Phorbol Ester and Dioctanoylglycerol 
Stimulate Membrane Association of Protein 
Kinase C and Have a Negative Inotropic 
Effect Mediated by Changes in Cytosolic Ca\(^{2+}\) 
in Adult Rat Cardiac Myocytes

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We used left ventricular myocytes from adult rats to investigate the effect of 4\(\beta\)-phorbol 12-myristate 13-acetate (PMA) and of sn-1,2-dioctanoylglycerol (DiC-8) on the membrane association of protein kinase C (PKC), cytosolic [Ca\(^{2+}\)], (Ca\(_\text{a}\)) homeostasis, and the contractile properties of single cardiac cells. Because PKC activity is known to be highly Ca\(^{2+}\) sensitive, the K\(^+\) concentration of the bathing medium was raised from 5 to 30 mM in some experiments, a perturbation known to depolarize the cell and increase Ca\(_a\). In cell suspensions both PMA (3\(\times\)10\(^{-10}\) and 3\(\times\)10\(^{-7}\) M) and DiC-8 (10\(^{-5}\) and 10\(^{-4}\) M) increased membrane association of PKC. The effect of PMA (10\(^{-7}\) M) on PKC translocation was enhanced in 30 mM KCI compared with 5 mM KCl. During steady field stimulation at 1 Hz in 1 mM bathing [Ca\(^{2+}\)], both PMA (10\(^{-7}\) M) and DiC-8 (10\(^{-5}\) M) decreased twitch amplitude to approximately 60% of control in 5 mM KCl, and the negative inotropic effect of either drug was more pronounced in 30 mM KCl than in 5 mM KCl. In single cardiac myocytes loaded with the Ca\(^{2+}\) indicator indo-1 and bathed in 5 mM KCl, we simultaneously measured cell length and Ca\(_a\). The myofilament responsiveness to Ca\(^{2+}\) was assessed by the relation between contraction amplitude and the peak of the Ca\(_a\) transient. The negative inotropic effect of both PMA and DiC-8 was related to a diminished amplitude of the Ca\(_a\) transient and not to a decreased myofilament responsiveness to Ca\(^{2+}\). In the absence of electrical stimulation, PMA (10\(^{-7}\) M) and DiC-8 (10\(^{-5}\) M) decreased the frequency of contractile waves due to spontaneous Ca\(^{2+}\) release from the sarcoplasmic reticulum, and DiC-8 also decreased resting Ca\(_a\). Thus, activation of PKC, which is thought to occur as part of the response of cardiac muscle to \(\alpha_1\)-adrenergic stimulation, is associated with a negative inotropic action due to a smaller Ca\(_a\) transient rather than to a decrease in the myofilament responsiveness to Ca\(^{2+}\). These effects on the membrane association of PKC and on contractility are enhanced by cell depolarization achieved by raising [KCl] in the bathing medium. (Circulation Research 1990;66:1143–1155)

Studies in a variety of tissues have shown that \(\alpha_1\)-adrenergic stimulation causes the hydrolysis of phosphatidylinositol 4,5-bisphosphate in the plasma membrane and leads to the production of two second messengers, inositol 1,4,5-trisphosphate (IP\(_3\)) and 1,2-diacylglycerol (DAG).\(^{1,2}\) Phosphoinositide metabolism also occurs in the heart during \(\alpha_1\)-adrenergic stimulation,\(^{3-5}\) but it is unclear whether IP\(_3\), DAG, or their metabolites play a role in modulating myocardial cell function.

An effect of IP\(_3\) to increase myofilament sensitivity to Ca\(^{2+}\) has been shown in skeletal muscle\(^6\) but not in the heart,\(^9\) and it is still questionable whether IP\(_3\) releases Ca\(^{2+}\) from the cardiac sarcoplasmic reticulum (SR).\(^9,13\)

The effect of DAG is even less understood. DAG increases the Ca\(^{2+}\) and phospholipid affinity of protein kinase C (PKC), thus promoting the activation of

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this enzyme.\textsuperscript{14,15} Tumor-promoting phorbol esters, which can substitute for DAG and directly activate PKC,\textsuperscript{15} have been used to study the functional sequelae of PKC activation in cardiac muscle. A negative inotropic effect of 4\beta-phorbol 12-myristate 13-acetate (PMA) has been shown in cultured chick\textsuperscript{16} and rat\textsuperscript{17} myocardial cells and in rat papillary muscles,\textsuperscript{18} and both PMA and DAG analogues decrease contractility in the perfused beating rat heart.\textsuperscript{19} In contrast, a recent study\textsuperscript{6} has shown that the phorbol ester phorbol 12,13-dibutyrate has no effect on the contractile force of rat papillary muscles.

Two general mechanisms through which the contractility of the cardiac myocytes can be decreased involve either a decrease in the amplitude of the cytosolic [Ca\textsuperscript{2+}], (Ca\textsubscript{a}) transient associated with the contraction or a diminution in the myocardial contractile response to Ca\textsubscript{a}. The effect of DAG and PMA on these mechanisms is unknown. Specifically, although there have been reports of the ability of PMA to decrease resting Ca\textsubscript{a} in suspensions of adult myocytes\textsuperscript{18} and time-averaged Ca\textsubscript{a} in spontaneously contracting embryonic chick cultured cells,\textsuperscript{16} the Ca\textsubscript{a} transient occurring in response to electrical stimulation has not been studied, and no Ca\textsubscript{a} measurements of any type have been reported after DAG administration. Similarly, there have been no studies of whether DAG or PMA decreases contractile strength by diminishing myofilament responsiveness to Ca\textsuperscript{2+}.

