Diacylglycerol and fatty acids synergistically increase cardiomyocyte contraction via activation of PKC

YEQING PI AND JEFFERY W. WALKER
Department of Physiology, University of Wisconsin, Madison, Wisconsin 53706
Received 27 August 1999; accepted in final form 29 December 1999

Pi, YeQing, and Jeffery W. Walker. Diacylglycerol and fatty acids synergistically increase cardiomyocyte contraction via activation of PKC. Am J Physiol Heart Circ Physiol 279: H26–H34, 2000.—Lipid signaling pathways are thought to play a prominent role in transducing extracellular signals into contractile responses in cardiac muscle. Two putative lipid messengers, diacylglycerol and arachidonic acid, can be generated via distinct phospholipases in separate signaling pathways, but certain stimuli cause them to be elevated in parallel. We tested the hypothesis that these lipids function as comessengers in ventricular myocytes by activating protein kinase C (PKC). In previous work, we demonstrated that the diacylglycerol analog dioctanoylglycerol (diC8) can be stimulatory or inhibitory toward myocyte twitches depending on how it is applied. Here we report that arachidonic acid and other cis-unsaturated fatty acids (UFA), at concentrations too low for direct effects, synergistically enhance the stimulatory effects of diC8 and convert inhibitory effects of diC8 into stimulation of myocyte twitches. Intracellular Ca2+ transients changed in parallel with twitch amplitude, suggesting regulation of Ca2+ homeostasis by these lipids. cis-UFA also interacted synergistically with the PKC activator phorbol 12-myristate 13-acetate to promote positive inotropic responses. Responses were blocked by the PKC antagonists chelerythrine chloride, bisindolylmaleimide, and Go-6976. DiC8 and arachidonic acid also synergistically translocated PKC-ε and PKC-α in intact myocytes. We propose that PKC integrates diacylglycerol and cis-UFA signals in the heart, resulting in preferential activation of positive inotropic mechanisms.

1) Positive inotropy; calcium; cis-unsaturated fatty acids; arachidonic acid; phorbol esters; protein kinase C

PROTEIN KINASE C (PKC) is a large family of serine/threonine protein kinases capable of modifying the activity of many cellular proteins by phosphorylation (23, 27). PKC is highly expressed in adult myocardium and has been implicated in the regulation of contractility, gene expression, and growth (27). PKC signaling may also contribute to the response of myocardium to ischemia (1, 9) and may play a role in the development of cardiac disease states such as hypertrophy and heart failure (3). Considerable uncertainty remains concerning the precise mechanisms of contractile regulation by PKC even under normal physiological conditions. There is growing evidence that this pathway can mediate inhibitory (negative inotropic) or stimulatory (positive inotropic) effects on cardiac muscle contraction, with the final inotropic state determined by a balance between inhibitory and stimulatory influences (21, 26, 30, 40). The subcellular mechanisms underlying these opposing responses, including the lipid signals involved and the target proteins phosphorylated, are not well understood. The physiological significance of this dual regulation is also unclear.

Key lipid cofactors of PKC are diacylglycerols, a class of neutral lipids generated within cell membranes by phospholipase C (PLC) under the control of G proteins, coupled cell surface receptors, tyrosine kinases, or Ca2+ (34). Diacylglycerols act in conjunction with phosphatidylserine and in some cases Ca2+ to activate PKC isoforms containing a cysteine-rich C-1 domain (25). Other lipid species such as fatty acids, lysophospholipids, and phosphatidylinositols also modulate the activity of certain PKC isoforms (2, 13, 16, 37, 38). Several PKC family members displaying responsiveness to diacylglycerol and cis-unsaturated fatty acids (cis-UFA) are expressed in adult cardiac myocytes (9, 13), but their precise functional roles are not yet clearly defined.

cis-UFA are elevated in the heart in response to physiological and pathological stimuli (39), presumably as a result of activation of phospholipase A2 (PLA2). Fatty acids generated by PLA2, particularly arachidonic acid, have been shown to influence a variety of cellular targets including ion channels, phospholipases, phosphatases, and PKC (7, 12, 13, 15). cis-UFA have also been shown to potently activate PKC in the presence of diacylglycerol because of a synergistic interaction between these ligands (19, 37). The mechanism of synergism between cis-UFA and diacylglycerol is unknown but may arise from binding at distinct sites on the protein surface (25) or may result from the formation of unique membrane structures (10). Of particular interest is the possibility that this lipid combination may circumvent the need for other cofactors to activate PKC such as phosphatidylserine and Ca2+. Synergistic effects of these lipids have also been demonstrated in vivo. Diacylglycerol and cis-UFA treatment stimulate phosphorylation of cellular proteins

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
and secretion in platelets (41), modulate synaptic function in neurons (4, 19), and promote PKC translocation in tumor cell lines (28, 38). However, there have been no detailed studies of the combined effects of diacylglycerol and cis-UF A on cardiac cell function.

