Tumor-Promoting Phorbol Esters Inhibit Cardiac Functions and Induce Redistribution of Protein Kinase C in Perfused Beating Rat Heart

Shaohua Yuan, Fred A. Sunahara, and Amar K. Sen

Activation of protein kinase C has been implicated in the regulation of a variety of cellular reactions. Although we, and others, have found protein kinase C and its substrate proteins to be present in both membrane and cytosolic fractions in the heart, the physiologic role of this kinase in the regulation of cardiac functions remains unknown. In the present study, we found that in isolated perfused rat heart, administration of phorbol esters 4/3-phorbol 12,13-dibutyrate (PDBu) and 12-0-tetradecanoyl-phorbol 13-acetate (TPA), which are specific activators of protein kinase C, produced marked dose-dependent negative changes in inotropy and chronotropy. A dose-dependent decrease in coronary flow was also observed. The diacylglycerol analogues, 1,2-oleoylacetate-glycerol and 1,2-dioctanoyl-glycerol, produced similar effects as the active phorbol esters on these isolated perfused hearts. An inactive analogue of phorbol ester, 4a-phorbol, failed to produce any effect. Protein kinase C activity in both membrane and cytosolic fractions prepared from rat heart could be activated by TPA and PDBu at the same concentration range as used in the experiments with perfused hearts. Following perfusion of the hearts with PDBu, a rapid translocation of protein kinase C from cytosolic to membrane fractions was also observed. Our findings provide the first direct evidence that protein kinase C may play a potentially important role in the regulation of cardiac functions. (Circulation Research 1987;61:372–378)
Isolation and Perfusion of Rat Hearts

Experiments were carried out on male Wistar rats (210–290 g, Charles River, Canada). The animals were anesthetized with ether and heparin was administered (2,000 U i.v.). The heart was quickly excised and chilled in normal saline solution at 4°C. Within 30 seconds after excision, the heart was perfused by the Langendorff method as described previously using a modified Krebs-Ringer bicarbonate solution containing (in mM) NaHCO₃ 25, NaCl 118, KCl 5.36, NaH₂PO₄ 1.17, MgSO₄ 1.2, CaCl₂ 1.25, and glucose 11.1 at 35°C gassed with 95% O₂ -5% CO₂. The container with the perfusate was placed at a level above the heart to achieve a pressure of 50 mm Hg. A low resistance needle valve was placed in the inflow line to establish an initial 10 mm Hg gradient between the container and the heart. The change of the coronary flow was reflected by a change in differential pressure, which was measured by a Statham P23AC transducer at high gain. The apex of the ventricle was attached to a Grass FT03C strain gauge at an initial tension of 2 g to record the force of contraction and the derived tension of the rate of ventricular contraction (dT/dt) on a Grass 7 PCPA polygraph. Also, changes in heart rate were recorded with a Grass CP4A cardiotachometer.

Preparation of Membrane and Cytosolic Fractions

Hearts were isolated and perfused with normal saline solution containing 0.1% ethanol (control) or phorbol esters in 0.1% ethanol for the time indicated in individual figure legends. Then, they were quickly thawed and homogenized in 20 ml of buffer A (20 mM Tris/HCl, 0.25 M sucrose, 5 mM EDTA, and 0.005% Triton X-100). The homogenate was centrifuged at 14,000g for 10 minutes, and the resulting supernatants were saved as cytosolic fraction, frozen in liquid nitrogen, and stored at −70°C until use. The supernatants were rehomogenized in 20 ml of buffer A (1.0–1.5 mg/ml), frozen in liquid nitrogen, and stored at −70°C until use.

(Na⁺,K⁺)ATPase activity (as a plasma membrane marker) in the membrane fraction was 18.4 ± 1.39 μmol P/mg protein/hr (mean ± SEM, n = 3), which represents sixfold to sevenfold increase compared with that in the homogenates (2.5 ± 0.1 μmol P/mg protein/hr, mean ± SEM, n = 3).