We used the single myocyte preparation from adult rats, which is free from contamination with other cell types, to study the effect of PMA and of a DAG analogue, sn-1,2-dioctanoylglycerol (DiC-8), on PKC translocation, on contractility, and on Ca\textsubscript{a} at rest and during electrical stimulation. From the simultaneous measurement of cell length and Ca\textsubscript{a} during the twitch, we could assess the myofilament responsiveness to Ca\textsuperscript{2+}. Additionally, since the mechanisms that underlie the negative inotropic effect of the DAG/PKC pathway are likely activated during \alpha\textsubscript{1}-adrenergic stimulation of the heart and since a negative contractile effect of \alpha\textsubscript{1}-adrenergic stimulation indeed occurs under conditions that favor enhanced cell Ca\textsuperscript{2+} loading,\textsuperscript{20,21} we repeated some of the above studies after Ca\textsubscript{a} was raised either by increasing the bathing [Ca\textsuperscript{2+}] (Ca\textsubscript{a}) or by adding KCl to the superfusate.\textsuperscript{22}

Some aspects of this work have been presented in abstract form.\textsuperscript{23–25}

\textbf{Materials and Methods}

\textbf{Myocyte Isolation Procedure}

Left ventricular myocytes were enzymatically dissociated, and their contractile properties were assessed, as previously described.\textsuperscript{26} Briefly, 2–4-month-old male Wistar rats from the Gerontology Research Center Colony were killed by decapitation; the heart was quickly removed and retrogradely perfused with 25 ml of a nominally Ca\textsuperscript{2+}-free buffer of the following composition (mM): NaCl 116.4, NaHCO\textsubscript{3} 26.2, NaH\textsubscript{2}PO\textsubscript{4} 10.1, KCl 5.4, MgSO\textsubscript{4} 0.8, and d-glucose 5.5. This medium was not recirculated and was continuously gassed with 95% O\textsubscript{2}-5% CO\textsubscript{2} to keep the pH at 7.35±0.05. Temperature was 36±1°C. The perfusate was then switched to a similar solution to which collagenase and CaCl\textsubscript{2} had been added to achieve a final concentration of 160 units/ml and 60 \muM, respectively. After 20–40 minutes of perfusion with this medium, the left ventricle was isolated, and single cardiac myocytes were mechanically disaggregated and resuspended in a bicarbonate buffer with 1.8 mM Ca\textsubscript{a}.

\textbf{PKC Assay}

The viability of myocytes used for PKC assay was determined by microscopic observation 30 minutes after resuspension of the cells in 1.8 mM Ca\textsubscript{a}. Myocytes were considered viable when they had a rod-shaped appearance and showed no evidence of blebs or granulations. Only cell suspensions with a viability greater than 70% were used for PKC determination.

Myocytes used in assessing subcellular distribution of PKC were suspended at 8×10\textsuperscript{4} to 2×10\textsuperscript{5} cells/ml in Earle’s balanced salt solution containing 1 mM MgCl\textsubscript{2}, 1 mM CaCl\textsubscript{2}, 15 mM glucose, and 0.5% bovine serum albumin in siliconized 25-ml Erlenmeyer flasks and gassed with 95% O\textsubscript{2}-5% CO\textsubscript{2} at 37°C. After treatment with PMA (Sigma Chemical, St. Louis, Missouri) or DiC-8 (Avanti Polar Lipids, Birmingham, Alabama), 0.8–1.0 ml cell suspension was pelleted by centrifugation in a microfuge (Beckman Instruments, Fullerton, California) for 3–5 seconds, the medium was removed, and cells were quickly suspended and homogenized with a Teflon-glass homogenizer at 10\textsuperscript{4} cell/ml in cold buffer containing 0.25 M sucrose, 20 mM N\textsubscript{2}-2-hydroxyethylpiperazine-N\textprime\prime-2-ethanesulfonic acid (HEPES), 5 mM ethyleneglycol-bis-(\beta-aminoethly ether)-N,N,N\prime,N\prime\prime-tetraacetic acid (EGTA), 5 mM dithiothreitol (DTT), 1 mg/ml fatty acid-free bovine serum albumin (Miles Laboratories, Elkhart, Indiana), and 50 \muM/ml leupeptin (Sigma Chemical) (pH 7.5). The homogenate was centrifuged at 27,000g for 15 minutes at 4°C; the cytosolic supernatant was saved, and the particulate fraction was resuspended in an equal volume of homogenizing buffer. Both cytosolic and particulate fractions were treated with 0.4% Triton X-100 for 1 hour on ice and then diluted with extraction buffer (1:3) immediately before assay. PKC activity was assayed by measuring Ca\textsuperscript{2+} and phospholipid-dependent transfer of 32P from [\textsuperscript{32}P]ATP (Du Pont/New England Nuclear, Boston, Massachusetts) to histone H1. The reaction mixture contained 25 mM HEPES, 10 mM MgCl\textsubscript{2}, 5 mM DTT, 20 \muM histone, 40 \muM [\textsuperscript{32}P]ATP (0.5 \muCl), and 0.5 mM EGTA plus or minus 2.5 \muM phosphatidyserine (Avanti Polar Lipids), 50 ng diolein, 0.75 mM CaCl\textsubscript{2}, and 10 \mul of sample in a total volume of 100 \mul (pH 7.5). Phosphorylated histone H1 was isolated and measured as previously described.\textsuperscript{7} PKC activity was calculated as the increase in activity produced by lipids and excess Ca\textsuperscript{2+} above activity observed with EGTA alone.
Translocation of PKC (i.e., redistribution from cytosol to membranes) was measured by determining the ratio of particulate activity/total activity (total activity is soluble plus particulate activity).

