Phorbol Ester Increases Calcium Current and Simulates the Effects of Angiotensin II on Cultured Neonatal Rat Heart Myocytes

Ayse Dosemeci, Ravinder S. Dhallan, Neri M. Cohen, W.J. Lederer, and Terry B. Rogers

The effects of increased protein kinase C activity were studied in neonatal rat myocytes grown in primary culture. The changes in mechanical and electrical behavior, as well as protein phosphorylation, that followed the apparent activation of protein kinase C by the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) were examined. As spontaneous beating frequency was increased minimally by 10 nM TPA and by 100% with 85 nM TPA, shortening amplitude, shortening velocity, and relaxation velocity decreased concomitantly. In contrast, 4-α-phorbol-12,13-didecanoate (α-PDD), which does not activate protein kinase C, had no effect on beating behavior at 800 nM. In voltage-clamped single myocytes, both steady-state and transient components of the cadmium-sensitive calcium current were increased by the addition of TPA (65 nM). Neither the time constant for the inactivation of the transient component of this calcium current nor the reversal potential was altered by TPA. The phosphorylation state of a discrete set of proteins, with apparent molecular weights of 32 and 83 kDa, was enhanced when TPA was added to intact myocytes. Angiotensin II enhances the phosphorylation state of the same set of proteins as observed with TPA. We conclude that activation of protein kinase C can modify mechanical behavior and increase L-type Ca2+ channel activity in cultured neonatal rat ventricular myocytes. The remarkable similarity in mechanical, electrical, and protein phosphorylation responses of cultured neonatal myocytes following TPA or angiotensin II application indicate that protein kinase C may mediate the action of angiotensin II. (Circulation Research 1988;62:347–357)

Angiotensin II (angII) is a peptide hormone that stimulates the hydrolysis of phosphoinositides in several target tissues, including adrenal cortex, liver, and vascular smooth muscle. There is accumulating evidence that the heart is also a target tissue for angII. Recently, we have characterized the high affinity receptors for angII on cultured neonatal rat ventricular myocytes. AngII, in the nanomolar range, increases contractile frequency in spontaneously beating cultured cells and augments calcium current, I Ca, in voltage-clamped cells. AngII does not increase cAMP accumulation, but the hormone was found to stimulate phosphoinositide hydrolysis in these cultured cells. The functional significance of the latter observation, however, has not been assessed.

The stimulation of phosphoinositide hydrolysis by agonists is an important signal transduction mechanism in many tissues. Activation of this pathway leads to the production of multiple potential second messengers, including (1,4,5)inositol-trisphosphate (IP3) and 1,2-diacylglycerol (DAG). However, in many tissues, it is not clear how these and other parallel intracellular signals are integrated to produce the functional response. For example, both muscarinic and α-adrenergic agonists have been shown to stimulate phosphoinositide hydrolysis in adult rat ventricular myocytes; yet, the functional responses of cardiac tissues to the two classes of agonists are markedly different. There is relatively little information on the functional significance of these messenger molecules in the heart.

The present study was initiated to investigate underlying biochemical mechanisms of angII responses by examining the functional significance of one of the metabolites of the phosphoinositide pathway, DAG, the second messenger that activates protein kinase C. This particular class of protein kinase has been identified in heart tissue. The phorbol ester, 12-O-tetradecanoylphorbol-13-acetate (TPA), an activator of protein kinase C, has been used to mimic the effects of DAG in intact cardiac cultures. We report for the first time that TPA, like angII, stimulates cadmium-sensitive I Ca in voltage-clamped cultured neonatal rat myocytes and causes an increase in contractile frequency in these cells. Furthermore, TPA and angII stimulate endogenous protein phosphorylation into the same set of proteins (MW 32 and 83 kDa). These observations support the hypothesis that some of the effects of angII on cardiac cells are mediated by protein kinase C. A preliminary report of some of the results have been published recently.

Materials and Methods

Tissue Culture

Primary cultures of neonatal rat cardiac ventricular myocytes were prepared as previously described.
Briefly, the hearts of decapitated 1–2-day-old Sprague-Dawley rats were removed aseptically and minced. The cells were then isolated by successive cycles (3–4) of 0.025% trypsin digestion. The isolated cells were incubated in growth medium (Dulbecco’s Modified Eagle Medium [DMEM], 10% fetal calf serum, 1% penicillin, and 1% streptomycin) in a humidified atmosphere of 95% air–5% CO₂ at 37°C for 3–12 days. For both the protein phosphorylation and the contractile measurement experiments, cells were grown in four-well (1.7-cm) tissue culture dishes. For the electrophysiological experiments, cells were grown on plastic coverslips in 35-mm culture dishes.