In the present study, adult rat ventricular myocytes were treated with a combination of fatty acids and diacylglycerol at low and subthreshold doses while twitch contractions and intracellular calcium transients were monitored. The goals of this study were to determine 1) whether synergism between cis-UF A and diacylglycerol promotes positive or negative inotropy, 2) whether alterations in contractility are mediated by changes in intracellular Ca\(^{2+}\) transients, and 3) whether this lipid combination activates one or more PKC isoforms. The results indicate that cis-UF A and diacylglycerol may serve as comessengers acting via PKC-ε and PKC-α to preferentially activate positive inotropic mechanisms in the adult heart. Some of this work has been presented in preliminary form (32).

MATERIALS AND METHODS

Reagents. All reagents were purchased from Sigma Chemical (St. Louis, MO) unless otherwise noted. Fatty acids including oleic, linoleic, linolenic, arachidonic, stearic, and myristic acids were prepared fresh in pure ethanol. PKC inhibitors were purchased from Calbiochem (La Jolla, CA). Stock solutions of chelerythrine chloride were prepared in distilled water, and G6-6976 and bisindolylmaleimide I (BIM) were prepared in dimethyl sulfoxide (DMSO). Both 1,2-di-sn-dioctanoylglycerol (diC8), obtained from Avanti Polar Lipids (Alabaster, AL), and active phorbol 12-myristate 13-acetate (PMA), from Calbiochem, were prepared fresh in DMSO. Fluoro 3 was obtained in its acetoxyethyl ester (AM) form from Molecular Probes (Eugene, OR) and prepared in DMSO. Caged diC8 was the α-carboxyl-2,4-dinitrobenzyl form synthesized and purified as previously described (14). Monoclonal anti-PKC-α (catalytic domain) was purchased from Upstate Biotechnology (Lake Placid, NY). Polyclonal anti-PKC-δ was from GIBCO BRL (Grand Island, NY). With the use of affinity purification, monoclonal anti-PKC-ε was prepared from anti-PKC-ε serum of rabbits immunized with a peptide derived from the COOH terminus of PKC-ε (13). Goat anti-rabbit or anti-mouse IgGs conjugated with horse-radish peroxidase were from GIBCO BRL or Boehringer Mannheim (Indianapolis, IN). The enhanced chemiluminescence (ECL) Western blot detection reagents, nitrocellulose transfer membrane (Hybond, ECL), and high-performance luminescence detection film (Hyperfilm, ECL) were from Amersham (Arlington Heights, IL). Collagenase II was from Worthington Biochemical (Freehold, NJ).

Isolation of ventricular myocytes. Left ventricular myocytes were enzymatically dissociated from adult male rat hearts as previously described (30). The yield was ~1–3 x 10\(^6\) cells per heart, and >80% were rod shaped and Ca\(^{2+}\) tolerant. CaC10 was added to Ca\(^{2+}\)-free Ringer buffer to achieve the indicated Ca\(^{2+}\) concentration. Ca\(^{2+}\)-free Ringer buffer had the following composition (in mM): 125 NaCl, 2 NaH\(_2\)PO\(_4\), 1.2 MgSO\(_4\), 5 KCI, 25 HEPES (pH 7.4), 5 pyruvate, and 11 d-glucose.

Ca\(^{2+}\) and cell twitch measurement. Cells were loaded with the fluorescent Ca\(^{2+}\) indicator fluo 3-AM, and intracellular Ca\(^{2+}\) was determined with a DeltaScan D-140 Microscopic Photometer system (Photo Technology, New Jersey City, NJ) as previously described (31). Cell twitches were initiated by electric field stimulation at 0.4 Hz with the use of a Grass SD9 stimulator (Queiny, MA) in a modified PH1 chamber (Warner Instrument, Hamden, CT) mounted on a Nikon Diaphot inverted microscope. The temperature was controlled at 30–32°C by a TC-344A Dual Heater Controller (Warner Instrument). 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 to 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. Cell shortening was also recorded and analyzed with a Pentium personal computer using Felix software (Photon Technology).