All values are reported as mean±SEM.

Contrastive Measurements

After the dissociation procedure, 1 ml cell suspension was plated in each of several 35-mm plastic Petri dishes that had been pretreated with a medium supplemented with 5% fetal calf serum to favor attachment of only the viable myocytes. To measure cell length, a Petri dish was placed on the stage of an inverted microscope equipped with phase-contrast optics, and the cells were continuously superfused with HEPES buffer of the following composition (mM): NaCl 137, MgSO4 1.2, CaCl2 1, KCl 5, HEPES 20, and d-glucose 16 (pH 7.4). In some experiments, CaCl2 was increased to 4 mM or KCl was increased to 30 mM. Solutions were preheated to keep the temperature in the bath between 35° and 37° C. Two platinum electrodes placed in the bathing fluid and connected to a stimulator (model SD9, Grass Instrument, Quincy, Massachusetts) were used to field-stimulate the myocyte to twitch with pulses of 2–4 msec. A TV camera (model WV-1850, Panasonic, Matsushita Communication Industrial, Yokohama, Japan) was used to project the image of the individual cell under study to a monitor (model WV-5200, Panasonic), and changes in cell length were quantified through edge-motion detection by use of a video dimension analyzer (model 303, Instrumentation for Physiology and Medicine, San Diego, California). The signal was then transmitted to a chart recorder (Brush 220, Gould, Cleveland, Ohio) and to a computer (model RD5500, Raytheon, Santa Ana, California) for on-line analysis of peak twitch amplitude. The extent of shortening was expressed as percent of the resting cell length (ES%). All averaged data are reported as mean±SEM.

Simultaneous Measurements of Cell Length and Ca2+

In another series of experiments, cell length and Ca2+ were measured simultaneously as recently described.27 Briefly, dissociated myocytes bathed in HEPES-buffered medium were loaded at 23° C with the ester derivative (AM form) of the Ca2+ probe indo-1,28 dissolved in dimethylsulfoxide and mixed with fetal calf serum and a dispersing agent (Pluronic F-12, BASF Wyandotte, Wyandotte, MI).29 Experiments with these cells always started at least 1 hour after loading. Previous studies have shown that after exposing the cells to indo-1 AM there may be changes in the Ca2+ transient and in the twitch that reach steady state 30–60 minutes after washout of the indicator.27 Indo-1 fluorescence was excited by epi-illumination with 10-μsec flashes of 350±5 nm light. Paired photomultipliers collected indo-1 emission by simultaneously measuring spectral windows of 391–434 and 457–507 nm selected by bandpass interference filters. The ratio of indo-1 emission at the two wavelengths was calculated as a measure of Ca2+ by using a pair of fast integrator sample-and-hold circuits under the control of a VAX 11/730 computer. Cell length was monitored from the bright field image of the cell, which was projected onto a photodiode array (1024SAQ/RC1024 LNA Starlight, Reticon, Sunnyvale, California) with a 5-msec time-scan rate. By using red light (650–750 nm) for the bright field image and a dichroic mirror (600-nm shortpass) to transmit the fluorescent light (395–510 nm), cell length and Ca2+ were measured simultaneously without cross talk. Because leakage of the Ca2+ indicator from the cells is favored when the temperature is raised between 23° and 36° C,27 these experiments were done at room temperature.

It has recently been shown that, when cardiac myocytes are loaded with the ester derivative of indo-1, significant compartmentalization of the indicator occurs in the mitochondria.27 Because the degree of compartmentalization is not identical in all myocytes, this prevents the use of a standard calibration curve to determine the absolute value of Ca2+. For this reason, the indo-1 signals shown in this study are not calibrated; their purpose is to show directional changes in indo-1 fluorescence ratio, which is taken as an indicator of Ca2+.

Statistical Analysis

Statistical analysis was performed by either paired t test or analysis of variance. A value of p<0.05 was taken to indicate significance.

Results

Effects of PMA and DiC-8 on PKC Translocation

Translocation of PKC in rat myocytes caused by PMA is shown in Figure 1. Measurements of the ratio of particulate/total activity were obtained after a 10-minute exposure to the drug, a time that in preliminary experiments had proven sufficient for maximal membrane association of PKC. The total activity of PKC averaged 24.5 pmol/min/106 cells, and PMA (10−7 M) caused a threefold increase in P/S+P, with negligible loss of activity and over 60% of total PKC present in the particulate fraction; no further enhancement of the response was obtained at higher concentrations of PMA (not shown).

