Concentration-Dependent Effects of Protein Kinase C–Activating and –Nonactivating Phorbol Esters on Myocardial Contractility, Coronary Resistance, Energy Metabolism, Prostacyclin Synthesis, and Ultrastructure in Isolated Rat Hearts

Effects of Amiloride

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An extensive investigation of the cardiac actions of phorbol esters and the potential role of the Na⁺-H⁺ exchanger in those actions was carried out using isolated rat hearts. Sixty minutes of perfusion with 10⁻⁹ M phorbol 12-myristate 13-acetate (PMA) or 10⁻⁹ M phorbol 12,13-dibutyrate (PDBu) produced marked cardiac dysfunction associated with depressed contractility, coronary constriction, and elevated resting tension, the latter being particularly evident with PMA. These effects were also associated with disturbances in tissue levels of energy metabolites manifested primarily by a reduction in ATP and an elevation in lactate. Furthermore, both phorbols produced a sustained stimulation of the release of 6-ketoprostaglandin F₁α (6-keto PGF₁α), the hydrolysis product of prostacyclin (prostaglandin I₂). Amiloride, an inhibitor of the Na⁺-H⁺ exchanger, significantly attenuated the loss in contractility and elevation in coronary pressure as well as the stimulated release of 6-keto PGF₁α, but was without effect on elevations in resting tension or on changes in energy metabolites. Increasing concentrations of PMA or PDBu 10-fold resulted in a much more rapid and severe (>80% loss in contractile function after 30 minutes) effect that was nonetheless qualitatively identical to that seen with the lower concentrations of phorbol. However, the effects were not prevented by amiloride. Surprisingly, 4α-phorbol 12,13-didecanoate (α-PDD, 10⁻⁶ M), which does not activate protein kinase C, was found to be a potent inhibitor of cardiac function (>80% loss in contractility and 50% increase in resting tension) after 30 minutes of perfusion, although these effects were not associated with changes in levels of energy metabolites or with elevations in coronary pressure. Similarly, none of the actions of this compound were attenuated by amiloride. In contrast to the sustained effects of other phorbols on 6-keto PGF₁α release, the effect of α-PDD was transient (<10 minutes). In all hearts studied, the marked depression in contractile function caused by all phorbol esters occurred in the absence of any ultrastructural changes. 4α-Phorbol (10⁻⁶ M), which does not activate protein kinase C, was without effect on any parameter studied. Our results demonstrate very complex effects of phorbol esters on numerous parameters of cardiac function, including an amiloride-sensitive component that occurs at low concentrations. The latter observation suggests the involvement of Na⁺-H⁺ exchange activation, possibly occurring as a consequence of protein kinase C stimulation, in mediation of the effects of phorbol esters at low concentrations. In addition, the inability of amiloride to attenuate the cardiac actions of high concentrations of phorbol esters, coupled with the prominent cardiodepressant effects of α-PDD, suggest concentration-dependent, non-protein kinase C–dependent actions of phorbol esters in cardiac tissues. (Circulation Research 1991;69:1114–1131)
Phorbol esters are diacylglycerol analogues that have been reported to depress cardiac contractility. This effect has been observed after their administration to isolated hearts as well as to myocyte preparations.\(^1\)\(^2\) The mechanisms underlying phorbol ester–induced depression of contractility are unknown but have been widely attributed to the ability of these agents to stimulate the activity of protein kinase C (PKC). This property has led to their widespread use for the investigation of PKC-mediated cellular events such as may occur as a consequence of phosphoinositide hydrolysis leading to the formation of diacylglycerol.\(^3\) The implication of PKC activation in phorbol ester–induced cardiac depression stems from observations showing that phorbol esters devoid of PKC-stimulating ability fail to alter cardiac function.\(^3\) In addition, we have recently reported that (±)-1-O-hexadecyl-2-O-acetyl-glycerol, a PKC inhibitor, significantly reduced the loss in cardiac function produced by a very low concentration (10\(^{-10}\) M) of phorbol 12-myristate 13-acetate (PMA) in working rat hearts.\(^2\)

Despite the generally accepted involvement of PKC in phorbol ester–induced cardiac changes, the precise mechanisms by which they exert these effects still remain elusive. Reports in the literature suggest a number of potential mechanisms, primarily involving changes in cell Ca\(^{2+}\) handling, although these are of a substantially divergent nature. Thus, decreased as well as increased transsarcolemmal Ca\(^{2+}\) influx have both been suggested as possible mediators of phorbol ester–induced loss in heart function.\(^1\)\(^5\) Furthermore, others have implicated defective sarcoplasmic reticular Ca\(^{2+}\) handling as a potential mechanism for phorbol ester–induced effects.\(^7\)

In view of the above studies and given the fact that phorbol esters produce a multitude of cellular effects in various tissues,\(^6\)\(^8\) it is reasonable to suggest that their cardiac effects are likely to be substantially more complex than originally considered. Moreover, the basis for their actions is unlikely to be attributable to a single underlying mechanism. The purpose of the present study was to examine and compare concentration-dependent effects of “active” phorbol esters, that is, those known to possess PKC-activating ability, as well as two phorbol esters known to be devoid of such properties, on myocardial and coronary responses in isolated rat hearts. We also examined the consequences of such treatments on myocardial energy production and ultrastructural changes in an attempt to determine if changes in these parameters could be related to changes in cardiac function. Phorbol esters have also been shown to stimulate production of prostaglandins (PGs) in various tissues;\(^9\)\(^12\) therefore, we also studied the effects of these compounds on the production of prostacyclin (PGI\(_2\)), the primary cardiac PG, as assessed by efflux of 6-keto PGF\(_{1\alpha}\). Since phorbol esters are potent stimulants of cardiac Na\(^{+}\)-H\(^{+}\) exchange activity and in view of the fact that the activity of this exchanger may be important to cardiac function under both normal and pathological conditions, we examined indirectly the contribution of this exchanger to phorbol ester–induced alterations by determining the influence of amiloride, a Na\(^{+}\)-H\(^{+}\) exchange inhibitor, on various changes produced by administration of phorbol esters.

**Materials and Methods**

**Animals**

Male Sprague-Dawley rats weighing 275–300 g were obtained from Canadian Hybrid Farms, Centreville, Canada, and from Charles River Canada Ltd., St. Constant, Canada. The rats were maintained in the animal holding facilities of the University of Western Ontario in accordance with the guidelines issued by the Canadian Council on Animal Care, Ottawa, Canada.