Subcellular fractionation of cardiac myocytes. Cytosol, filament, and membrane fractions were obtained from the cells by using a modified version of the method previously described (13). In brief, isolated myocytes in 0.5 mM Ca\(^{2+}\) Ringer solution were incubated with test compounds for the indicated times at 37°C. Myocytes were quickly pelleted by centrifugation and 50 μl of each pellet was resuspended in fresh Ringer buffer. Lysis buffers containing 20 mM Tris (pH 7.4), 137 mM NaCl, 50 mM NaF, 5 mM EDTA, 1 mM PMSF, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml soybean trypsin inhibitor, and either 0.05% digitonin or 1% Triton X-100 were prepared fresh and chilled on ice. Digitonin lysis buffer was added to cell pellet in 1.5-ml microcentrifuge tubes, and pellets were dispersed by pipetting the solution up and down. The tubes were rocked for 5 min at 4°C. Samples were then centrifuged for 2.5 min at 15,000 g. The supernatants containing the cytosol and membrane fractions were transferred to other tubes and centrifuged at 40,000 g for 30 min at 4°C, producing a pellet designated the membrane fraction. The supernatant was designated the cytosol fraction. The 15,000 g pellet was designated the filament/nuclear fraction and washed twice with digitonin lysis buffer. Triton X-100 lysis buffer was added to each remaining pellet, which was then lightly vortexed and allowed to stand on ice for 60 min. Tubes were then vortexed for 1 min and centrifuged at 40,000 g for 30 min at 4°C. Supernatants containing membrane or filament fraction were transferred to prechilled microcentrifuge tubes and placed on ice. Aliquots of cytosol, filament/nuclear, and membrane fractions were diluted with equal volumes of sample buffer containing 2% SDS, 20% glycerol, 100 mM dithiothreitol, 0.1% bromphenol blue, and 200 mM Tris (pH 6.8), boiled for 5 min, and then stored at −20°C before Western analysis was completed. The protein concentration was determined using the bicinchoninic acid assay (Sigma Chemical).

Western blotting. Immunoblot analysis was carried out on each of the three subcellular fractions: cytosol, filament/nuclear, and membrane. Equal amounts of protein (typically 20–30 μg) from each fraction were electrophoresed on 12% SDS-PAGE gels and then transferred to nitrocellulose membranes. Blots were then stained with 0.1% Ponceau S solution to visualize protein bands and confirm both consistent protein loading among wells and complete transfer of protein to blots. The nitrocellulose membranes were incubated in blocking buffer containing 20 mM Tris (pH 7.6), 137 mM NaCl, and 0.1% Tween 20 (TBS-T) as well as 5% nonfat milk for 1 h at room temperature (RT). After being washed in TBS-T solution twice (10 min each), filters were separately incubated with primary antibodies (1:500–1:1,000 dilution in TBS-T solution) for 1 h at RT or overnight at 4°C with rotation. After being washed in TBS-T solution three times (10 min each), filters were incubated for 1 h at RT with...
horseradish peroxidase-linked secondary antibody (1:5,000 dilution in TBS-T solution containing 0.03% nonfat milk). Filters were again washed in TBS-T solution four times (10 min each). Bound antibodies were detected by the ECL method according to the manufacturer's (Amersham) instructions. Signals were quantified using a Bio-Rad GS-670 imaging densitometer.

Statistics. Data are presented as means ± SE with the number of observations given for each experiment. A Student’s paired or unpaired t-test was used to test statistical difference, and P < 0.05 was taken as significant unless otherwise indicated. For groups of three or more, a one-way ANOVA was used (with a Bonferroni t-test for multiple comparisons) to determine significance, with P values given in legends for Figs. 2, 3, and 5.

