, 10 μM digitonin, only 45% of the lactate dehydrogenase was released, yet the cells were readily permeable (>92%) to ³²P. Similar results were obtained with saponin (data not shown). The effects of 10 μM digitonin treatment on levels of intracellular PKC activity were also examined. Relative to intact cells, 38.8 ± 8% (n = 3) of the PKC activity remained in whole cell homogenates of permeabilized cells. Therefore, this concentration of digitonin was used in the experiments described below, which was a compromise between complete permeabilization and minimal loss of cytosolic proteins and intracellular damage.

Functional Studies—Experiments were performed to assess the functional status of SR in the permeabilized cardiocytes. It is well documented that phasic contractions of chemically and mechanically skinned cardiac cells, at submicromolar free Ca²⁺, result from the sequential loading and release of Ca²⁺. The concentration of Ca²⁺ below 1 μM is in the linear range. Enzyme activity was defined as the resulting supernatant was subjected to a chromatographic separation of the range of 35-50 pg/well. All of the data were normalized to protein.

Protein Kinase C Assay—PKC activity was measured in whole cell homogenates by methods published previously with minor modifications (13). Cardiocytes were removed from culture dishes and homogenized in 0.5 ml of ice-cold 10 mM Tris-HCl, 0.5 mM ECTA, 2 mM EDTA, 0.1 mM bacitracin, 25 μl/ml leupeptin, 1% Nonidet P-40. After the homogenate was centrifuged at 100,000 × g for 10 min, the resulting supernatant was subjected to a chromatographic separation on a 1-ml DEAE-cellulose column. The PKC fraction was eluted as described (13). PKC activity was measured using 10-20 μg of protein, which was in the linear range. Enzyme activity was defined as the incorporation of [γ-³²P]ATP into histone type III.

The effects of digitonin on contractile activity of cultured neonatal rat heart cells. Cells were cultured as described under “Experimental Procedures.” Beating behavior was monitored by following the motion of the edge of a cell using an optical video dimension analysis system as described under “Experimental Procedures.” Superfusion solution was switched from control medium to medium supplemented with 10 μM ryanodine. Typical sample record taken from a single cell is shown.

Fig. 1. The effects of ryanodine on contractile activity of cultured neonatal rat heart cells. Cells were cultured as described under “Experimental Procedures.” Beating behavior was monitored by following the motion of the edge of a cell using an optical video dimension analysis system as described under “Experimental Procedures.” Superfusion solution was switched from control medium to medium supplemented with 10 μM ryanodine.
Data points are the means of three or four experiments ± S.E. In many cases, the standard error is not evident because the error bars do not extend beyond the size of the symbols. See "Experimental Procedures" for details.

The effects of phorbol esters on the phasic contractile behavior of digitonin-permeabilized myocytes were examined. Cells were exposed to 80 nM TPA, conditions that we have shown previously result in negative inotropic responses in intact cultures of these cells (10). As shown in the record from one cell (Fig. 4), application of phorbol ester resulted in a 37% decrease in contraction frequency. The results from a series of experiments showed that TPA decreased frequency by 26 ± 3.3% (± S.E., n = 8) of control. Further, it was clear that TPA treatment did not alter sensitivity of the SR to ryanodine. After the response to 80 nM TPA reached a plateau, contractions were still completely inhibited by 10 μM ryanodine (data not shown). These data are consistent with the view that activation of PKC results in reduced Ca2+ transport by the SR and provide a functional rationale to pursue the molecular studies described below.