Assay of Protein Kinase C Activity

Protein kinase C activity was determined by a modification of the method of Katoh and Kuo as described previously. Protein kinase C activity was assayed by measuring the incorporation of ³²P from [³²P]ATP into lysine-rich histone. Sixty micrograms membrane protein were incubated for 1 hour on ice with 0.075% Triton X-100 (vol/vol) in buffer A in a final volume of 100 μl. Following this preincubation period, protein kinase C was assayed in the presence of 10 mM MgCl₂, 10 mM dithiothreitol, 8.8 mM KH₂PO₄, 30.4 mM NaHPO₄, 50 μg histone, and 0.5 mM Na₂[³²P]ATP (0.8–1.2 × 10⁶ cpm) with or without 0.5 mM CaCl₂, 10 μg PS, and different concentrations of phorbol esters in a final volume of 250 μl. Protein kinase C activity in the cytosolic fraction was detected without any preincubation with Triton X-100. The incubation was carried out at 30°C for 5 minutes and was terminated by the addition of 5 ml of 10% trichloroacetic acid. The incubation solution was filtered through a Scatron Filtermates 7031 12-well cell harvester (Skatron Inc., Sterling, Va.). The filter papers were washed twice each with 5 ml trichloroacetic acid solution, were put into scintillation vials containing 10 ml aquasol, and were counted in a liquid scintillation spectrometer. Kinase activity was expressed as pmol P/mg protein/min. Specific protein kinase C activity was calculated by subtracting the protein kinase activity in the presence of 0.5 mM CaCl₂ alone from the kinase activity in the presence of 0.5 mM CaCl₂, 10 μg PS, and 1 μM TPA.

(Na⁺,K⁺)ATPase activity assay was assayed according to Post and Sen. The final millimolar concentrations in the assay mixture were NaCl 100, KCl 25, MgCl₂ 4.0, Na₂ATP 3.0, and imidazole-glycylglycine 30 (pH 7.6) with or without ouabain 5.0. Twenty micrograms protein were incubated for 10 minutes at 37°C in a final volume of 1.0 ml. The difference in the amount of P, liberated in the presence and absence of ouabain was taken as (Na⁺,K⁺)ATPase activity, which was expressed as mmol P/mg protein/hr.

Protein concentration was determined according to Lowry et al using bovine serum albumin as standard.

Results

Effect of Phorbol Esters and DAG Analogues on Cardiac Functions in Perfused Beating Rat Heart

Figure 1 shows that when hearts were continually perfused with PDBu 10⁻⁶ M at a perfusion rate of 0.1 ml/min, both coronary flow and heart rate decreased within 1 minute, reached a minimum in about 5 minutes, and persisted over the 20-minute perfusion
FIGURE 1. Time course of effect of PDBu on rat cardiac functions. Rat hearts were prepared as described in "Materials and Methods" and were constantly perfused with PDBu 10^{-6} M at perfusion rate of 0.1 ml/min for 20 minutes. Control values were heart rate, 237.0 ± 6.9 beats/min; coronary flow, 6.0 ± 0.5 ml/min; developed tension of contraction, 7.3 ± 0.3 g/heart; dT/dt max, 141.4 ± 11.0 g/sec. Each point represents mean ± SEM, n = 5.

period. However, the force of contraction and dT/dt diminished within 1 minute, reached a minimum at 2.5 minutes, and then recovered gradually. The force of contraction and dT/dt recovered to about 80 and 50%, respectively, at 7 minutes and persisted at this stage during the remainder of the perfusion period.

The dose-dependent effects of phorbol esters on isolated perfused rat heart are shown in Figure 2. When hearts were perfused with TPA or PDBu at concentrations ranging from 10^{-7} to 10^{-4} M at a perfusion rate of 0.1 ml/min for 5 minutes, a dose-dependent negative chronotropy (Figure 2A), a negative inotropy (Figure 2C), and coronary vasoconstriction (Figure 2B) were observed. With the above changes, the rate of ventricular contraction (dT/dt) was also decreased (Figure 2D). The effect of TPA started gradually during the 5-minute perfusion, and its inhibitory effect did not recover after the drug perfusion was stopped. In contrast, after perfusion with PDBu for 5 minutes, all of the changes in cardiac function recovered to control level after the drug perfusion was stopped for 5--20 minutes, depending on the dose used (data not shown). 4α-Phorbol (an inactive analogue) did not inhibit cardiac functions at the same concentration range as TPA and PDBu (Figure 2).