RESULTS

Figure 1 shows the comessenger actions of the diacylglycerol analog diC₈ and the cis-UFA oleic acid in rat ventricular myocytes. A 5-s pulse of diC₈ released intracellularly from a caged compound was below the threshold for initiating a change in twitch amplitude (Fig. 1A). Similarly, 25 μM oleic acid was below its threshold for changing inotropic state (Fig. 1B). However, when combined together these two lipid messengers produced a strong positive inotropic response even at these subthreshold concentrations (Fig. 1C). This observation represents clear evidence for a synergistic interaction between these signaling molecules because the twitch response to the combination was greater than the sum of the individual effects. In addition to oleic acid, other cis-UFA tested, including linoleic acid (Fig. 1, D and E), linolenic acid (Fig. 1, F and G), and arachidonic acid (30), gave similar results, but the saturated fatty acids stearic acid or myristic acid were ineffective (not shown). Overall, the positive inotropic response observed with the combination of diC₈ and cis-UFA represented a 200% increase over basal twitch amplitude that developed with a half-time of 3 min (Table 1).

In contrast to the results of Fig. 1, which show positive inotropic effects of lipid PKC activators, most investigators have concluded that diacylglycerol analogs and phorbol esters promote negative inotropic responses in cardiac (ventricular) preparations (5, 24, 26, 30, 32, 36, 40). Indeed, bath application of 25 μM diC₈ onto the same rat ventricular myocytes paced under the same conditions gave rise to an inhibitory response (Fig. 2A). This response was on average a 29 ± 2% decrease in twitch amplitude that developed with a half-time of 7 min (Table 1). The mechanism of inhibition appeared to involve changes in intracellular Ca²⁺ because a parallel reduction in the magnitude of systolic Ca²⁺ transients was also observed (Fig. 2A). The phorbol ester PMA also gave rise to negative inotropic responses (see below), showing that this myocyte preparation gave the expected physiological responses to these traditional PKC activators (when applied in the bath).
We next asked whether synergy between diC₈ and cis-UFA might enhance this negative inotropic response. Surprisingly, bath application of 25 μM diC₈ (which on its own gave a 29% inhibition of twitches) plus 25 μM arachidonic acid (a subthreshold dose) converted the negative inotropic response into a substantial positive inotropic response (Fig. 2A). The mechanism appeared to involve changes in intracellular Ca²⁺ because peak systolic Ca²⁺ was increased in parallel. This change in the fundamental nature of the response to diacyglycerol was also observed in the presence of subthreshold doses of other cis-UFA (Table 1), but not in the presence of stearic or myristic acid. Positive inotropic responses reached approximately a 100% increase over basal twitch amplitude and developed with a half-time of 3 min (Table 1). The time course of the increase in twitch amplitude was similar for bath-applied and photoreleased diC₈.

The involvement of PKC in this new cellular response to lipid messengers was tested with a battery of organic inhibitors of PKC. In separate experiments, 500 nM BIM (Fig. 3), 100 nM Gö-6976 (Fig. 3), and 4 μM chelerythrine (not shown) each blocked the positive inotropic response by >90%. Gö-6976 was the most effective and consistent of the inhibitors employed. It did not influence basal twitch amplitude, a phenomenon occasionally observed with BIM and chelerythrine (see also Ref. 26). Gö-6976 is of particular interest because it has been reported to preferentially inhibit non-organic inhibitors of PKC. In separate experiments, the response to lipid messengers was tested with a battery of inhibitors used above was able to block the inhibitory response to bath-applied diC₈ (Fig. 3). This observation suggests the possibility of additional mechanisms of inhibition by this neutral lipid besides via PKC. It may also explain why cis-UFA did not synergistically enhance the negative inotropic response to diC₈. To address this concern, we used phorbol esters because there is good evidence that negative inotropic responses to phorbol esters are mediated by PKC. We examined the response of cardiac myocytes to the widely used PMA with and without cis-UFA. As expected, PMA alone caused a concentration-dependent