Figure 2 shows the time course of the membrane association of PKC in response to 10−7 M PMA. In 5 mM KCl, PKC translocation increases rapidly within the initial 30 seconds of exposure and appears to plateau within 5 minutes. At high [KCl], the initial rate of membrane association of PKC is more rapid, and at 10 minutes, particulate/total activity is significantly greater than in the lower [KCl]. Time controls at both values of [KCl] indicate that, in the absence of PMA, particulate/total activity does not vary with time. The inactive PMA analogue 4β-phorbol (10−9–10−7 M) had no effect on PKC translocation (not shown).
DiC-8 was also tested to determine whether it induced membrane association of PKC in rat cardiac myocytes. In five experiments, PKC translocation was measured in control and 10 minutes after exposure to either $10^{-5}$ or $10^{-4}$ M DiC-8 in 5 mM KCl. Total PKC activity (10.2 pmol/min/10^6 cells) was unchanged 10 minutes after exposure to $10^{-4}$ or $10^{-5}$ M DiC-8 or $10^{-3}$ M PMA. The ratio of particulate/total activity was 0.089±0.011 in control and increased to 0.126±0.011 at $10^{-3}$ M DiC-8 ($p<0.01$) and to 0.213±0.011 at $10^{-4}$ M DiC-8 ($p<0.01$).

**Contractile Studies**

Figure 3A shows the effect of $10^{-7}$ M PMA on the twitch in a representative single cardiac myocyte not loaded with indo-1. After exposure to the drug, there is a rapid decrease in twitch amplitude. The lower traces in the same panel show twitches at fast chart speed obtained at the time indicated in the upper continuous tracing. Figure 3B shows the average effect of $10^{-7}$ M PMA on contraction in cells bathed in either 1 or 4 mM Ca_{o} and in control baths at 1 mM Ca_{o}. The relative magnitude and time course of the negative inotropic effect of PMA are similar at the two values of Ca_{o} and there is no time effect of steady field stimulation on twitch amplitude. Under conditions of higher cell Ca_{o} loading, obtained by increasing [KCl] to 30 mM in the bathing medium, the effect of PMA on the twitch is enhanced (Figure 4). The upper tracing of Figure 4A shows the marked negative effect of PMA on twitch amplitude in a representative rat myocyte continuously stimulated in a buffer containing 30 mM KCl. The lower tracings in Figure 4A show individual twitches at a fast chart speed obtained in control and when the effect of the drug was maximal (see legend to Figure 4). Figure 4B shows average results for cells electrically stimulated at 1 Hz in 30 mM KCl and 1 mM Ca_{o}. PMA ($10^{-7}$ M) causes an average decrease in twitch amplitude to approximately 30% of control, and this is already maximal after a 5-minute exposure to the drug. Comparison of this result with that from cells studied under similar conditions but in 5 mM KCl (continuous line with open circles) shows that at the higher [KCl] the effect of the PMA is faster and of greater magnitude. Figure 4B also shows that the inactive PMA analogue 4β-phorbol does not have an effect on contractility and that twitch amplitude does not vary with time under these conditions in the absence of PMA.

The effects of DiC-8, a membrane-permeant diacylglycerol, on the twitch of single cardiac myocytes is shown in Figures 5 and 6. The experiments in Figure 5 were in 5 mM KCl, and both the tracings from a representative cell (panel A) and the average data (panel B) show that DiC-8 ($10^{-5}$ M) has a negative inotropic effect of magnitude and a time course similar to that of $10^{-7}$ M PMA in cells studied under similar conditions. Figure 6 shows that in single cardiac myocytes, regularly stimulated at 1 Hz in 30 mM KCl, DiC-8 ($10^{-5}$ M) reduces twitch amplitude with a time course and to an extent similar to that obtained with PMA under similar conditions (Figure 4). This figure also shows that the decrease in twitch amplitude determined by DiC-8, unlike that of PMA, is at least partially reversible with washout of the drug.

From the data presented so far it is evident that both PMA and DiC-8 have a negative inotropic effect on adult rat cardiac myocytes. In the subsequent experiments, we have used cells loaded with the Ca^{2+} probe indo-1 to assess whether the changes in Ca_{i} underlie the effect of PMA and DiC-8 on twitch amplitude. Figure 7 shows that the negative inotropic
FIGURE 2. Graph showing effect of $10^{-7} \text{M} \text{4B-phorbol 12-myristate 13-acetate}$ on the time course of the translocation of protein kinase C (PK-C) in suspensions of rat left ventricular myocytes bathed in either 5 mM KCl ($\bullet$; n=6) or 30 mM KCl ($\triangle$; n=4). The points at 10 minutes were obtained from 13 and 11 determinations made in 5 and 30 mM KCl, respectively. As in Figure 1, total PK-C activity remained stable throughout the course of the experiment. Statistical analysis of the effect of 4B-phorbol 12-myristate 13-acetate, across all time points, at 5 and 30 mM KCl by a two-way repeated-measures mixed model analysis of variance shows a significant difference between the two groups ($p=0.0107$). A separate analysis at each point in time (one-way analysis of variance) shows a significant difference (asterisks) at 30 seconds ($p=0.0192$), at 1 minute ($p=0.0067$), and at 10 minutes ($p=0.0015$). Time controls in 5 mM KCl ($\text{■}; n=14$) and 30 mM KCl ($\text{□}; n=11$) show no change in the ratio of particulate activity soluble plus particulate activity ($P/S + P$).