Since phorbol esters are not naturally present, we decided to examine the effect of DAG analogues on cardiac function. The results are shown in Figure 3. The DAG analogues OAG and DiC8 mimicked the effects of tumor-promoting phorbol esters. DiC8 and OAG at concentrations from 10^{-5} M to 6.0 × 10^{-5} M at a perfusion rate of 0.1 ml/min for 5 minutes significantly inhibited the force of ventricular contraction and dT/dt. Heart rate and coronary flow were also
Possible Role of Protein Kinase C in Cardiac Functions

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Effect of DAG analogues on cardiac functions in perfused beating rat heart. Rat hearts were prepared and perfused as described under "Materials and Methods." Diagrams show effect of DAG analogues OAG (-----) and DiC8 (-- — ) on heart rate (A), coronary flow (B), force of contraction (C), and dT/dt (D). Control values were heart rate, 202.3 ± 12.5 beats/min; coronary flow, 6.1 ± 0.28 ml/min; force of contraction, 8.0 ± 0.7 g/heart; dT/dt max, 109 ± 11.8 g/sec. When hearts were perfused with vehicle DMSO (0.8%) at perfusion rate of 0.1 ml/min for 5–20 minutes, a change of 1–3% from control values was observed. All points were measured after hearts were perfused with OAG or DiC8 at indicated concentrations for 5 minutes. Each point represents mean ± SEM, n = 3.

Activation of Protein Kinase C Activity by Phorbol Esters in Rat Heart Membrane and Cytosolic Fractions In Vitro

To demonstrate further that the changes in cardiac function induced by phorbol esters were mediated through the activation of protein kinase C, the effect of phorbol esters on protein kinase C activity in vitro was also tested. After hearts were perfused with the vehicle (0.1% ethanol) for 10 minutes, they were freeze-clamped, and the membrane and cytosolic fractions were prepared. The results shown in Figure 4 demonstrate that TPA and PDBu, at the same concentration range used in the perfusion experiments, activated protein kinase C in a concentration-dependent manner. Activation of protein kinase C by these two tumor-promoting phorbol esters occurred in both the membrane (Figure 4A) and cytosolic fraction (Figure 4B). Figure 4 also shows that inactive analogue 4α-phorbol did not activate protein kinase C to any significant extent.

Table 1 shows the distribution of protein kinase C in rat heart membrane and cytosolic fractions. As we reported previously in canine cardiac tissue, the specific activity of protein kinase C in rat heart membrane fraction was higher than that in the cytosolic fraction; also, the total protein kinase C activity in the cytosol was higher (1,880.0 pmol P/min) compared with that in the membrane fraction (440.0 pmol P/min).

Redistribution of Protein Kinase C Activity in Membrane and Cytosolic Fractions Following PDBu Perfusion of Rat Heart

Figure 5 shows that after hearts were perfused with PDBu (10⁻⁶ M), protein kinase C specific activity in the membrane of isolated rat heart increased significantly by about 50% within 3 minutes. There was an equally rapid decrease in cytosolic protein kinase C activity. Protein kinase C activity in the membrane increased by

Figure 4. Activation of protein kinase C activity by phorbol esters in rat heart membrane and cytosolic fractions in vitro. Rat hearts were perfused with 0.1% ethanol for 10 minutes and then were quickly freeze-clamped. Membrane and cytosolic fractions were prepared and protein kinase activity was assayed as described in "Materials and Methods." Different concentrations of TPA, PDBu, and 4α-phorbol were used as indicated in x axis. Panel A: effect of phorbol esters on membrane fraction. Panel B: effect of phorbol esters on cytosolic fraction. Values are mean ± SEM, n = 4.
Protein kinase C activity in the homogenates alone. Each value represents mean ± SEM, n = 3.