**Chemicals**

The following drugs were obtained from Sigma Chemical Co., St. Louis, Mo.: PMA, phorbol 12,13-dibutyrate (PDBu); 4α-phorbol 12,13-didecanoate (α-PDD), 4α-phorbol, and amiloride. Phorbol esters were stored at −20°C in ethanol at stock concentrations of either 1 or 10 mM, and dilutions were made in Krebs-Henseleit buffer before addition to the perfusate. Phorbol esters are photosensitive; therefore, stock concentrations, dilutions, and perfusion equipment were covered with foil to prevent their degradation. Amiloride was dissolved in water. The antibody to 6-keto PGF\(_{1\alpha}\) was acquired from Dr. Lawrence Levine of Brandeis University, Waltham, Mass. Radiolabeled 6-keto PGF\(_{1\alpha}\) was obtained from NEN Research Products, Mississauga, Canada, and normal rabbit serum as well as goat anti-rabbit immunoglobulin G were from ICN Biomedicals, Inc., Mississauga, Canada.

**Perfusion Protocol**

Rats were lightly anesthetized with sodium pentobarbital and killed by decapitation. The hearts were quickly excised and placed in a crucible containing ice-cold Krebs-Henseleit buffer to produce immediate cessation of contraction. Hearts were squeezed a few times to dislodge any clot in the coronary vasculature and mounted by the aorta on a stainless-steel cannula for retrograde perfusion according to the Langendorff method.\(^12\) The atria were removed, and the hearts were paced with a stimulator (model S44, Grass

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Instrument Co., Quincy, Mass.) at three times threshold voltage at a rate of 5 Hz with fine-gauge platinum electrodes. The Krebs-Henseleit perfusion buffer had the following composition (mM): NaCl 120, NaHCO3 20, MgCl2 1.2, KCl 4.63, KH2PO4 1.17, CaCl2 1.25, and glucose 8. The buffer was gassed continuously with a mixture of 95% O2-5% CO2, and pH of the buffer was 7.4. A Watson-Marlow peristaltic pump was used to maintain a constant flow rate of 10 ml/min, and a system of water jackets kept the preparation at 37°C for the duration of the experiment. Developed force was measured as apicobasal displacement obtained by attaching the ventricular apex to a Grass FT.03 force displacement transducer. A diastolic tension preload was initially set at 2 g. This parameter has been referred to as resting tension throughout the text. The signal from the force transducer was relayed to a differentiator for the measurement of rate of contraction (+dF/dt) and rate of relaxation (−dF/dt). Coronary perfusion pressure was obtained by connecting a Statham Gould P23ID or Spectramed P22XL pressure transducer to a side arm off the cannula. All recordings were made using a Grass model 7D polygraph.

**Experimental Protocol**

After a 30-minute stabilization period, one of the following phorbol esters—PMA (10⁻⁹ or 10⁻⁷ M), PDBu (10⁻⁸ or 10⁻⁶ M), 4α-phorbol (10⁻⁶ M), or α-PDD (10⁻⁶ or 10⁻⁴ M)—was added to the buffer (time of addition was designated time zero), and any effects on contractile function were observed over 30 or 60 minutes. Functional parameters were assessed, and samples of coronary effluent were collected at regular intervals as shown in “Results.” The coronary effluent was collected and stored at −20°C for subsequent assay of PGF₁α content by specific radioimmunoassay of its stable hydrolysis product 6-keto PGF₁α. In studies to assess the role of Na⁺-H⁺ exchange, amiloride (40 or 80 µg/ml) was added 30 minutes before phorbol ester addition and was present in the buffer for the entire perfusion period. At the end of each experiment, the heart was clamped while on the cannula by using Wollenberger tongs that had been precooled in liquid nitrogen. Hearts were then stored in liquid nitrogen for subsequent assay for ATP, creatine phosphate, ADP, AMP, and lactate.

**Radioimmunoassay for 6-Keto PGF₁α**

Measurement of 6-keto PGF₁α was carried out using a double antibody radioimmunoassay procedure as described previously. Briefly, to 0.5 ml sample was added 0.1 ml [3H]6-keto PGF₁α (0.02 µCi) and 0.1 ml 6-keto PGF₁α antibody (1:4,000 dilution). The mixture was then incubated at 37°C for 1 hour, after which 0.1 ml each of normal rabbit serum (1:25 dilution) and goat anti-rabbit immunoglobulin G (1:3 dilution) was added. The incubation mixture was kept at 4°C overnight. After incubating, the tubes were centrifuged at 1,600g for 30 minutes, the supernatant was poured off, and the excess moisture was wiped off using a small piece of Eaton-Dikeman grade 615 (fast speed) filter paper; care was taken not to disturb the pellet. The pellet was dissolved with 0.2 ml of 0.1 M NaOH dissolved in scintillation cocktail, and radioactivity was measured using a liquid scintillation counter (model LS 7500, Beckman Instruments, Inc., Fullerton, Calif.). The amount of 6-keto PGF₁α in each tube was obtained from a standard curve. Sensitivity of the assay was 10 pg, and cross-reactivity of the antibody with other eicosanoids was <1%.

**Metabolite Assays**

Energy metabolites in 6% perchloric acid extracts were determined enzymatically using a Beckman DU-65 spectrophotometer according to Bergmeyer. These assays are based on changes in extinction at 340 nm indicative of formation or decrease in NADH or NADPH after the initiation of the specific reaction. Specifically, ATP and creatine phosphate were determined by measuring increases in NADPH formation after addition of hexokinase and creatine kinase, respectively. ADP and AMP contents were assessed by monitoring reduction in NADH after adding pyruvate kinase and myokinase, respectively. Lastly, lactate was determined by monitoring formation of NADH after administration of lactate dehydrogenase.

**Electron Microscopy**

At least two hearts from each experiment were transferred at the end of the perfusion period with attached cannula to a perfusion system kept at a constant perfusion pressure of 80 cm H₂O₂ and perfusion-fixed with 150 ml fixative (pH 7.4) containing 0.08 M sodium cacodylate, 2% glutaraldehyde, and 1% paraformaldehyde. After fixation, various sections measuring ~1 cm×1 mm ×1 mm were cut from the left and right ventricle and intraventricular septum, placed in a small vial containing cold fixative, and stored at 4°C. Further processing and sectioning of tissue were performed by the pathology department at University Hospital, London, Canada. Briefly, postfixation, dehydration, and infiltration of tissue were done using a Lynx automatic tissue processor. Tissues were rinsed with 0.1 M sodium cacodylate buffer, postfixed with 1% osmium tetroxide, and dehydrated using a graded series of ethanol and acetone rinses. The final absolute ethanol/acetone solution was replaced by “en bloc” stain consisting of a uranyl nitrate/saturated lead acetate solution in a 3:7 ratio and left for 1 hour. Tissues were infiltrated and embedded in Epon Araldite resin and polymerized overnight in a 70°C oven. Thin (60–90-nm-thick) sections were cut using a diamond microtome knife, stained with uranyl acetate and lead citrate, and viewed with an electron microscope (model 109, Carl Zeiss, Inc., Thornwood, N.Y.).