---

**Table 1. Summary of inotropic effects of diC₈, cis-UFAs, and PMA**

<table>
<thead>
<tr>
<th>Observation</th>
<th>Basal</th>
<th></th>
<th>t₁/₂, min</th>
</tr>
</thead>
<tbody>
<tr>
<td>5-s diC₈ + 25 μM C18:1</td>
<td>21</td>
<td>170 ± 30</td>
<td>3.1 ± 0.2</td>
</tr>
<tr>
<td>5-s diC₈ + 50 μM C18:2</td>
<td>10</td>
<td>204 ± 35</td>
<td>2.4 ± 0.3</td>
</tr>
<tr>
<td>5-s diC₈ + 50 μM C18:3</td>
<td>9</td>
<td>202 ± 28</td>
<td>2.2 ± 0.5</td>
</tr>
<tr>
<td>5-s diC₈ + 25 μM C20:4</td>
<td>29</td>
<td>193 ± 14</td>
<td>3.2 ± 0.2</td>
</tr>
<tr>
<td>25 μM free diC₈</td>
<td>11</td>
<td>29 ± 2</td>
<td>6.9 ± 0.9</td>
</tr>
<tr>
<td>25 μM free diC₈ + 25 μM C18:1</td>
<td>7</td>
<td>84 ± 12</td>
<td>2.9 ± 0.3</td>
</tr>
<tr>
<td>25 μM free diC₈ + 50 μM C18:2</td>
<td>7</td>
<td>118 ± 28</td>
<td>3.3 ± 0.2</td>
</tr>
<tr>
<td>25 μM free diC₈ + 50 μM C18:3</td>
<td>10</td>
<td>117 ± 20</td>
<td>2.7 ± 0.2</td>
</tr>
<tr>
<td>25 μM free diC₈ + 25 μM C20:4</td>
<td>14</td>
<td>119 ± 12</td>
<td>2.3 ± 0.3</td>
</tr>
<tr>
<td>100 nM PMA</td>
<td>15</td>
<td>40 ± 5</td>
<td>5.8 ± 0.7</td>
</tr>
<tr>
<td>1 nM PMA</td>
<td>7</td>
<td>17 ± 4</td>
<td>6.1 ± 1.5</td>
</tr>
<tr>
<td>0.25 nM PMA</td>
<td>5</td>
<td>42 ± 13</td>
<td>4.5 ± 1.3</td>
</tr>
<tr>
<td>100 nM PMA + 25 μM C20:4</td>
<td>7</td>
<td>3.7 ± 4</td>
<td>6.3 ± 2.7</td>
</tr>
<tr>
<td>1 nM PMA + 25 μM C20:4</td>
<td>4</td>
<td>17 ± 10</td>
<td>2.0 ± 1.5</td>
</tr>
<tr>
<td>0.25 nM PMA + 25 μM C20:4</td>
<td>8</td>
<td>74 ± 8</td>
<td>6.0 ± 0.8</td>
</tr>
</tbody>
</table>

Data are expressed as means ± SE. Each cis-unsaturated fatty acid (UFA), C18:1 (oleic), C18:2 (linoleic), C18:3 (linolenic), and C20:4 (arachidonic), was used at a subthreshold dose. Negative inotropic responses are indicated by a minus sign. DiC₈, diastanoloylglycerol; PMA, phorbol 12-myristate 13-acetate; t₁/₂, time required to reach one-half of the maximum inotropic effect. *P < 0.01 vs. 25 μM free diC₈; † and ‡ significant difference among all groups revealed by ANOVA (P = 0.00003 and 0.00004, respectively); §significant difference between PMA alone and PMA + 25 μM C20:4 revealed by unpaired t-test (100 nM PMA vs. PMA + 25 μM C20:4, $P = 0.0019; 1 nM PMA vs. PMA + 25 μM C20:4, P = 0.0003; and 0.25 nM PMA vs. PMA + 25 μM C20:4, P = 0.047).

---

We next turned our attention to the involvement of PKC in the negative inotropic response of ventricular myocytes to diC₈, which has been called into question (5, 36, 40). Indeed, in our hands none of the three PKC inhibitors used above was able to block the inhibitory response to bath-applied diC₈ (Fig. 3). This observation suggests the possibility of additional mechanisms of inhibition by this neutral lipid besides via PKC. It may also explain why cis-UFA did not synergistically enhance the negative inotropic response to diC₈. To address this concern, we used phorbol esters because there is good evidence that negative inotropic responses to phorbol esters are mediated by PKC. We examined the response of cardiac myocytes to the widely used PMA with and without cis-UFA. As expected, PMA alone caused a concentration-dependent