action of PMA is associated with a diminution in the amplitude of the Ca$_i$ transient without an effect on either the time course of the contraction or of indo-1 fluorescence (panel C). This figure also shows that PMA decreases the diastolic value for the 410/490 nm ratio of indo-1 fluorescence and that it increases diastolic cell length. In similar experiments with indo-1 loaded myocytes, the effect of DiC-8 on the amplitude of the Ca$_i$ transient, on the diastolic and peak systolic values for Ca$_i$ and cell length, and on the time course of both the light and length signals (Figure 8) is comparable with that described for PMA in the previous figure. DiC-8 decreases the systolic and diastolic 410/490 nm ratio of indo-1 fluorescence and twitch amplitude; diastolic cell length increases without a significant change in the duration of the Ca$_i$ transient or the twitch. Thus, a decrease in the amplitude of the Ca$_i$ transient underlies the negative inotropic effect of PMA and DiC-8.

The other general mechanism that could contribute to the negative effect of PMA and DiC-8 is a decrease in the myofilament responsiveness to Ca$^{2+}$. To evaluate this possibility, contractions and Ca$_i$ transients of different amplitude, before and after exposure to either PMA or DiC-8, were obtained by continuous field stimulation of the myocyte in varying Ca$_o$ (not shown). Alternatively, stimulation was resumed after a period of rest, an intervention that in rat myocardial tissue produces a descending staircase in contractility and in Ca$_o$ (Figure 9A). Because both PMA and DiC-8 have a negative inotropic action, the range of Ca$_o$ used in the presence of these agents must be higher than in control. This method makes it possible to obtain a similar spectrum of Ca$_i$ transients before and after exposure to PMA or DiC-8. Subse-
Because different mechanisms are responsible for Ca$_i$ homeostasis during the twitch and at rest, when cardiac myocytes have a membrane potential that is more negative than $-70$ mV, we determined the effects of PMA and DiC-8 on spontaneous contractile waves in the absence of electrical stimulation. In the unstimulated state, Ca$_{i}^{2+}$-tolerant rat myocytes bathed in physiological Ca$_{o}$ exhibit spontaneous localized Ca$_{i}^{2+}$ release from the SR, which then diffuses along the length of the cell and induces further Ca$_{i}^{2+}$ release from the SR. The consequent localized Ca$_{i}^{2+}$ myofilament interaction produces a band of contracted sarcomeres that propagates along the long axis of the myocyte as a contractile wave. Although the molecular mechanisms that initiate spontaneous Ca$_{i}^{2+}$ release are unknown, it is clear that the frequency of spontaneous SR Ca$_{i}^{2+}$ oscillations can be increased by perturbations that
raise Ca\textsuperscript{2+}\textsuperscript{2,31-34} or that alter SR Ca\textsuperscript{2+} release (e.g., caffeine).\textsuperscript{30}

Figure 10 depicts the effect of PMA on the frequency of spontaneous contractile waves in the absence of field stimulation. Panel A shows tracings from a representative rat myocyte in 4 mM Cao, and panel B shows the time course of the average effect of PMA on wave frequency in either 1 or 4 mM Cao. The phorbol ester reduces wave frequency to 50% of control, and as is the case for twitch amplitude (see Figure 3B), both the time course and the magnitude of the effect are similar at 1 and 4 mM Cao. This figure also shows that in the absence of the drug the frequency of spontaneous contractile waves does not vary with time.

Under similar experimental conditions, DiC-8 had an effect comparable with that of PMA. In the unstimulated state, in 1 mM Cao, the frequency of the spontaneous contractile waves decreases to 65.0±6.17% of control 10–20 minutes after exposure to 10\textsuperscript{-5} M DiC-8 (n=4; wave frequency in control was 3.1±0.9/min). In indo-1 loaded rat myocytes after addition of DiC-8, there is a decrease in Cao and a small increase in cell length (Figure 11).

**Discussion**

Our data show that both PMA and DiC-8 stimulate membrane association of PKC in rat cardiac myocytes and that, during cell depolarization with high [KCl], the effect of PMA on the rate and association of cytosolic PKC onto a membrane fraction is enhanced. In normal [KCl], PMA and DiC-8 have a significant negative inotropic effect on the electrically stimulated twitches: this action is attributable to a smaller Cao transient and not to a decrease in myofilament responsiveness to Ca\textsuperscript{2+}. In higher [KCl], the negative effect of PMA and DiC-8 on twitch amplitude is enhanced. In the unstimulated state, both PMA and DiC-8 decrease the frequency.
of spontaneous SR Ca\(^{2+}\) oscillations, which in single cardiomyocytes are manifest as spontaneous contractile waves; DiC-8 also decreases Ca\(_{\text{c}}\). Thus, two agents that determine membrane association of PKC in cardiac muscle have a negative inotropic effect.