Calcium Transport Studies—The functional responses of the myocytes to phorbol ester may be explained as a result of reduced Ca2+ uptake by the Ca2+ ATPase or altered Ca2+ efflux via the Ca2+ release channel or a combination of both. The net Ca2+ transport activity of the SR was assessed by measuring the velocity of oxalate-supported 45Ca2+ accumulation into permeabilized myocytes in the presence of mitochondrial inhibitors and exogenous ATP. Under the conditions used, oxalate-stimulated 45Ca2+ uptake was observed only when the cells were permeabilized (Fig. 5A). The time course for 45Ca2+ accumulation was also examined (Fig. 5B). The uptake was biphasic, with an initial rapid phase, which was independent of oxalate, followed by a period of 20–30 min in which the oxalate-supported uptake was linear. In many of the experiments described below, the velocity of 45Ca2+ accumulation was measured in this linear range.

It has been shown previously that cAMP increases Ca2+ pump activity in isolated cardiac SR vesicles and in the SR compartment of chemically skinned adult rat myocytes (24, 26, 27). In order to validate further the permeabilized neonatal myocyte system, the effects of cAMP on oxalate-stimulated 45Ca2+ transport were examined. As shown in Fig. 6, addition of 5 μM cAMP increased the velocity of 45Ca2+ uptake 2-fold, from 0.140 to 0.265 nmol of 45Ca2+/min/mg of protein. These results reveal that analogous to its properties in adult heart cells, the Ca2+ pump activity in these permeabilized cultures can be activated by cAMP. Further, it is clear that these permeabilized myocytes still contain sufficient cAMP-
Protein Kinase C Inhibits Cardiac SR Ca\(^{2+}\) Transport

Fig. 5. Characterization of oxalate-stimulated transport of \(^{45}\)Ca\(^{2+}\) in permeabilized myocytes. In panel A, myocytes were treated with increasing concentrations of digitonin for 10 min. Following permeabilization, the amount of \(^{45}\)Ca\(^{2+}\) uptake was measured in incubations of 10 min in pCa 6.5 medium either supplemented with (●) or in the absence of (○) 5 mM potassium oxalate. The points represent the means of triplicates with a variation of 10% or less. Nearly identical results were obtained in two additional experiments. Panel B shows a time course for \(^{45}\)Ca\(^{2+}\) accumulation in digitonin-permeabilized myocytes (10 μM detergent) incubated in pCa 6.5 medium either in the presence (●) or absence (○) of 5 mM potassium oxalate. The results are from a typical experiment performed in triplicate ± SE. The experimental protocols are described in detail under “Experimental Procedures.”

Effects of Phorbol Esters on Calcium Transport—In the next series of experiments, the effects of phorbol ester on Ca\(^{2+}\) transport activity were examined. Initial oxalate-stimulated Ca\(^{2+}\) transport experiments were performed at pCa 6.5, a Ca\(^{2+}\) concentration at which phasic contractions were attenuated by TPA (Fig. 4). As shown in the kinetic studies in Fig. 7, TPA (80 nM) and PDBu (5 μM) reduced the velocity of the oxalate-stimulated \(^{45}\)Ca\(^{2+}\) transport by 45 and 38%, respectively. The effects of these phorbols were specific for the oxalate-stimulated component of the Ca\(^{2+}\) uptake. Although not shown in Fig. 7, results from many independent experiments revealed that neither TPA nor PDBu had any effect on the basal (oxalate-free) component of Ca\(^{2+}\) accumulation. The specificity of the effects was examined further by the application of biologically inactive phorbol analogues, α-TPA and α-PDBu. As shown in Fig. 8, the α-analogues had no effect on the velocity of \(^{45}\)Ca\(^{2+}\) uptake. The results presented in Figs. 7 and 8 are from representative experiments since the magnitude of the velocities of Ca\(^{2+}\) uptake varied between cultures. However, the relative effects of the phorbols were always the same. A summary of normalized results from a large number of experiments is shown in Table I. Taken together, these data support the view that activation of PKC leads to a reduction in Ca\(^{2+}\) transport rates in cardiac SR.