---

**Fig. 2. Synergistic effects of arachidonic acid (AA) plus bath-applied diC₈ on myocyte twitches and Ca²⁺ transients. A: diC₈ and AA were added to the bath by perfusion of the myocyte stimulation chamber. Representative twitch amplitude (top traces) and corresponding fluo 3 fluorescence records (bottom traces) are shown for 3 individual cells before and 5 min after lipid treatments. To facilitate comparison of records from different cells, twitch records were normalized and plotted as a percentage of diastolic cell length (%max). Fluorescence signals were calibrated and converted to intracellular Ca²⁺ concentration ([Ca²⁺]) as described previously (28). Cell 1: control (C) and 25 μM diC₈. Cell 2: control and 25 μM AA. Cell 3: control and 25 μM diC₈ + 25 μM AA. B: summary of lipid effects on twitches (left) and Ca²⁺ transients (right). Cell shortening data (twitches) are presented relative to control arbitrarily set at 100%. Data represent means ± SE for n = 11 myocytes. By paired t-test: *P < 0.05; **P < 0.01 vs. control. By one-way ANOVA lipid treatments were all different with P < 0.05.
negative inotropic response (Fig. 4A and Table 1). At 100 nM PMA, twitch amplitude was inhibited by 40 ± 5%, whereas at 1 nM PMA it was inhibited by only 17 ± 4% (Table 1). These inhibitory effects of PMA were effectively blocked by PKC inhibitors (Fig. 4B), confirming the involvement of PKC. Inclusion of a subthreshold dose of arachidonic acid (25 μM) converted the 1 nM PMA negative inotropic response into a positive response (Table 1); this response was also blocked by PKC inhibitors (not shown). At the higher concentration of 100 nM PMA, arachidonic acid significantly attenuated the inhibitory effect of PMA but could not convert it into a positive inotropic response (Table 1).

Interestingly, at the lower dose of 0.25 nM PMA, the response to PMA alone was not inhibitory but was instead stimulatory, resulting in a 42 ± 13% increase in twitch amplitude (Table 1). Such a biphasic concentration dependence for PMA, with positive inotropic effects at low concentrations and negative inotropic effects at high concentrations, was reported previously (40). Inclusion of arachidonic acid at the very low PMA concentration of 0.25 nM enhanced the positive inotropic response to PMA (Fig. 4C and Table 1). This response to the combination of PMA and arachidonic acid was mediated by PKC because treatment with Go-6976 or BIM blocked the response (Fig. 4D). Overall, these results suggest that synergistic activation of PKC either by PMA/arachidonic acid or by diC8/arachidonic acid favors positive inotropic over negative inotropic mechanisms in isolated cardiac myocytes.

To further confirm that PKC was the primary mediator of the synergistic effects of diC8/arachidonic acid, we carried out Western blot analysis of PKC translocation. The three main isoforms present in these cells were PKC-ε, PKC-α, and PKC-δ. In Fig. 5A, blots with isoform-specific antibodies revealed that 25 μM diC8 alone typically caused only a small degree of translo-
cation, and 25 µM arachidonic acid alone was essentially ineffective at causing PKC translocation. However, the combined action of activators caused a substantial translocation of PKC-ε from the cytosol to particulate fractions (Fig. 5A). Similarly, translocation of PKC-α was minimal with diC₈ or arachidonic acid alone but was quite robust when diC₈ and arachidonic acid were combined (Fig. 5A). Translocation of PKC-δ in response to these lipid combinations was much more subtle (Fig. 5A) and, in fact, was not statistically significant (Fig. 5B and C). Redistribution of PKC-ε was the most pronounced of the three isoforms investigated with ~33% of total PKC-ε being lost from the cytosol (Fig. 5C). Concomitantly, 19% of the total PKC-ε appeared in the filament fraction and the remaining 14% in the membrane fraction (Fig. 5C). PKC-α moved primarily from cytosol (11% of total) to the membrane fraction (10% of total) (Fig. 5C). PKC translocation is thought to be an index of PKC activation (albeit an indirect one). Measurements of this phenomenon in rat cardiac myocytes revealed synergistic effects of cis-UFA and diC₈ on ε- and α-isoforms of PKC.

**DISCUSSION**

The main findings of this study are that cis-UFA and diacylglycerol synergistically increase intracellular Ca²⁺ transients and contractility in adult rat cardiac myocytes. PKC appeared to mediate the inotropic effects of this combination of lipid messengers because PKC antagonists blocked these effects. Moreover, the translocation of PKC from cytosol to particulate fractions responded synergistically to these lipids. It is well documented that diacylglycerol and cis-UFA can synergistically activate PKC in vitro (37), so the simplest interpretation of our results is that diacylglycerol and fatty acids interact directly with PKC to bring about these cellular changes. The precise protein substrates targeted by PKC activated in this manner remain to be identified. However, we also found that intracellular Ca²⁺ transients were increased in parallel with the positive inotropic effect, suggesting regulation of Ca²⁺ homeostasis. A similar conclusion was reached in studies of the positive inotropic action of diacylglycerol alone (31). In several studies, the L-type Ca²⁺
channel responsible for carrying trigger Ca$^{2+}$ for excitation-contraction coupling has been identified as a major target of PKC (8, 11, 17, 30).