It should be noted that PMA, in the dosage range studied, has a far greater effect on the membrane association of PKC than does DiC-8. The translocation by 10\(^{-5}\) M DiC-8 is much less than that caused by 10\(^{-7}\) M PMA, yet 10\(^{-5}\) M DiC-8 has a negative inotropic effect of magnitude and time course similar to that of 10\(^{-7}\) M PMA. The most likely cause for this discrepancy may reside in a relatively weak and reversible membrane association of PKC caused by DiC-8, in contrast to a tight and irreversible association known to be caused by PMA.\(^{35}\) This may result in an underestimate of in situ PKC membrane association caused by DiC-8 and preclude a strict correlation between PKC translocation by DiC-8 and PMA and their effect on contractility.

Other possibilities could be considered. The physiological response to PMA and DiC-8 might saturate at levels of PKC translocation below the maximal level achieved with 10\(^{-7}\) M PMA. Alternatively, it is possible that only one of different PKC subspecies\(^{36}\) may be responsible for the physiological response described in this paper. In this case, biochemical determinations of PKC levels without identification of the different kinases involved could mask a strong correlation between the biochemical and physiological response to PMA and DiC-8.

It is noteworthy that the negative inotropic action of PMA and DiC-8 on twitch amplitude and the effect of PMA on the membrane association of PKC are augmented after exposure to high [KCl], a perturbation known to increase cell Ca\(^{2+}\) through depolarization of the cells. This effect of KCl could be

FIGURE 9. Tracings and plots showing effect of 4\(\beta\)-phorbol 12-myristate 13-acetate (PMA) and sn-1,2-diacetyl glycerol (DiC-8) on myofilament responsiveness to Ca\(^{2+}\). This was assessed as the relation between the percent of resting cell length (ES%) and peak systolic 410/490 nm ratio of indo-1 fluorescence for twitches obtained before and after exposure to the drug. Panel A: Example of one of the protocols used to obtain contractions and Ca\(_{\text{c}}\) transients of different amplitude (see text). In control, this representative rat myocyte was stimulated from rest at 0.5 Hz in 0.5 mM Ca\(_{\text{c}}\) (left tracing). The stimulation protocol was repeated in the presence of 10\(^{-5}\) M DiC-8 in 0.5 mM Ca\(_{\text{c}}\) (middle tracing) and in 4 mM Ca\(_{\text{c}}\) (right tracing). It is noteworthy that DiC-8 had a negative inotropic effect that was overcome by raising Ca\(_{\text{c}}\). When Ca\(_{\text{c}}\) transients of the same amplitude in the absence and presence of the drug are selected, the contraction is enhanced in DiC-8 (e.g., the second Ca\(_{\text{c}}\) transient in the left tracing is similar to the fifth Ca\(_{\text{c}}\) transient in the right tracing). These Ca\(_{\text{c}}\) transients and the associated twitches are superimposed in the right section of panel A. It is apparent that contractility is increased in the presence of DiC-8. Panels B-E: The effects of 10\(^{-7}\) M PMA and 10\(^{-5}\) M DiC-8 were assessed in six and four myocytes, respectively (C, control; X, PMA in panels B and C and DiC-8 in panels D and E). Both PMA and DiC-8 shifted to the left the relation between ES% and peak 410/490 nm indo-1 fluorescence in three and two myocytes, respectively (representative examples in panels B and D); in the remaining cells from each group, there was no effect (representative examples in panels C and E). Neither drug shifted to the right the relation between ES% and peak systolic ratio of indo-1 fluorescence, indicating that neither PMA nor DiC-8 causes a decrease in myofilament responsiveness to Ca\(^{2+}\) under our experimental conditions.

FIGURE 10. Tracings and graphs showing effect of 10\(^{-7}\) M 4\(\beta\)-phorbol 12-myristate 13-acetate (PMA) on spontaneous contractile wave frequency in the absence of electrical stimulation. Panel A: Upper tracing shows a decrease in wave frequency after addition of PMA in a representative rat myocyte in 4 mM Ca\(_{\text{c}}\). The two lower tracings are at a faster chart speed and were obtained before and after the addition of PMA at the times indicated in the upper tracing. Panel B: Time course of the average effect of PMA on wave frequency in rat myocytes in either 1 mM Ca\(_{\text{c}}\) (A; n=4; control wave frequency=3.5±0.4/min) or 4 mM Ca\(_{\text{c}}\) (C; n=17; control wave frequency=14.3±2.1/min). Time control in 1 mM Ca\(_{\text{c}}\) shows that spontaneous contractile wave frequency remains stable over the course of the experiment (●; n=3; wave frequency at time 0=2.3±0.96/min).
mediated by the increase in cell Ca\textsuperscript{2+} loading and the known dependence of PKC activity\textsuperscript{14} on free Ca\textsuperscript{2+} in the range of 10\textsuperscript{-7} to 10\textsuperscript{-5} M. However, the relative decrease in twitch amplitude and wave frequency by PMA were similar in 1 and 4 mM Ca\textsubscript{0}, suggesting that a higher degree of cell Ca\textsuperscript{2+} loading than that caused by increasing Ca\textsubscript{0} from 1 to 4 mM is required for the Ca\textsuperscript{2+} dependence of PKC activity to become evident. Alternatively, the marked changes in twitch amplitude observed in high [KCl] might be related to some other factor in addition to PKC activity.