**Expression of Results and Statistical Analysis**

Functional data were expressed as a percentage of the readings observed immediately before phorbol ester addition to the perfusion buffer (initial or predrug
values). Metabolite content and 6-keto PGF$_2$α release were expressed as mean±SEM. Raw data were analyzed using unpaired or paired Student's t test or analysis of variance followed by the Student-Newman-Keuls test as appropriate. Correlation coefficients were calculated by simple correlation analysis. Differences were regarded significant at $p<0.05$.

**Results**

**Contractile Function and Energy Metabolism**

The effects of perfusion with 10$^{-9}$ M PMA are shown in Figure 1. Developed force decreased by a total of 77% after 60 minutes of perfusion with PMA as compared with predrug (0-minute) values. In contrast, developed force in control hearts perfused with 0.01% ethanol decreased by 43% over the same time period. The latter effect is likely due to the ability of ethanol to depress contractility over time, since, in the absence of ethanol, loss in function over the total perfusion period was <15% (not shown). The decrease in contractile force produced by 10$^{-9}$ M PMA was significantly different from control after 30, 45, and 60 minutes of perfusion (Figure 1, top left panel). Figure 1 (top right panel) illustrates the changes in +dF/dt and −dF/dt during perfusion with 10$^{-9}$ M PMA. Both +dF/dt and −dF/dt declined in PMA-treated hearts by a total of 75% and 80%, respectively, as compared with predrug values, whereas control hearts decreased by 43% and 51%, respectively. The decrease in +dF/dt with PMA treatment was significant compared with control values after 30, 45, and 60 minutes, and the decrease in −dF/dt with PMA treatment was significantly different from control after 15, 30, 45, and 60 minutes. In addition, +dF/dt and −dF/dt were significantly different from each other in control hearts after 30, 45, and 60 minutes of normal Krebs' buffer perfusion, as indicated by “a” in the top right panel of Figure 1. Both parameters decreased over time; however, −dF/dt decreased to a greater extent than did +dF/dt in control hearts. When +dF/dt and −dF/dt were compared in hearts treated with 10$^{-9}$ M PMA, −dF/dt was significantly different from +dF/dt at all time points after PMA addition, as indicated by “b” in the top right panel of Figure 1. Thus, 10$^{-9}$ M PMA preferentially depressed −dF/dt, an effect clearly

![Graphs showing effects of 10$^{-9}$ M phorbol 12-myristate 13-acetate (PMA) on developed force, rates of contraction and relaxation, and resting tension over 60 minutes of perfusion as well as tissue energy metabolites at the end of the perfusion period.](image-url)
evident after 5, 10, and 15 minutes of perfusion, where \(-dF/dt\) was found to be significantly lower than \(+dF/dt\) in PMA-perfused hearts but not in control hearts.

A reduction in resting tension was observed in control hearts compared with ethanol-treated hearts after 10 and 15 minutes of perfusion with 10\(^{-9}\) M PMA (Figure 1, bottom left panel). After 15 minutes of perfusion with 10\(^{-9}\) M PMA, however, resting tension started to increase and was significantly higher than that in untreated controls after 45 and 60 minutes.

Examination of energy metabolite content after 60 minutes of perfusion showed that, in hearts treated with 10\(^{-9}\) M PMA, ATP values were significantly reduced whereas lactate content was significantly increased (Figure 1, bottom right panel). There were no significant effects on creatine phosphate, ADP, or AMP content after PMA treatment.

Hearts perfused with 10\(^{-8}\) M PDBu displayed changes similar in contractile function and energy metabolite content to changes in hearts perfused with 10\(^{-9}\) M PMA, although PDBu produced a more rapid onset of loss in function (Figure 2). Developed force decreased by 66% from predrug values after 60 minutes of perfusion (Figure 2, top left panel), and differences between control and PDBu-treated hearts were significant after only 5 minutes of perfusion. Perfusion with 10\(^{-8}\) M PDBu resulted in a rapid decline in both \(+dF/dt\) and \(-dF/dt\) (Figure 2, top right panel). After 60 minutes, \(+dF/dt\) and \(-dF/dt\) had decreased by 62% and 70%, respectively, from predrug values. Both \(+dF/dt\) and \(-dF/dt\) were significantly different from their respective controls at all time points measured. As was found with PMA (10\(^{-9}\) M), \(+dF/dt\) and \(-dF/dt\) were significantly different from each other in hearts treated with 10\(^{-8}\) M PDBu; this was especially evident during the first 15 minutes of perfusion, as indicated by “b” in the top right panel of Figure 2.

As seen with 10\(^{-9}\) M PMA, 10\(^{-8}\) M PDBu caused an initial decline in resting tension (Figure 2, bottom left panel) in the first 10 minutes of perfusion, followed by a gradual increase, although, unlike the results with PMA, the increase was not significant at any time.

As was observed with PMA-treated hearts, ATP content was significantly decreased after 60 minutes
of PDBu perfusion, while lactate content was significantly increased (Figure 2, bottom right panel). PDBu had no effect on creatine phosphate, ADP, or AMP content.

Because both 4α-phorbol and α-PDD have been demonstrated to be ineffective in activating PKC, we indirectly assessed the contribution PKC in the cardiac actions of phorbol esters by examining the effects of both of the above compounds. Their effects on contractile function and energy metabolism are shown in Figure 3. This figure clearly shows that α-PDD (10⁻⁶ M) produced a marked loss in developed force (79%) after only 30 minutes of perfusion, whereas 4α-phorbol (10⁻⁶ M) produced no effect (Figure 3, top left panel). Because of the marked cardiac dysfunction seen after only 30 minutes of treatment with α-PDD, the experiments were stopped, and hearts were freeze-clamped at that point. For some experiments, the perfusion period with 4α-phorbol was extended to 60 minutes without a significant difference arising in any contractile parameter when compared with corresponding control hearts (data not shown). Neither +dF/dt nor −dF/dt was significantly affected by 4α-phorbol.