A variety of extracellular stimuli cause diacglycerol and arachidonic acid to be elevated in parallel in cardiac tissues. Angiotensin II treatment of neonatal myocytes caused diacglycerol elevation via PLC and arachidonic acid production via PLA$_2$, possibly by activating separate receptor subtypes (20). With regard to adrenergic receptors, it is well established that $\alpha_1$-receptors couple to PLC and generate diacglycerol, whereas $\beta_2$-receptors can couple to PLA$_2$, leading to formation of arachidonic acid (29). Thus extracellular norepinephrine may produce the necessary intracellular combination of lipid comessengers by simultaneously activating separate receptor subtypes (20). With regard to mechanical perturbations (35) or oxygen stress (1, 9) can lead to activation of complex signaling cascades that produce both diacylglycerols and arachidonic acid in ventricular myocytes. The results of the present study suggest that costimulation of PLC and PLA$_2$ to produce diacglycerol and arachidonic acid in the heart will increase the probability of switching on PKC isoforms that mediate positive inotropic responses.

These observations shed light on a long-standing controversy over whether PKC mediates negative or positive inotropic responses in cardiac muscle. Recent work has demonstrated that the nature of the inotropic response to PKC activation depends on a number of experimental parameters including lipid species, lipid concentration, and how lipids are applied (6, 13, 26, 30, 40). For example, the diacglycerol analog diC$_8$ gives a negative inotropic effect when applied in the bath surrounding the myocytes but gives a robust positive inotropic response when released intracellularly from a caged compound (30). Phorbol esters such as PMA typically give strong inhibitory effects on contraction (8, 24, 30, 36, 40), but a systematic concentration dependence revealed stimulatory effects at very low concentrations (40). A robust stimulatory effect of PMA has also been reported (21). Finally, fatty acids alone can serve as cell-permeable PKC activators (2, 38) promoting positive inotropic responses in cardiac myocytes (6, 13). Overall, it is apparent that PKC can mediate either a positive or negative inotropic response in ventricular myocytes. The reasons for this are not entirely clear; however, the present work demonstrates that when a cis-UFA is part of the activating lipid signal the response is positively inotropic in nature.

MacLeod and Harding (21) suggested that treatment of isolated myocytes with growth factors during isolation or during dye loading could alter signaling mechanisms and the nature of the response to phorbol esters. Lasley et al. (18) showed that pretreatment of cardiac tissues with adenosine can alter the response to PKC activators. In this regard, we were careful to avoid exposure of myocytes to hormones (e.g., insulin), growth factors (e.g., serum), or other agonists (e.g., adenosine) during isolation and handling. The observation reported here that cis-UFA can convert one type of inotropic response into another may provide an explanation for how hormones, growth factors, and other agents might alter the response to PKC activators. If such extracellular agents mobilize intracellular cis-UFA, negative inotropic responses could be attenuated or converted into positive inotropic responses following subsequent treatment with diacyglycerol analogs or phorbol esters.

Several reports have indicated that metabolites of arachidonic acid can activate PKC or may participate in certain inotropic responses (Ref. 6 and the references therein). Arachidonic acid is thought to be quite stable and not subject to extensive metabolism in cardiac tissues (39), and the positive inotropic response to arachidonic acid is not influenced by inhibition of cyclooxygenase (13). In the present work, the sensitizing effect of arachidonic acid on the diC$_8$ response was not unique to arachidonic acid but was shared by other cis-UFA including oleic acid, linoleic acid, and linolenic acid. These results suggest that synergy is due to the unique “kinked” conformation of cis-UFA and is not dependent on metabolism of arachidonic acid or other cis-UFA to oxygenated species. However, we cannot completely rule out the possibility that lipoxygenase or cytochrome P-450 metabolites contribute to the observed responses.