Other recent studies have described a negative inotropic action of phorbol esters\textsuperscript{16-19} and DAG analogues\textsuperscript{19} in multicellular cardiac tissues. However, those preparations\textsuperscript{18,19} contain nerve terminals with stored catecholamines. Under those experimental conditions, the PMA and DAG analogues could have mediated desensitization of cardiac adrenoceptors,\textsuperscript{57} which might have contributed to the negative effects observed. In the perfused, beating rat heart, PMA increased the membrane association of PKC, and both PMA and DAG analogues decreased the force of contraction and coronary flow.\textsuperscript{19} Whether the negative inotropic effect was related to a direct myocardial action of the drugs or was mediated by hypoxia secondary to the decrease in coronary flow could not be ascertained from that study.

Here we have shown that a decrease in the Ca\textsubscript{i} transient underlies the negative inotropic effect of PMA and DiC-8 and that, in the unstimulated state, DiC-8 causes a diminution in Ca\textsubscript{i}. The mechanisms through which the decrease in Ca\textsubscript{i} occurs are still unclear. During electrical stimulation, an effect on the ionic currents that are activated during depolarization could contribute to the observed decrease in the Ca\textsubscript{i} transient. However, recent experiments\textsuperscript{17,38} with cultures of neonatal rat ventricular cells have yielded contrasting results on the effects of PMA on the L-type Ca\textsuperscript{2+} current. In one study,\textsuperscript{17} PMA increased the magnitude of both the transient and steady-state components of the Ca\textsuperscript{2+} current, even though it produced a negative inotropic effect. In the other study,\textsuperscript{38} PMA had a biphasic effect, initially stimulating and subsequently inhibiting Ca\textsuperscript{2+} channel activity, respectively, at 5 seconds and at 20 minutes after exposure to the drug. In the same study,\textsuperscript{38} DiC-8 increased Ca\textsuperscript{2+} influx both at 5 seconds and at 20 minutes. Similarly, contrasting reports on the effect of phorbol esters and DAG analogues on Ca\textsuperscript{2+} current have been described in other cell types. In Aplysia neurons, phorbol esters enhance Ca\textsuperscript{2+} current,\textsuperscript{39} probably through recruitment of a previously covert class of Ca\textsuperscript{2+} channels.\textsuperscript{40} In embryonic chicken dorsal root ganglion neurons, both a DAG analogue and a phorbol ester decrease the Ca\textsuperscript{2+} current,\textsuperscript{41} and a DAG analogue decreases both the T- and L-type currents in GH\textsubscript{3} cells.\textsuperscript{42} Finally, in myocardial tissue, PMA has been reported to increase the amplitude of the delayed rectifier K\textsuperscript{+} current.\textsuperscript{43} It is unclear whether this effect, by shortening the duration of the action potential, could decrease the amplitude of the Ca\textsubscript{i} transient.

The negative effect of PMA and DiC-8 on the Ca\textsubscript{i} transient associated with the twitch could also result from changes in resting Ca\textsubscript{i} and not require an effect on the membrane currents that become activated during the action potential. Indeed, during field stimulation, both PMA and DiC-8 decrease diastolic Ca\textsubscript{i} and increase diastolic cell length (Figures 7 and 8), and in the absence of electrical stimulation, DiC-8 decreases Ca\textsubscript{i} (Figure 11). A similar effect has been reported for PMA in suspensions of adult rat cardiac myocytes\textsuperscript{18} loaded with fura-2 AM as well as in some other cell types studied in the absence of depolarization.\textsuperscript{44-49} The decrease in resting Ca\textsubscript{i} could occur either through enhanced Ca\textsuperscript{2+} extrusion across the sarcolemma or increased SR Ca\textsuperscript{2+} uptake. In cardiac sarcolemma, PKC phosphorylates, among other proteins,\textsuperscript{50,51} a protein with electrophoretic characteristics similar to phospholamban in the SR;\textsuperscript{52} this could be responsible for modulating Ca\textsuperscript{2+} pumping activity across the membrane. Whether such a protein represents a contaminant from the SR is still under debate.\textsuperscript{50} Additionally, a study in neutrophils relates the change in cell Ca\textsuperscript{2+} induced by PMA to stimulation of an ATP-dependent Ca\textsuperscript{2+} transport in the plasma membrane.\textsuperscript{53} PKC has also been shown to phosphorylate the SR and to stimulate Ca\textsuperscript{2+} uptake by this organelle.\textsuperscript{54} This finding has later been confirmed,\textsuperscript{55} and the protein phosphorylated has been identified as phospholamban. However, an increase in SR Ca\textsuperscript{2+} content would lead to a greater Ca\textsubscript{i} transient during the electrically stimulated twitch, a result opposite to what has been observed (Figures 7 and 8). Thus, increased Ca\textsuperscript{2+} extrusion across the sarcolemma that overrides any enhancement in SR Ca\textsuperscript{2+} uptake appears the most likely mechanism for the decrease in contractile wave frequency and in resting Ca\textsubscript{i} and for the negative inotropic action of PMA and DiC-8. A recent report\textsuperscript{56} has shown that in rat papillary muscle PMA