α-PDD produced a significant increase in resting tension after only 10 minutes of treatment (Figure 3, bottom left panel). It is interesting to point out that no initial decrease in resting tension, as was observed previously with either PMA or PDBu, was evident. Resting tension was not affected by 4α-phorbol treatment. There was no effect on energy metabolite content with either 4α-phorbol or α-PDD (Figure 3, bottom right panel).

We also examined a lower (10⁻⁸ M) concentration of α-PDD on cardiac function and found that after 60 minutes of perfusion, no significant effects on contractile function, resting tension, energy metabolite content, coronary perfusion pressure, or 6-keto PGF₁α release were evident (data not shown).

**Coronary Perfusion Pressure**

A marked increase in coronary perfusion pressure was observed over the entire perfusion period after addition of either 10⁻⁶ M PMA or 10⁻⁸ M PDBu (up to 300% and 225% of predrug values, respectively) as shown in Figures 4A and 4B. Coronary perfusion pressure in PMA-treated hearts increased rapidly after 10 minutes of perfusion and was significantly

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**FIGURE 3.** Graphs showing effects of 10⁻⁶ M of either 4α-phorbol or 4α-phorbol 12,13-didecanoate (α-PDD) on developed force, rates of contraction and relaxation, and resting tension over 30 minutes of perfusion as well as tissue energy metabolites at the end of the perfusion period. +dF/dt, rate of contraction; −dF/dt, rate of relaxation; CP, creatine phosphate. Data represent mean ± SEM of five separate experiments and, for functional data, indicate percent of prephorbol addition. Asterisks indicate significant differences (p<0.05) between the two treatment groups. Note that for reasons of clarity control function data are not shown, although these were not different from values seen in the presence of 4α-phorbol.
different from controls at ≥10 minutes (Figure 4A). A significant effect was observed earlier (5 minutes) in PDBu-treated hearts, and pressures were significantly higher from control over the subsequent perfusion period (Figure 4B). Neither 4α-phorbol nor α-PDD had any effect on coronary perfusion pressure, although the latter produced a moderate and transient, although not statistically significant, reduction in coronary perfusion pressure (Figure 4C).

**Release of 6-Keto PGF₁α**

We assessed the ability of various phorbol esters to stimulate PGI₂ production as determined by the release of 6-keto PGF₁α, its stable hydrolysis product. As demonstrated in Figure 5, increased 6-keto PGF₁α efflux was observed from hearts treated with 10⁻⁸ M PMA, 10⁻⁸ M PDBu, and 10⁻⁶ M α-PDD. Both PMA (Figure 5A) and PDBu (Figure 5B) produced a sustained increase in 6-keto PGF₁α release that was...
significant over most of the treatment period, and this effect was similar for both compounds (Figures 5A and 5B). In contrast, perfusion with α-PDD produced only an early transient, albeit significant, increase in release after 5 minutes. 4α-Phorbol failed to have any effect on 6-keto PGF<sub>1α</sub> release (Figure 5C).

**Ultrastructural Analysis**

Electron micrographs of all hearts studied, irrespective of treatments, revealed totally normal ultrastructural morphology. Thus, for all experiments, sarcomeres, sarclemma, mitochondria, and nucleus appear normal. A particularly striking example of a lack of ultrastructural damage despite depressed cardiac function was found with higher phorbol ester concentrations, which showed profound and rapid depression in cardiac function (see below).

**Effects of Amiloride**

Direct effects of amiloride. To investigate the possible contribution of the Na<sup>+</sup>-H<sup>+</sup> exchanger to the phorbol ester effects, hearts were pretreated with 40 μg/ml amiloride before the addition of phorbol ester. By itself, amiloride produced a slight, nonsignificant decrease in developed force over time; however, both +dF/dt and −dF/dt were significantly less than control values after 90 minutes of amiloride treatment (data not shown). Amiloride increased resting tension slightly but had no direct effects on either coronary perfusion pressure or energy metabolite content. Release of 6-keto PGF<sub>1α</sub> was reduced by amiloride, but this effect was not significantly different compared with controls (data not shown).

Influence of amiloride on phorbol ester-induced functional changes and metabolic changes. Table 1 summarizes the effects of amiloride on the functional changes caused by 10<sup>−9</sup> M PMA and 10<sup>−8</sup> M PDBu. The depression in contractile function in terms of force and +dF/dt and −dF/dt produced by either phorbol ester was significantly attenuated by amiloride. However, amiloride had no effect on the phorbol ester–induced changes in resting tension, nor did it exert any influence on the changes in energy metabolites produced by either PDBu or PMA.

At a concentration of 40 μg/ml, amiloride failed to have any effect on cardiac depression produced by 10<sup>−6</sup> M α-PDD (not shown). Given the fact that a substantially higher concentration of α-PDD was used compared with other phorbol esters, the amiloride concentration was doubled to 80 μg/ml. However, even at that concentration, amiloride failed to alter contractile dysfunction produced by α-PDD. Furthermore, amiloride did not block the increase in resting tension induced by α-PDD and did not affect energy metabolite content after 30 minutes of perfusion (data not shown).

Coronary perfusion pressure. The effects of amiloride on the coronary perfusion pressure changes evoked by phorbol esters are shown in Figure 6. Amiloride significantly attenuated the initial increases in coronary perfusion pressure observed with both 10<sup>−9</sup> M PMA and 10<sup>−8</sup> M PDBu but had no effect with continued phorbol ester treatment (Figures 6A and 6B). In addition, amiloride failed to affect coronary pressure in the presence of 10<sup>−6</sup> M α-PDD (Figure 6C).

Release of 6-keto PGF<sub>1α</sub>. Figures 7A and 7B show that amiloride pretreatment significantly blocked the stimulated release of 6-keto PGF<sub>1α</sub> observed with 10<sup>−9</sup> M PMA and 10<sup>−8</sup> M PDBu, respectively. Although 6-keto PGF<sub>1α</sub> release in hearts treated with both amiloride and α-PDD was not significantly different from that in hearts treated with α-PDD alone (except at time 0), a trend did appear that was suggestive of reduced release with amiloride (Figure 7C).

Effects of High Phorbol Ester Concentrations

Studies reported in the literature have used much higher concentrations (from 10<sup>−7</sup> to 10<sup>−6</sup> M) of...
phorbol esters\textsuperscript{3,17-19} than were used in the present study. In an attempt to relate the findings in those studies to the model used in the present study, a series of experiments was performed to examine whether 10^{-5} M PMA and 10^{-6} M PDBu possessed similar amiloride-sensitive effects as in the previous experiments. These results are summarized in Table 2 with respect to functional parameters and energy metabolism.