**Measurement of Contractile Properties**

The contraction of a single cell in a spontaneously beating multicellular network was measured using an optical-video motion detection system as previously described.²² Briefly, the four-well culture dish was mounted on the stage of an inverted phase microscope, and the cells in a single well were superfused with control media (DMEM, 25 mM HEPES, 0.1% dimethylsulfoxide [DMSO], pH 7.4). The superfusion media was gassed with 95% O₂-5% CO₂ and maintained at 35 ± 0.3°C. The image of the beating cells was monitored using a video camera (Cohu, San Diego, California), and the video signal was recorded on tape and processed by a video dimension analyzer (Instrumentation for Physiology and Medicine, San Diego, California). The video dimension analyzer provides an analog output proportional to the position of the moving edge of a cell. The contraction and relaxation velocities were obtained by electronically differentiating the analog position versus time output of the video dimension analyzer as described previously.²³ In experiments in which the cells were driven at a constant beating rate, the cells were field-stimulated with 50-V pulses of 1-msec duration at frequencies indicated in the figure legends. For all the experiments, the cells were superfused with either control media, control media containing TPA (Sigma Chemical, St. Louis, Missouri), or control media containing 4-a-phorbol-12,13-didecanoate (a-PDD) (Sigma) as indicated. The phorbol esters were added from stock solutions in DMSO, and all of the superfusion media contained a final DMSO concentration of 0.1%. Control experiments indicated that this solvent (0.1%) had no effect on contractile behavior. Each culture well was exposed to a single dose of either TPA or a-PDD at the indicated concentration, and each experimental result was expressed as the mean of at least three trials.

**Measurement of I_{Ca}**

Cadmium-sensitive calcium currents, I_{Ca}, in single cultured neonatal rat heart cells were measured using the whole-cell voltage-clamp configuration as described previously.²² Briefly, plastic coverslips with adherent cells were removed from the culture dish 3–4 days after plating, transferred to the experimental chamber, and superfused at a flow rate of 1 ml/min with a modified Tyrode’s solution (in mM: NaCl 145, KCl 4, CaCl₂ 1, MgCl₂ 1, glucose 10, and HEPES 10, pH 7.36) containing 10 μM tetrodotoxin (TTX) (Sigma). TPA was applied in the same buffer containing 0.1% DMSO. Electrodes were pulled from borosilicate glass capillaries (1.5 mm o.d., World Precision Instruments, New Haven, Connecticut) to a tip diameter of approximately 1 μm. The electrodes had a resistance of 2–4 MΩ when filled with 140 mM cesium glutamate, 1 mM NaCl, and 10 μM EGTA. Possible voltage errors resulting from pipette series resistance were corrected for using dynamic series-resistance control compensation; however, 2–3 MΩ of uncompensated series resistance usually remained. Sodium channels were blocked by external TTX and potassium channels were blocked by internal cesium. Cadmium (0.1 mM) was added before the end of each experiment, and I_{Ca} was measured as the cadmium-sensitive current.

Analysis of the voltage-clamp experiments was performed as previously described.²³ The amplitude and time constant for inactivation of the transient component of I_{Ca} were measured by fitting the current records with a nonlinear least-squares method to a single exponential function.²³ Steady-state activation (d_a) was measured using a standard voltage protocol. The tail current (I_{tail}) was measured on returning to the holding potential (−50 mV) from a 10-msec step to a test potential (V_{test}). To measure the steady-state inactivation variable (f_a), a 200-msec prepulse to the test potential (V_{test}) was followed by a 10-msec step to the holding potential (−50 mV); then, the current (I_{tail}) elicited by a depolarization to the evaluation potential (+5 mV) for 200 msec was measured. To control for I_{r} rundown, all trials to V_{test} were alternated with trials to V_{hold} (the test potential that gives the largest current transient at the evaluation potential, I_{transient}), and the ratio of the resulting currents (I_{tail}/I_{transient}) was determined. The data for the steady-state activation (d_a) and inactivation (f_a) variables were fit to the following equations:

\[ d_a = 1 + \exp(-V - V_a/k) \]  
\[ f_a = 1 + \exp((V - V_a)/k) \]  

where V is the membrane potential, and V_a and k are constants.

**Protein Phosphorylation**

The effect of angII and TPA on protein phosphorylation in intact neonatal rat heart cells was examined using sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis of samples from cells that had been prelabeled with [³²P]orthophosphate. Culture medium was removed from the wells, and the monolayers were washed once with phosphate-free DMEM medium; then, 0.39 ml of the same medium was added that contained carrier-free [³²P]orthophosphate (New England Nuclear Research Products, Boston, Massachusetts) (4 μCi/well). The culture plates were incubated in a humidified atmosphere of 5% CO₂-95% O₂ at 37°C for 1.5–2 hours. After the preincubation with [³²P]orthophosphate, the indicated reagents were added...
to the wells. Both control and treated culture wells contained 0.1% DMSO in experiments with phorbol esters or 0.1 mM bacitracin in experiments with angII. Following mixing, the culture plates were returned to the incubator for the indicated time intervals. At the end of the incubation, [32P]-containing DMEM was removed by aspiration, and the cells were immediately solubilized in 0.2 ml of 2% SDS solution. The SDS-treated samples were recovered from wells, transferred to tubes, and stored overnight at -20°C. After thawing, they were incubated in a water bath at 85°C for 5 minutes, and aliquots were removed for protein estimation.24 The volumes of the solubilized samples were adjusted with 2% SDS to normalize the protein concentrations, and electrophoresis buffer was added in a ratio of 1:3 (vol/vol) of original sample to yield the following final concentrations of reagents: 2% SDS, 7.5% sucrose, 1% mercaptoethanol, and 60 mM Tris-HCl, pH 6.8.