![Figure 11. Tracings showing effect of 10\textsuperscript{-5} M sn-1,2-dioctanoylglycerol (DiC-8) on Ca\textsubscript{i} as the 410/490 nm ratio of indo-1 fluorescence and on cell length in the absence of field stimulation in a representative rat myocyte in 1 mM Ca\textsubscript{0}. After addition of DiC-8, there is a decrease in Ca\textsubscript{i} and a minor increase in cell length.](http://circres.ahajournals.org/lookup/doi/10.1161/01.RES.66.4.1152)
reduces intracellular Na⁺ activity and increases Ca²⁺ efflux in rat myocytes. These effects, which could result from activation of the Na⁺-K⁺ pump and/or Na⁺-Ca²⁺ exchange, may provide an explanation for the negative action of PMA.

Our results also exclude a decreased myofilament responsiveness to Ca²⁺ after exposure to PMA or DiC-8 as a cause for the negative inotropic action of these drugs. PKC has been shown to phosphorylate cardiac troponin I and T,⁵⁷ and although the physiological significance of phosphorylation of troponin T is unknown, there is evidence to suggest that phosphorylation of troponin I leads to a decrease in the myofilament sensitivity to Ca²⁺.⁵⁸⁻⁶¹ In many nonmyocardial tissues, PKC activates Na-H exchange,⁶²⁻⁶⁴ with extrusion of H⁺ ions from the cell, secondary intracellular alkalinization, and accumulation of intracellular sodium ions. Such an increase in intracellular pH, which has been shown to occur also in rat ventricular myocytes loaded with a fluorescent pH indicator and exposed to PMA and a DAG analogue,⁶⁵ would lead to an increase in myofilament sensitivity to Ca²⁺⁶⁶ and cause the well-known positive inotropic effect of alkalosis in cardiac muscle.⁶⁷ However, it should be considered that, if phosphorylation of troponin I and intracellular alkalosis both occurred after exposure to PMA or the DAG analogue, their opposing effects on myofilament sensitivity to Ca²⁺ could balance each other and prevent an increase in myofilament responsiveness to Ca²⁺ (Figures 9C and 9E). The variation in effect shown in Figure 9 could be due to a difference in intracellular pH among cells, and further studies are required to elucidate this problem.

Because PKC activation occurs during α-adrenergic stimulation of the myocardium,⁶⁸ it is of interest to discuss the experimental results described in this paper in the context of the known physiological effects of α-agonists on myocardial contractility. Although the positive inotropic action of α-adrenergic stimulation has been extensively described and characterized,⁶⁻⁷⁰⁻⁷⁵ several reports have indicated that α-adrenergic agonists can also have a persistent negative inotropic action.²⁰,²¹,⁷⁵ The positive effect of α₁-adrenergic stimulation appears secondary, at least to a considerable extent, to an increase in the myofilament sensitivity to Ca²⁺⁶⁹,⁷⁰,⁷⁶ and does not reflect only an augmentation of the Ca⁺ transient; contrasting reports have suggested either an enhancement⁷¹ or no effect⁷²,⁷³ of α₁-stimulation on the slow inward Ca²⁺ current (Iᵢ). However, regardless of the cause of the positive effect of α₁-receptor stimulation, it seems that under certain conditions a negative feedback loop may predominate. A transient decrease in developed force is known to occur as part of the response of myocardial tissue to stimulation of α₁ receptors⁷⁴: it precedes the increase in contractility, and this short-lived decrease in developed force can be prevented by preexposure of the tissue to PMA.⁶,¹⁸ A persistent negative effect of α-adrenergic stimulation has been described in rabbit papillary muscles stimulated at high frequency,⁷⁵ in rat hearts bathed in high Ca₀²¹ and, more recently, in single cardiac myocytes in high Ca₀.²⁰ Our results (the present study and Reference 20) and the findings of others¹⁶⁻¹⁹,²¹,⁷⁵ are consistent with the hypothesis that, during α₁-adrenergic stimulation of cardiac muscle, at least two pathways may be activated with opposite effects on contractility. As cell Ca²⁺ loading exceeds a set point before α₁-adrenergic stimulation, the mechanism that has a negative effect on the inotropy of the heart and that could relate to activation of the Ca²⁺-sensitive PKC by DAG may predominate over the mechanism that enhances contractility; thus, an explanation would be provided for reports²⁰,²¹,⁷⁵ of a negative contractile effect of α₁-adrenergic stimulation of the myocardium under conditions that tend to increase cell Ca²⁺ loading.

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**KEY WORDS** • diocanoylglycerol • phorbol ester • \(\alpha_1\)-adrenergic stimulation • cardiac myocytes • cytosolic Ca\(^{2+}\) • protein kinase C
Phorbol ester and dioctanoylglycerol stimulate membrane association of protein kinase C and have a negative inotropic effect mediated by changes in cytosolic Ca2+ in adult rat cardiac myocytes.

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