Table 1. Distribution of Protein Kinase C in Rat Heart Membrane and Cytosolic Fractions

<table>
<thead>
<tr>
<th></th>
<th>Membrane</th>
<th>Cytosol</th>
</tr>
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<tbody>
<tr>
<td>Total protein (mg)</td>
<td>5.6 ± 0.7</td>
<td>37.0 ± 3.2</td>
</tr>
<tr>
<td>Kinase activity (pmol P/mg protein/min)</td>
<td>48.9 ± 9.6</td>
<td>22.4 ± 8.3</td>
</tr>
<tr>
<td>Control</td>
<td>50.0 ± 6.2</td>
<td>23.5 ± 7.7</td>
</tr>
<tr>
<td>+Ca²⁺, PS, TPA</td>
<td>124.3 ± 9.3*</td>
<td>74.4 ± 7.2*</td>
</tr>
<tr>
<td>Specific activity (pmol P/mg protein/min)</td>
<td>74.3</td>
<td>50.9</td>
</tr>
<tr>
<td>Total activity (pmol P/min)</td>
<td>440.0</td>
<td>1,880.0</td>
</tr>
<tr>
<td>Total activity (%)</td>
<td>19</td>
<td>81</td>
</tr>
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*p < 0.001, †p < 0.05, n = 4.

Discussion

Experiments in the present study clearly demonstrate that tumor-promoting phorbol esters significantly inhibit cardiac functions in perfused spontaneously beating rat heart. The negative inotropy, the negative chronotropy, and coronary vasoconstriction induced by PDBu and TPA were dose dependent, occurred at nM–μM concentration range, and started very rapidly (within 1 minute). Furthermore, the cell-membrane-permeable DAGs also mimicked the effects of the active phorbol esters. The in vitro studies showed that the activation of protein kinase C activity by PDBu and TPA was very similar and correlated well with the doses required to the observed effects in vivo. In addition, the inactive analogue, 4a-phorbol, neither activated protein kinase C in vitro nor produced any effect in the perfused beating heart. Our experiments strongly suggest that the activation of protein kinase C by tumor-promoting phorbol esters could regulate cardiac functions.

Previous experiments have shown that tumor-promoting phorbol esters and several hormones induced the translocation of protein kinase C in several nonexcitable tissues and cell lines. In most cases, the translocation results in a decrease in the enzyme in the cytosolic fraction and an increase in the membrane fraction. It seems that the distribution ratio of this kinase varies with physiologic state of the tissue and relates with the early events of hormonal stimulation. In our experiments, we observed a rapid translocation of protein kinase C from the cytosol to the membrane after rat hearts were perfused with PDBu. Tumor-promoting phorbol ester-induced redistribution of the kinase in our experiments may also be responsible for mediating the inhibitory effect on cardiac functions in perfused hearts. It appears that there is a good correlation between the redistribution of protein kinase C and the decrease in heart rate. However, such correlation does not exist for the other functional changes observed following PDBu perfusion. If protein kinase C activation and redistribution is related to cardiac contraction, then it is necessary to postulate that the partial activation of protein kinase C may be sufficient to produce the observed negative inotropic effect. The reason for the reversed process of negative inotropic effect of PDBu is not clear; the prolonged perfusion of phorbol ester may result in some autocrine mechanism.

It has been realized recently that a major function of protein kinase C in a variety of tissues is related to a down regulation or feedback control of cell-surface receptors. This may be an important function of protein kinase C in the heart. It seems that the observed inhibitory effects of phorbol esters and DAGs might also be due to their direct effect on excitation–relaxation cycle or through a negative feedback control on Ca²⁺ mobilization in cardiac cells.

Protein kinase C has been implicated in the alteration of Ca²⁺ and myofibrillar protein interactions in the heart. Katoh et al have found that protein kinase C is the only kinase so far known to phosphorylate both cardiac troponin inhibitory subunit (troponin I) and troponymosin-binding subunit (troponin T) in vitro. Phosphorylation of troponin I is known to decrease the affinity of troponin complex for Ca²⁺. It has also been found that C protein of myocardial thick filament
was phosphorylated by protein kinase C, and that this phosphorylated C protein had a decreased effect in stimulating actin-activated myosin ATPase activity. However, since the native protein kinase C (M, 78,000) is activated only in the presence of phospholipid, it may not be functional in the cytosol. The formation and release of the soluble, irreversibly activated form of protein kinase C from the membrane may account for the phosphorylation of cytosolic proteins in the heart as has been shown in platelets treated with TPA.