With respect to function, Table 2 shows a marked (>80\%) reduction in contractility and elevations in coronary pressure and resting tension 30 minutes after addition of either 10^{-7} M PMA or 10^{-6} M PDBu. Amiloride failed to prevent these effects and, surprisingly, significantly enhanced PDBu-induced depression in both +dF/dt and -dF/dt. Amiloride also failed to attenuate the alterations in energy metabolites, such as reduction in high-energy phosphate and elevation in lactate content as a result of phorbol ester treatment.
Figure 8 shows that both $10^{-7}$ M PMA and $10^{-6}$ M PDBu increased 6-keto PGF$_{1\alpha}$ release. Although the effect was not significant, amiloride tended to depress 6-keto PGF$_{1\alpha}$ release after addition of high concentrations of either phorbol ester.

As described previously for lower phorbol concentrations, we also failed to observe any structural defects with higher levels of these compounds. A particular example is illustrated in Figure 9, which shows a myocyte with excellent ultrastructural integrity (Figure 9A) from a heart perfused for 30 minutes with $10^{-7}$ M PMA. This is particularly interesting, since this treatment produced a total cessation of contractility, marked increases in resting tension, and an elevation in coronary perfusion pressure (Figure 9B).

**Relation Between ATP, Lactate, and Contractile Depression**

Since reduction in ATP content and accumulation of tissue lactate accompanied phorbol ester–induced loss in force in some experiments, the correlation between these metabolic changes and force development was assessed (Figure 10). Sixty-minute (low phorbol ester concentrations, Figures 10A and 10B) and 30-minute experiments (higher phorbol ester concentrations, Figures 10C and 10D) were analyzed separately. The relation between the reduction in developed force and increased lactate and between reduced developed force and decreased ATP in 60-minute experiments was significant ($r = -0.85$ and $r = 0.84$, respectively; $p < 0.05$). In contrast, although similar trends were apparent with high phorbol ester concentrations, no significant relation was found between either developed force and lactate or developed force and ATP in these studies ($r = -0.62$ and $r = 0.57$, respectively). With respect to elevations in coronary perfusion pressure, these changes were
**Figure 9.** Typical example of normal electron micrograph (panel A) from an isolated heart perfused for 30 minutes with $10^{-7}$ M phorbol 12-myristate 13-acetate (PMA, panel B). Note that the sarcolemma (sl) and mitochondria (m) appear intact; also note the lack of contraction-band formation of sarcomeres (s) and the electron-dense and intact intercalated disc (id) despite the total loss of function, an elevation in resting tension, and an increase in coronary perfusion pressure (CPP). $+dF/dt$ and $-dF/dt$ indicate the rates of contraction and relaxation, respectively. Magnification of micrograph, $\times 9,750$. 
Figure 10. Graphs showing relations between tissue lactate or ATP levels with developed force in hearts perfused for 60 minutes (panels A and B) or 30 minutes (panels C and D) as described in “Results.” The data were taken from all groups of hearts used in this study and each point represents mean±SEM of five to 11 experiments. Asterisks beside correlation coefficient indicate a significant relation between metabolite changes and developed force.

Discussion

Overall Summary of Results

The ability of phorbol esters to depress cardiac contractility has been demonstrated in studies using numerous experimental models (see below). The present report supports those observations and demonstrates novel aspects of these concentration- and time-dependent effects. These include the observations that phorbol ester–induced cardiac depression is associated with coronary vasoconstriction, elevation in resting tension, stimulated release of PGI₂, and disturbed energy metabolism, particularly with respect to depressed ATP and elevated lactate contents. Our studies further show that, even under conditions of severe contractile dysfunction, no apparent ultrastructural damage was evident. To elucidate potential mechanisms of phorbol ester–induced dysfunction, we have shown that Na⁺-H⁺ exchange activation mediates, at least partially, cardiac effects produced by phorbol esters. A last major observation, which represents a totally unexpected finding, was the ability of a non-PKC–activating phorbol ester to potently depress cardiac contractility. The latter phenomenon is suggestive of a potential mechanism for phorbol ester effects unrelated to PKC activation. A summary of the divergent responses of the heart to various phorbols, which forms the basis for this discussion, is presented in Table 3.

Mechanisms for Phorbol Ester–Induced Depression in Contractility

PDBu (10⁻⁸ M) and PMA (10⁻⁹ M), two phorbol esters that have been demonstrated to activate PKC, produced similar changes in contractile function, energy metabolites, and PGI₂ release. The finding that two different concentrations produced similar effects is likely a reflection of the different potencies of these two phorbol esters. Previous studies have shown that PMA is more potent than PDBu in increasing PKC activity in various cell types. It has been suggested that the different potencies most likely reflect the higher lipophilicity of PMA than PDBu, which could then influence the ability of the phorbol to reach the site of action by affecting the rate of penetration into the cell membrane.

The finding that phorbol esters had negative inotropic effects agrees with reports in the literature using a variety of models, including isolated perfused rat heart, papillary muscle, cultured neonatal ventricular cells, and adult rat ventricular cells.

significantly related to reduced ATP and elevated lactate contents for all concentrations of phorbol esters studied (Figure 11).
The precise mechanisms, however, by which phorbol esters exert these effects are not entirely clear. Numerous mechanisms for such actions have been postulated including 1) both increased Ca\(^{2+}\) influx\(^1\) and decreased [Ca\(^{2+}\)]\(_{\text{c}}\)\(^4\) 2) direct effects on excitation/contraction coupling processes, such as phosphorylation of troponin proteins\(^24,25\) and phosphorylation of phospholamban,\(^7,26\) and 3) nonspecific effects unrelated to PKC activation, such as membrane perturbations.\(^27,28\) Based on the observation that amiloride, at a concentration well known to inhibit myocardial Na\(^+\)-H\(^+\) exchange,\(^29-32\) attenuated the contractile depression induced by 10\(^{-9}\) M PMA and 10\(^{-8}\) M PDBu, the present study suggests that a possible added mechanism for phorbol ester–induced cardio-depression is Na\(^+\)-H\(^+\) exchange activation, at least at low phorbol ester concentrations. It should be added that the implication of Na\(^+\)-H\(^+\) exchange is strength-

### Table 3. Summary of Cardiac Actions of Phorbols

<table>
<thead>
<tr>
<th></th>
<th>PMA 10(^{-9}) M</th>
<th>PDBu 10(^{-9}) M</th>
<th>(\alpha)-PDD 10(^{-8}) M</th>
<th>4(\alpha)-Phorbol 10(^{-6}) M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contractile depression</td>
<td>Yes(^*)</td>
<td>Yes(^*)</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Contracture</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td>Coronary constriction</td>
<td>Yes(^\dagger)</td>
<td>Yes(^\dagger)</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>ATP reduction</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Lactate elevation</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>Ultrastructural damage</td>
<td>No</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Stimulation of 6-keto PGF(_{1\alpha}) efflux</td>
<td>Yes(^*)</td>
<td>Yes(^*)</td>
<td>Yes</td>
<td>No</td>
</tr>
</tbody>
</table>

\(PMA,\) phorbol 12-myristate 13-acetate; PDBu, phorbol 12,13-dibutyrate; \(\alpha\)-PDD, 4\(\alpha\)-phorbol 12,13-didecanoate; 6-keto PGF\(_{1\alpha}\), 6-ketoprostaglandin F\(_{1\alpha}\).