Additional experiments were performed to examine further the specificity of the effects of phorbol esters on SR function.
PKC-depleted cardiocytes were prepared by long term treatment of the cultures with TPA. It is now well recognized that such treatment leads to down-regulation of PKC (28). When cultures were treated with 1 μM TPA for 24 h, PKC activity was decreased to 3.8% of control value. PKC activity in cell homogenates decreased from (in nmol of ^32P incorporated/min/mg of protein) 2.6 to 0.10. The effects of tumor-promoting phorbol esters on ^45Ca^2+ uptake were examined in these PKC-depleted cultures. The effects of TPA were examined in parallel cultures that had been incubated in the presence (Fig. 9B) or absence (Fig. 9A) of TPA for 24 h before the Ca^2+ transport experiments were performed. The magnitudes of oxalate-stimulated ^45Ca^2+ uptake velocity were identical in control and PKC-depleted cells. The velocities were 0.381 and 0.389 nmol of ^45Ca^2+/min/mg of protein, respectively (compare oxalate-stimulated ^45Ca^2+ uptake curves in Fig. 9, A and B). However, in contrast to control cells (Fig. 9A), Ca^2+ transport in the PKC-depleted cells was completely insensitive to TPA (Fig. 9B). The same results were obtained with PDBu. Table I includes a summary of the results for both phorbols. These data further support the interpretation that the effects of phorbol esters observed in this study are mediated by PKC.

One explanation for the reduced rate of Ca^2+ accumulation is that the action of PKC lowers the affinity of the Ca^2+ pump.

**Table I**

Effects of phorbol esters on the rate of oxalate-stimulated Ca^2+ accumulation in permeabilized cultured rat cardiocytes

<table>
<thead>
<tr>
<th>Phorbol ester used</th>
<th>Rate of ^45Ca^2+ accumulation</th>
<th>^45Ca^2+ uptake +5 mM oxalate (pmol/mg protein)</th>
<th>^45Ca^2+ uptake +5 mM oxalate phorbol ester (pmol/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal</td>
<td></td>
<td>+5 mM oxalate</td>
<td>+5 mM oxalate phorbol ester</td>
</tr>
<tr>
<td>PDBu (5 μM)</td>
<td></td>
<td>2.28 ± 0.29</td>
<td>1.14 ± 0.22</td>
</tr>
<tr>
<td>TPA (80 nM)</td>
<td></td>
<td>2.76 ± 0.48</td>
<td>1.63 ± 0.26</td>
</tr>
<tr>
<td>α-PDBu (25 μM)</td>
<td></td>
<td>3.24 ± 0.19</td>
<td>2.96 ± 0.18</td>
</tr>
<tr>
<td>α-TPA (200 nM)</td>
<td></td>
<td>2.81 ± 0.36</td>
<td>2.55 ± 0.25</td>
</tr>
<tr>
<td>PKC-depleted cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PDBu (5 μM)</td>
<td></td>
<td>3.94 ± 0.81</td>
<td>3.63 ± 0.50</td>
</tr>
<tr>
<td>TPA (80 nM)</td>
<td></td>
<td>4.21 ± 0.78</td>
<td>4.09 ± 0.66</td>
</tr>
</tbody>
</table>

* Mean of four independent experiments ± S.E.
* Mean of five independent experiments ± S.E.
* Mean of two independent experiments ± range/2.
* Mean of three independent experiments ± S.E.
Biochemical effects of phorbol esters have biological significance under physiological circumstances. The effects of phorbol esters on oxalate-stimulated Ca\(^{2+}\) accumulation in permeabilized cardiocytes. Cultured myocytes (days 4-5 in culture) were permeabilized with 10 \(\mu\)M digitonin for 10 min. \(^{45}\)Ca\(^{2+}\) transport was measured in these cells as described under "Experimental Procedures." The data points represent the specific oxalate-stimulated uptake over a 10-min interval, which was determined as the difference between \(^{45}\)Ca\(^{2+}\) accumulation in the presence of 5 mM potassium oxalate and basal uptake in the absence of oxalate. The amount of specific \(^{45}\)Ca\(^{2+}\) accumulation was measured in the presence of increasing free Ca\(^{2+}\) concentrations (A). In parallel experiments, cells were treated in an identical manner except that the uptake medium was supplemented with 80 nM TPA (B). In these latter experiments, TPA had no effect on basal Ca\(^{2+}\) accumulation values compared with control cells. The data are the means of five to eight independent experiments ± S.E.