How might cis-UFA convert inhibitory cellular responses into stimulatory responses by PKC activation? In the case of diC$_8$, the inhibitory response appears to be at least partly PKC independent. Therefore, cis-UFA may simply sensitize the cellular PKC to the existing level of diC$_8$, with the ensuing PKC-dependent positive inotropic response overcoming the inhibitory effects of diC$_8$. Another possibility is that the unique actions of cis-UFA may be related to their ability to influence the subcellular localization of PKC. Studies with green fluorescent protein (GFP)-tagged PKC in rat basophilic leukemia cells indicated that diC$_8$ or PMA alone induces translocation of PKC predominantly to the plasma membrane (28). Inclusion of arachidonic acid with diC$_8$, however, results in PKC being associated with intracellular structures (28). Consistent with this finding, PKC-\(e\) responded to arachidonic acid by anchoring preferentially to cardiac myofilament structures near the Z line rather than at the surface membrane (13). The ability of arachidonic acid and other cis-UFA to change the localization of PKC may contribute to its ability to convert one type of physiological response into another.

The combined actions of cis-UFA and diC$_8$ may also offer some selectivity at the level of which PKC isoform is activated. Western blot analysis revealed synergistic effects of these lipids on both PKC-\(e\) and PKC-\(\alpha\). Translocation was most pronounced for PKC-\(e\), less for PKC-\(\alpha\), and not statistically significant for PKC-\(\delta\). On this basis, we postulate that PKC-\(e\) and PKC-\(\alpha\) are geared toward producing positive inotropic effects in cardiac myocytes. This apparent PKC isoform selectivity contrasts with the finding that 100 nM PMA caused translocation of all three isoforms (\(e\), \(\alpha\), and \(\delta\)) to the particulate fraction by >80% (not shown). Overstimu-
lation of all PKC isoforms may be partly responsible for negative inotropism in this system.

In a recent study in a tumor cell line (38), synergism between diC₈ and arachidonic acid caused redistribution of GFP-tagged PKC-γ but not PKC-ε, in apparent contradiction with our results. A variety of factors may influence PKC translocation, including the lipid composition of membranes and the nature of anchoring proteins in various cellular compartments. Therefore, the effects of diC₈/arachidonic acid in one cell type may not translate to others. Moreover, there is considerable evidence that PKC-ε can be synergistically activated by diC₈/cis-UFA in vitro (16, 37, 33), so our finding of PKC-ε translocation in myocytes in response to these lipids is reasonable.

Phorbol esters and diacylglycerol analogs are powerful and widely used tools for studying PKC function in cells. Our results reinforce the need to exercise caution when using these reagents in cardiac myocytes. Phorbol esters may not initiate the same types of cellular mechanisms and physiological changes that endogenous pathways regulate, especially at high concentrations. It is also important to note that the inhibitory response to diC₈ may be fundamentally different from the negative inotropic response to PMA. Two previous reports (5, 36) suggested that diC₈ blocks L-type Ca²⁺ channel activity by a PKC-independent mechanism. The data presented here are consistent with this view. However, some of the actions of bath-applied diC₈ may be due to PKC because diC₈ does initiate some (albeit weak) PKC redistribution, and its negative inotropic effect was blocked by adenosine or BIM treatment in one study (24).

In conclusion, we have used the cell-permeable PKC activators diC₈, PMA, and cis-UFA to demonstrate both negative and positive inotropic effects of PKC on electrically paced rat ventricular myocytes. These contractile effects were mediated at least in part by changes in the cellular machinery that controls the intracellular Ca²⁺ transient. In accordance with earlier reports, diC₈ and PMA predominantly produced inhibitory responses when applied alone in the bath surrounding the myocytes. However, when combined with subthreshold doses of cis-UFA, both diC₈ and PMA gave robust positive inotropic responses (even when applied in the bath). Because diacylglycerol and cis-UFA are naturally occurring lipid messengers that can be elevated simultaneously in cardiac myocytes, the data suggest that these lipids may serve as messengers capable of activating PKC isoforms that promote stimulatory (rather than inhibitory) changes in cardiac Ca²⁺ homeostasis and contractility.

We thank Dr. XuPei Huang for insightful discussions and Seth Robia and Kara Kemnitz for comments on the manuscript.

This work was supported by National Heart, Lung, and Blood Institute (NHLBI) Grant P01-HL-47053. Y. Q. Pi received a Postdoctoral Fellowship from the American Heart Association-Wisconsin Affiliate, and J. W. Walker received a Career Development Award from NHLBI Grant K04-HL-03119.

REFERENCES

20. Lokuta AJ, Cooper C, Gaa ST, Wang HE, and Rogers TB. Angiotensin II stimulates the release of phospholipid-derived...