*Amiloride sensitive.

\(\dagger\)Initial response amiloride sensitive.

\(\dagger\)Transient stimulation only.
ened by our ability to confirm all effects observed with amiloride with the more potent and specific exchange inhibitor 5-((N,N-hexamethylene)-amiloride (data not shown). Indeed, based on our studies, we feel that phorbol ester–induced cardiac effects are mediated by numerous processes, each of which contributes to cardiac depression.

One way that stimulated Na\(^+-\)H\(^+\) exchange might influence contractile function is by increasing [Ca\(^{2+}\)], causing Ca\(^{2+}\) overload. This suggestion was made by Frelin et al.,\(^{29}\) who showed that, in rat cardiac cells, inhibition of Na\(^+-\)H\(^+\) exchange by amiloride markedly decreased the amount of Na\(^+\) accumulation produced after Na\(^+\),K\(^+\)-ATPase inhibition by ouabain. These authors suggested that increased Na\(^+\) accumulation via Na\(^+\)-H\(^+\) exchange activation may be extruded in exchange for Ca\(^{2+}\) by the Na\(^+\)-Ca\(^{2+}\) exchanger. Alternatively, an increased [Na\(^+\)] could reduce the amount of Ca\(^{2+}\) extruded by the Na\(^+\)-Ca\(^{2+}\) exchanger by decreasing the Na\(^+\) gradient.\(^{33}\) Either mechanism could result in increased [Ca\(^{2+}\)]. In this regard, Ikeda et al.\(^{34}\) have shown that, in mouse myocardial cells, PMA (from 10 nM to 1 \(\mu\)M) induced a concentration-dependent rise in [Ca\(^{2+}\)] above that observed with the Ca\(^{2+}\)-ionophore A23187 alone, an effect inhibited by amiloride. Kim and Smith\(^{4}\) indicated that Na\(^+-\)H\(^+\) exchange appeared to be involved in the increased Ca\(^{2+}\) content induced by reduction in pH\(_{o}\) in cultured chick ventricular cells, since amiloride inhibited this pH\(_{o}\)-induced uptake of Ca\(^{2+}\). Furthermore, using working rat hearts, we have recently shown that the decrease in contractile function induced with 10--10 \(\mu\)M PMA was blocked by the Na\(^+\)-H\(^+\) exchange inhibitors, amiloride, 5-((N,N-hexamethylene)-amiloride, and quinacrine, as well as by the Ca\(^{2+}\) channel blocker, nifedipine, further supporting a role for both Ca\(^{2+}\) and Na\(^+\)-H\(^+\) exchange in phorbol ester–induced cardiodepression.\(^{2}\)

It is important to note that, at least in cardiac tissue, the hypothesis of an increased [Ca\(^{2+}\)] via Na\(^+\)-Ca\(^{2+}\) exchange as a result of Na\(^+\)-H\(^+\) exchange activation was based on studies in which Na\(^+\),K\(^+\)-ATPase activity was depressed either by ischemia, hypoxia, or treatment with ouabain, thereby allowing Na\(^+\)\(_{o}\) to accumulate.\(^{34,35}\) It has been proposed\(^{30}\) that the exchanger might play an important role in pathophysiological conditions such as ischemia/reperfusion injury, which is known to be associated with intracellular acidification and inhibition of the Na\(^+\),K\(^+\)-ATPase pump: two conditions that would promote influx of Na\(^+\) and subsequent influx of Ca\(^{2+}\) via Na\(^+\)-Ca\(^{2+}\) exchange. Indeed, in the ischemic/reperfused heart, Na\(^+\)-H\(^+\) exchange inhibitors significantly increased recovery of function\(^{35,36}\) through a mechanism possibly involving decreased Na\(^+\) accumulation and, thereby, decreased exchange of Na\(^+\) for Ca\(^{2+}\) and a subsequent reduction in Ca\(^{2+}\) accumulation.\(^{35,36}\) Although a comparison of ischemia/reperfusion experiments to the present study should be made with caution, we have provided evidence supporting the notion of a similar Na\(^+\)-H\(^+\) exchange component mediating phorbol ester cardiac effects.

In this regard, we observed a distinct, amiloride-sensitive component of the negative inotropic effect produced by low concentrations of PMA and PDBu, indicating the possible involvement of Na\(^+\)-H\(^+\) exchange in this effect. It could be assumed that the Na\(^+\),K\(^+\)-ATPase was operational; however, it is possible that maximal activation of the Na\(^+\)-H\(^+\) exchanger with phorbol esters may have exceeded the capacity of the Na\(^+\),K\(^+\)-ATPase to buffer the increase in Na\(^+\)\(_{o}\) resulting in an increase in [Na\(^+\)]. It could also be suggested that, in view of diminished ATP levels after phorbol ester treatment, some reduction of Na\(^+\) pump activity could have occurred, further exacerbating the effect of Na\(^+\)-H\(^+\) exchange activation. Although we do not have direct proof for Ca\(^{2+}\) involvement, the considerable elevations in resting tension induced by PMA (10\(^{-9}\) and 10\(^{-7}\) M) and PDBu (10\(^{-6}\) M) suggest enhanced accumulation of Ca\(^{2+}\) after phorbol ester treatment. Also, the increased synthesis of PGI\(_{2}\), which could reflect a stimulation of Ca\(^{2+}\)-dependent enzymes such as phospholipase A\(_{2}\) (see below), would also support the concept of increased [Ca\(^{2+}\)].

A potential problem in implicating enhanced [Ca\(^{2+}\)] as a mediator of phorbol ester–induced myocardial depression was our failure to observe an expected transient positive inotropic effect before the loss of function. A possible explanation may lie in the fact that a number of cellular events may occur simultaneously, which could prevent a pure Ca\(^{2+}\)-mediated increase in contractile force at the concentration of phorbol esters used in the present study. Indeed, a recent preliminary report\(^{37}\) has shown a transient positive inotropic effect of 10\(^{-12}\) M PMA in guinea pig hearts, an effect associated with elevations in [Ca\(^{2+}\)].