For Ca\(^{2+}\). In order to test this hypothesis, the Ca\(^{2+}\) dependence of oxalate-supported Ca\(^{2+}\) accumulation was measured. As shown in Fig. 10, Ca\(^{2+}\) uptake was stimulated over a range of pCa from 6.8 to 5.8. The half-maximal velocity was observed at pCa ≈ 6.3. It is noteworthy that the frequency of phasic contractions was stimulated over the same Ca\(^{2+}\) concentration range (Fig. 3). These results are also consistent with the reported affinity of the SR Ca\(^{2+}\) ATPase for Ca\(^{2+}\) in isolated SR vesicles (29). As shown in Fig. 10, TPA (80 nM) did not change the position of the Ca\(^{2+}\) dependence relation but decreased the accumulation of \(^{45}\)Ca\(^{2+}\) over the entire concentration range examined. Therefore, the effects of PKC cannot be simply explained as a result of lowered affinity of the Ca\(^{2+}\) ATPase for Ca\(^{2+}\).

**DISCUSSION**

The widespread distribution of PKC activity in many tissues is well documented (1); yet the specific molecular processes mediated by PKC are largely unknown. Accumulating evidence in the literature indicates that activation of PKC by phorbol esters or cardioactive hormones leads to negative effects on contractile force in a variety of cardiac preparations (8-10). At present, the underlying mechanisms of the negative inotropic response in heart are not known. The goal of the present study was to identify such mechanisms. A preparation of permeabilized cultured neonatal rat myocytes was used to examine Ca\(^{2+}\) transport properties in cardiac SR and the effects of PKC activation on them. The main findings are that phorbol esters decrease the velocity of Ca\(^{2+}\) uptake into the SR and that these effects are mediated by the activation of PKC. Further, this permeabilized cell system was exploited in parallel functional studies that revealed that the biochemical effects of phorbol esters have biological significance under physiological circumstances.

In previous studies we have obtained considerable information concerning the changes in mechanical and electrical properties of cultured cardiocytes evoked by activation of PKC (5, 10). Although no information was derived in these earlier studies to support any particular mechanism, a simple explanation for the negative inotropic action of phorbol esters would be that the transport activity of the SR was depressed. Since there is evidence that neonatal rat myocytes might not have functionally significant levels of SR expression, the initial focus of this study was to determine if SR does contribute to excitation-contraction coupling in these cultures (17, 18). Several results presented here clearly demonstrate that the SR has a role in contractile behavior in these cultures. First, ryanodine decreased beating frequency and the relaxation rate in spontaneously beating intact cell cultures. Second, in the presence of ATP, permeabilized cells beat spontaneously when exposed to low free Ca\(^{2+}\) (pCa in the 6.5 range), and these phasic contractions were rapidly inhibited by ryanodine. Third, the Ca\(^{2+}\) concentration range for the stimulation of beating frequency was the same that activates the cardiac SR Ca\(^{2+}\) pump (24, 29). It is clear that the spontaneous beating behavior in permeabilized cultured cardiomyocytes is a functional measure of Ca\(^{2+}\) transport activity of the SR.

One important advantage of the permeabilized cell preparation over isolated SR vesicles is that it is possible to perform biochemical and functional experiments in parallel. A significant finding in the present study was that phorbol esters decreased the rate of phasic contractions in such cells. This negative effect on SR function provides a plausible explanation for the negative inotropic responses of intact cardiac preparations to phorbols. These results are also consistent with the observations reported here on the inhibitory effects of phorbol esters on oxalate-supported Ca\(^{2+}\) transport. Thus, the functional studies underscore the biological relevance of the biochemical effects of phorbols.