Although protein kinase C may act through phosphorylation of the myofibrillar proteins to produce negative inotropic effect, activated protein kinase C in the particulate fraction of cardiac tissue may also account for the effect of tumor-promoting phorbol esters in the perfused heart. The latter effects may be through the phosphorylation of transmembrane proteins such as channels, pumps, and ion exchange carriers in sarcolemma, sarcoplasmic reticulum (SR), and other organelles. It has been reported that in cardiac SR, protein kinase C phosphorylated a Mr 22,000 protein, phospholamban, resulting in an activation of (Ca²⁺, Mg²⁺)ATPase and an increase in Ca²⁺ uptake from cytosol to SR. Recent studies in some other cell types have shown that the activation of (Ca²⁺, Mg²⁺)ATPase by protein kinase C may be responsible for the rapid extrusion of Ca²⁺ after it mobilizes into the cytosol. Ca²⁺ channels in the cardiac sarcolemma and SR could also be modulated by protein kinase C as has been clearly shown in some other cells. Indeed, preliminary experiments reported by Leatherman et al showed that TPA produced a negative inotropic effect in the cultured chick embryo ventricular cells, which was accompanied by reduced Ca²⁺ influx rate. Hansford et al and Capogrossi et al reported recently that tumor-promoting phorbol ester inhibited the potassium chloride–induced increase in cell Ca²⁺ and the spontaneous release of Ca²⁺ from SR in rat cardiac myocytes. It will be important to correlate these functional changes with the changes in the phosphorylation–dephosphorylation state of the cardiac cells by protein kinase C. We and others have found several substrate proteins for protein kinase C to be present in cardiac sarcolemma.

The negative chronotropic effect of tumor-promoting phorbol esters could be due to the direct alterations in the electric activity of the SA node and the conduction system in the myocardium. More recently, Satoh and Hashimoto reported that TPA decreased the K⁺ conductance in rabbit SA node and inhibited Na⁺ inward current in canine Purkinje fibers. It will be very interesting to study further the effect of tumor-promoting phorbol esters on the cardiac electric activity and whether it could mimic to some extent the effect of parasympathetic stimulation.

The coronary arterial circulation is regulated by the complex mechanisms, which involve a variety of neural, hormonal, and local factors. Under normal conditions, however, the coronary flow is autoregulated according to the oxygen requirements of the cardiac cells. It is not clear whether the decreased coronary flow induced by phorbol esters in our experiments was due to the inhibited cardiac function. This seems unlikely based on the fact that PDBu-induced inhibition of the cardiac contractility recovered, but the coronary vasoconstriction persisted during the prolonged perfusion. Protein kinase C has been found to modulate the tonic phase of contraction in some vascular smooth muscles, but phorbol ester-induced coronary vasoconstriction in our experiments was rapid, suggesting that this vasoconstriction may act through a different mechanism. However, the mechanism is still unknown.

The role of protein kinase C in the cardiac functions remains largely unexplored. Since a number of hormones and neurotransmitters, as well as their related second messenger systems, appear to cause an increase in intracellular Ca²⁺, the inhibitory effect of protein kinase C on the cardiac functions reported here is of considerable interest. Many questions remain unanswered. For example, what role does protein kinase C play in the mechanism of action of α₁-adrenergic agonists and muscarinic cholinergic agonists? Both these agents are known to stimulate PI turnover, even though they produce opposite effects on cardiac function. Furthermore, even within the muscarinic agonists with similar functional effect on the heart, it has been reported that some stimulate PI turnover but others do not. And what role does other second messenger, such as cAMP, play in modulating the effect of protein kinase C, and vice versa? Thus, a clear understanding of these second messenger mechanisms will be important not only in the physiologic myocardial functions but also in the pathophysiologic states of the heart, such as cardiac failure, cardiac hypertrophy, and ischemic heart disease. Undoubtedly, the role of the second messenger signals generated by PI turnover in the heart will be a focus of cardiac research in the near future.

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