The findings that 1) PDBu increased resting tension significantly only at a concentration of 10\(^{-6}\) M and 2) \(\alpha\)-PDD (10\(^{-6}\) M) significantly increased resting tension indicate that this concentration-dependent effect may either reflect the lower potency of PDBu, as previously discussed, or may be a PKC-independent effect of these individual phorbol esters. As noted previously, \(\alpha\)-PDD does not activate PKC and, therefore, was not expected to have any significant effects on cardiac function. However, in addition to elevating resting tension, \(\alpha\)-PDD caused significant cardiodepression.

It was surprising that no morphological changes were observed under any treatment condition, particularly in view of the fact that we have suggested Ca\(^{2+}\) as a possible mediator of phorbol ester effects. Furthermore, as illustrated in Figure 9, it was very surprising that no evidence of contraction-band formation was ever observed, although elevations in resting tension were clearly evident. It is possible that the increase in [Ca\(^{2+}\)] was not substantial enough to damage the myocardium or that other mechanisms in addition to Ca\(^{2+}\) overload may have contributed to the negative inotropic effect. In addition, masking of
ultrastructural defects due to tissue processing artifacts cannot be totally excluded.

In the present study, PMA and PDBu have been shown to depress ATP content and increase lactate accumulation, whereas creatine phosphate, ADP, and AMP content were unaffected by any phorbol ester treatment. The fact that only ATP content was affected was surprising, but the results may suggest a specific direct action of phorbol esters on ATP synthesizing systems at the mitochondrial level. Neither 4α-phorbol nor α-PDD altered energy metabolite content, indicating that these metabolic changes may be mediated by PKC. On the other hand, given the observation that 4α-phorbol and α-PDD failed to produce coronary constriction as well as changes in energy metabolites suggests that metabolic changes, including ATP reduction and lactate accumulation, may occur as a consequence of regional ischemic areas resulting from elevation in coronary resistance. Indeed, the close correlation between changes in energy metabolites and coronary perfusion pressure supports such a view.

Protein Kinase C-Dependent and –Independent Phorbol Ester Effects

The stereochemistry of α-phorbols is different from that of the PKC-activating β-phorbols, and it is most likely the β-configuration that is responsible for PKC-stimulating ability. In this regard, there is substantial evidence that α-PDD and 4α-phorbol do not activate PKC and, thus, provide useful negative controls to confirm PKC-mediated effects.16,20,21,38 The potent cardiodepressant effect of 10-6 M α-PDD in the present study strongly suggests that PKC activation may not be necessary for cardiotoxic effects of all phorbol esters. Perhaps more importantly, these data demonstrate a multiplicity of phorbol ester actions that may or may not involve PKC activation. Indeed, Nishizuka27 noted that, although phorbol esters have been demonstrated to activate PKC, the possibility exists that PKC is not their sole target and that these compounds may act as membrane perturbers, particularly at higher concentrations. In light of that concept, one possible interpretation of these results is that membrane perturbation accounts for the negative inotropic effect of high concentrations of PMA, PDBu, and α-PDD, since these effects were reasonably similar for all phorbols and were not prevented by pharmacological pretreatments. For instance, because of the lipophilicity of these compounds, it is possible that they intercalate within the membrane and in so doing may physically interfere with the normal functioning of ion channels, pumps, and ATPases. PKC-independent effects of phorbols have been reported in other tissues, for instance on hippocampal Ca2+ currents,28 although ours represents the first such report in a cardiac preparation. Furthermore, it is important to point out that 4α-phorbol was totally without effect on any aspect of cardiac function. This could suggest that the presence of the ester moiety is critical for cardiotoxic properties of high concentrations of these compounds. The occurrence of nonspecific actions at relatively high concentrations (i.e., 10-7 or 10-6 M) of phorbol esters is important, because many studies with phorbol esters have used these concentrations.3,39,40 Although it has been generally assumed that these phorbol effects are mediated via PKC activation, there is at present insufficient evidence to implicate PKC in phorbol ester effects. This problem is compounded by the fact that few studies have reported the effects of PKC inhibition on the cardiac actions of phorbol esters, most likely because many PKC inhibitors are potent cardiodepressants, which confounds interpretation of the data. In our hands, we have found that (±)-1-O-hexadecyl-2-O-acetylglycerol significantly attenuated the effects of very low concentrations (10-10 M) of PMA, in spite of a direct negative inotropic effect of the PKC inhibitor.2 Therefore, it is possible that the effects of low concentrations of phorbol esters are indeed PKC-dependent, whereas this dependency is lost with increasing phorbol ester concentrations.

Possible Mechanisms for Phorbol Ester-Induced Coronary Constriction

The finding that amiloride failed to block the sustained vasoconstriction produced by phorbol ester in this study suggests that this effect is independent of Na+-H+ exchange activation. However, amiloride did have an attenuating effect on the initial increase in coronary perfusion pressure after 5 and 10 minutes of perfusion with PMA (10-9 M) and after 5 minutes of perfusion with PDBu (10-8 M). Therefore, although vasoconstrictive effects due to Na+-H+ exchange activation cannot be totally excluded, it is likely that this action of phorbol esters is due to other mechanisms.

β-Phorbol esters, but not α-phorbols, have been demonstrated to produce a slowly developing and sustained constriction of vascular smooth muscle39,41-45 implying a role for PKC in the sustained phase of smooth muscle constriction. The results of the present study are consistent with the concept of PKC involvement, since both α-PDD and 4α-phorbol failed to have a significant effect on coronary perfusion pressure.