Experimental evidence demonstrates that it was possible to measure Ca\(^{2+}\) transport activity of the SR in permeabilized cells. Oxalate stimulated the accumulation of Ca\(^{2+}\) in permeabilized cells in the presence of mitochondrial inhibitors. Further, this Ca\(^{2+}\) uptake required ATP and was activated over a pCa range of 7.0–5.5. These properties are characteristic of active Ca\(^{2+}\) transport by the Ca\(^{2+}\) pump in cardiac SR, both in isolated SR fractions and in skinned adult myocytes (24, 29, 30). It is now well established that cAMP stimulates the Ca\(^{2+}\) pump in isolated cardiac vesicles via phosphorylation of phospholamban (26, 27). cAMP stimulates oxalate-supported Ca\(^{2+}\) transport in the present study as well. Yet despite the many similarities, several results indicate that the SR level is less prominent in neonatal cultures compared with adult myocytes. The magnitude of oxalate-stimulated uptake reported here is 3-fold less than that reported in adult myocytes (24). Further, the effects of ryanodine on contraction are more marked in adult myocytes compared with the neonatal myocyte results (21, 23). However, taken together, these results reveal that oxalate-stimulated Ca\(^{2+}\) uptake in neonatal cells has properties comparable to those in adult rat heart cells and is a valid index of Ca\(^{2+}\) transport by the SR in these cultures.

An important finding in the present study was that physiologically active doses of phorbol esters decrease the rate of Ca\(^{2+}\) transport into cardiac SR. Experimental evidence presented here reveals that the effects of phorbols are mediated by PKC. The biologically inactive \(\alpha\) analogues were not effective. Additionally, the phorbols did not alter Ca\(^{2+}\) transport in cells that had been depleted of PKC. These latter control experiments also showed that phorbol esters did not act through indirect mechanisms such as (a) nonspecific permeabilization of the SR; (b) interference with Ca\(^{2+}\)-oxalate complex formation reaction; or (c) inhibition of the uptake of...
Protein Kinase C Inhibits Cardiac SR Ca²⁺ Transport

oxalate by the SR compartment. Taken together, these data support the view that the action of phorbol esters in these experiments results from the activation of PKC in these cells.

It is crucial to know the specific site of action of PKC on the SR. One likely possibility is that the activity of the Ca²⁺ pump is inhibited. It is important to note that under the conditions used, the oxalate-stimulated 45Ca²⁺ accumulation may not be a direct measure of Ca²⁺ pump activity but is rather a net value due to active transport minus passive efflux of Ca²⁺ through the release channels. It has already been seen that ruthenium red and ryanodine (at high doses) can increase Ca²⁺ accumulation in isolated cardiac SR and that caffeine, a release channel activator, can reduce oxalate-stimulated Ca²⁺ accumulation in skinned adult rat myocytes (24, 30). Thus, one cannot exclude the possibility that the inhibitory effects of PKC result from increased Ca²⁺ efflux through the channel.

We have found that ruthenium red, a Ca²⁺ efflux blocker, does not reverse the inhibitory action of phorbol esters, suggesting that the pump is the major site of action of PKC. However, the potential effects of PKC on the release channel may be independent of the action of ruthenium red, or phorbol esters may interfere with the action of this agent. Thus, further experiments in which Ca²⁺ release can be measured directly are required to resolve this issue.

Regardless of the particular Ca²⁺ transport system involved, these studies do reveal that cardiac SR is a target for PKC. Further, the permeabilized cell system was further exploited to provide direct evidence that PKC alters not only transport properties but also functional properties of the cells. Finally, the results reported here yield insight into the role of PKC in the regulation of cardiac function and provide a mechanism for the negative inotropic action of phorbol esters, which is consistent with the results from studies in many cardiac preparations.

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