There is substantial evidence that phorbol esters constrict vascular smooth muscle through mechanisms postulated to involve activation of Na+-Ca2+ exchange to increase [Ca2+]i,45 activation of Na+, K+-ATPase,46 and Ca2+ influx via L-type Ca2+ channels.22,47,48 There are a number of studies implicating Ca2+ influx via L-type Ca2+ channels in sustained vasoconstriction, although their involvement is still controversial. Indeed, both an activation of the Ca2+ current47 as well as an inhibition of the Ca2+ transient produced by other agonists49 have been reported in studies in which phorbol esters were added to vascular smooth muscle cells. Based on studies carried out using Ca2+-free buffer or in the presence nifedipine, the study of Chiu et al22 showed that PDBu-induced constriction of rat aorta depended ~40% on Ca2+.
influx via the slow inward $\mathrm{Ca}^{2+}$ current. The residual constriction, they suggested, may have been the result of PKC phosphorylation of other kinases. The complexity underlying phorbol-induced vascular contraction, particularly with regard to $\mathrm{Ca}^{2+}$ requirement, has been further highlighted by studies of Jiang and Morgan, who reported a lack of elevation in $[\mathrm{Ca}^{2+}]$ despite marked constriction after phorbol ester addition to isolated rat aorta. In addition, these authors, as well as others, have demonstrated the ability of various phorbol esters to produce contraction of rat aorta or porcine coronary arteries in the absence of extracellular $\mathrm{Ca}^{2+}$, suggesting that sensitization of contractile proteins to $\mathrm{Ca}^{2+}$ may, at least in part, account for the vascular contraction produced by phorbol esters. Taken together, there is evidence both from the literature as well as the present study that there is a component of the sustained contraction of smooth muscle in response to phorbol esters that is dependent on $\mathrm{Ca}^{2+}$ influx; however, other mechanisms including phosphorylation of other kinases and sensitization of myosin are also likely involved. Because of the inability of PKC-nonactivating phorbols to increase coronary pressure, it seems likely that phorbol ester–induced coronary constriction is PKC-mediated. This concept is strengthened by a recent study that showed that a synthetic peptide PKC inhibitor attenuated PDBu-induced constriction of isolated porcine coronary arteries. Although there may be a minor influence of $\mathrm{Na}^+\cdot\mathrm{H}^+$ exchange activity on contraction, the predominant effects, particularly with respect to sustained constriction, are likely independent of $\mathrm{Na}^+\cdot\mathrm{H}^+$ exchange activity. The precise role of $\mathrm{Ca}^{2+}$ in mediating phorbol ester–induced constriction still remains to be fully elucidated.

**Stimulation of Prostacyclin Efflux**

Although stimulation of PGF$_2\alpha$ synthesis has been shown to occur with phorbol esters, this is the first report showing this property in cardiac tissue. The inability of either 4α-phorbol or α-PDD to stimulate sustained PG release suggests that this effect is likely PKC dependent and involves the availability of free arachidonic acid. How this occurs is uncertain, although it has been proposed that it is due to inhibition of reacylation as a result of inhibition of arachidonoyl coenzyme A synthase and lysophosphatidate acyltransferase activities. Others have questioned this and proposed a $\mathrm{Ca}^{2+}$-dependent phospholipase A$_2$ activation as the primary contributing factor. The finding that amiloride significantly inhibited phorbol ester–induced PG release suggests that this phenomenon was also dependent on $\mathrm{Na}^+\cdot\mathrm{H}^+$ exchange activity. In fact, a role for $\mathrm{Na}^+\cdot\mathrm{H}^+$ exchange in phospholipase A$_2$ activation has been suggested to occur in platelets and in rat mast cells. In this regard, the suggestion has been made that, because phospholipase A$_2$ activity shows a pH optimum in the neutral to alkaline range, the enzyme might be stimulated in situations where the $\mathrm{Na}^+\cdot\mathrm{H}^+$ exchanger is maximally activated, for example, during reperfusion after an ischemic insult. Indeed, it has been previously demonstrated that inhibition of $\mathrm{Na}^+\cdot\mathrm{H}^+$ activity with amiloride attenuated PG efflux on reperfusion, a phenomenon associated with reduced injury. An interesting finding in the present study was that α-PDD, at $10^{-6}$ M, caused a significant but transient increase in PGF$_2\alpha$ release. Because α-PDD does not activate PKC, it is possible that the α-PDD structure had direct disruptive effects on the plasma membrane, resulting in release of arachidonic acid from the membrane and subsequent metabolism to PGF$_2\alpha$. As mentioned previously, it is generally thought that the availability of arachidonic acid is the limiting factor in eicosanoid release; therefore, any stimulus that increases arachidonic acid can potentially increase release of 6-keto PGF$_{1\alpha}$ in addition, the time course of release of 6-keto PGF$_{1\alpha}$ was different from that of both PMA and PDBu; therefore, it is most likely that sustained stimulation in PG production requires PKC activation. Despite the substantial evidence that phorbol esters can directly stimulate PG synthesis in various tissues, the possibility that the enhanced 6-keto PGF$_{1\alpha}$ efflux observed in the present study was, in part, due to regional myocardial ischemia as a consequence of coronary vasoconstricting actions of phorbol esters cannot be ruled out. However, unpublished studies in our laboratory using a variety of vasoconstrictors, such as phenylephrine or peptido-leukotrienes, have routinely resulted in a very modest (10–15%) increase in 6-keto PGF$_{1\alpha}$ efflux from isolated rat hearts, substantially less than the marked stimulation of efflux seen in the present study after phorbol ester addition. Therefore, it is likely that the bulk of stimulated release of 6-keto PGF$_{1\alpha}$ occurred as a consequence of phorbol ester–induced stimulation, although the subsequent regional ischemia could have represented a minor contribution.

Since the mechanisms for phorbol ester–mediated stimulation of 6-keto PGF$_{1\alpha}$ release likely reflect enhanced phospholipase A$_2$ activity, it can reasonably be assumed that this phenomenon was associated with the release of other arachidonic acid metabolites. However, although eicosanoids possess complex cardiac actions, none can be related to the marked depression in contractile function or the coronary constriction associated with phorbol ester administration; indeed, prostacyclin exerts coronary dilating effects. Furthermore, it should be noted that we were unable to modulate cardiac responses to any phorbol with various cyclooxygenase inhibitors, including indomethacin, aspirin, or ibuprofen, despite an almost total inhibition of 6-keto PGF$_{1\alpha}$ efflux (data not shown).

**Conclusions**

In summary, we have demonstrated very complex effects of phorbol esters on various aspects of cardiac function. This complexity is most likely a reflection of diverse actions of these compounds on cellular ho-
meostasis that are concentration dependent and in many instances independent of PKC activation. Na⁻⁻H⁺ exchange apparently plays a contributing role in cardiac depression produced by low concentrations of phorbol esters and is likely of importance in mediating phorbol ester–stimulated PG synthesis.

In contrast, the exchanger is unlikely to participate in sustained coronary constriction by phorbols. Such a diversity of actions necessitates further studies to define specific mechanisms of the actions of phorbol esters on various aspects of heart function.

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Key words: heart · contractility · coronary resistance · phorbol esters · protein kinase C · Na⁺-H⁺ exchange · calcium · energy metabolism · prostaglandins
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