**SDS Polyacrylamide Gel Electrophoresis**

A discontinuous SDS gel electrophoresis system described by Laemmli23 was used with minor modifications.9 The separating gel had a 7–17% linear gradient of acrylamide, and the stacking gel consisted of 3% acrylamide. Samples containing 20–50 μg of protein in 100 μl were applied. Following electrophoresis, the gels were stained in 0.125% Coomassie blue in methanol/acetic acid/water (55:7:43), destained in methanol/acetic acid/water (4:1:6), and dried under vacuum. The dried gels were exposed to Kodak X-Omat AR film for 2–3 days. After developing, the autoradiographs were scanned using a Guiford Response spectrophotometer. The apparent molecular weights of the electrophoretic bands were estimated using the following molecular weight standards: bovine serum albumin, rabbit muscle phosphorylase-b, β-lactoglobulin B, bovine carbonic anhydrase, α-glycerophosphate dehydrogenase, and fumarase.

**Two-dimensional Gel Electrophoresis**

Two-dimensional electrophoretic separation of proteins was performed in a “Mini-Gel” apparatus (Biorad, Richmond, California) adapting the methods of O’Farrell.26 Cells were labeled with [32P]orthophosphate at 14 μCi/well, otherwise as described above. The incubation was terminated by removing [32P]-containing medium and adding 0.1 ml of 1% SDS, 10% mercaptoethanol. The solubilized samples were recovered from wells, transferred to tubes, and stored overnight at -20°C. Separation on the first dimension was performed on isoelectric focusing (IEF) gels cast in capillary tubes (1 mm i.d., 7.5-cm long) containing 8 M urea, 2% ampholines (LKB) (1 part pH 3.5–10, and 3 parts pH 5–7). To each sample was added: 0.26 g urea; 100 μl 10% NP-40; 54 μl ampholines, pH 5–7; and 6 μl ampholines, pH 3.5–10; 200 μl glycerol. Aliquots (40 μl) of the sample preparation were applied to the isoelectric focusing gels and were overlayed with 20 μl of a solution containing 5 M urea, 2% NP-40, and 2% ampholines. The proteins were resolved on the gels at a constant voltage (500 V for initial 10 minutes and 1,500 V for another 2 hours). IEF gels were then removed from capillary tubes and were equilibrated for 30 minutes in an SDS reducing buffer (5% mercaptoethanol, 3% SDS, 0.01% bromophenol blue in 0.06 M Tris-HCl, pH 6.8). The second dimension was performed on SDS mini-slab gels with a resolving gel (6×8×0.1 cm) of 12% acrylamide concentration and a stacking gel (0.7×8×0.1 cm) of 4% acrylamide concentration.

**Results**

**Effect of TPA on Contractile Properties**

The initial focus of these experiments was to assess the possible significance of protein kinase C in regulating cardiac cell function. Accordingly, the effects of the phorbol ester TPA, a known activator of protein kinase C,20 were studied in spontaneously beating cultured neonatal rat myocytes. There was a significant increase in the beating rate of spontaneously contracting heart cells when the cultures were superfused with 80 nM TPA (Figure 1A). There also was a concurrent decrease in the amplitude of contraction, maximum velocity of contraction, and maximum velocity of relaxation. The average maximal increase in spontaneous beating in 3 experiments where 80 nM TPA was added was 86±12% (n=5, mean ± SEM) above control values. The average maximal decrease in amplitude was 46±6% below control values (n=5, mean ± SEM).
Figure 2. Time course for action of phorbol ester, TPA, on contractile activity of cultured neonatal rat myocytes. Spontaneous beating rate, amplitude of contraction, and velocities of contraction and relaxation were measured as described in "Materials and Methods." Spontaneously beating cells were exposed to TPA (80 nM) for period indicated by solid bar. Each point represents average value over 1-minute interval. Mean ± SEM). These effects were constant for at least 20 minutes after reaching a plateau. A threshold dose of 10 nM TPA was observed for these effects. In contrast, the phorbol ester α-PDD, which does not activate protein kinase C, had no effects on any of these parameters at doses as high as 800 nM (Figure 1B).

The rate of onset in the functional responses was studied in detail. Figure 2 shows a representative time course of the effect of 80 nM TPA on spontaneous beating frequency, twitch amplitude, twitch velocity, and velocity of relaxation. There was a lag time of 4.2 ± 0.8 minutes (n = 4) before the onset of the responses. In all experiments with TPA, the fall in the amplitude of contraction began at the same time and had the same time course of development as the increase in spontaneous beating rate. Further, the effects of TPA were not readily reversible. Typically, only after 30–60 minutes of washout of TPA (80 nM) would the spontaneous beating rate and twitch amplitude begin to return toward control levels (data not shown).

It is possible that the TPA-mediated decreases in both shortening velocity and shortening amplitude are functions of the increase in contractile frequency evoked by TPA. Accordingly, the effects of TPA were examined on cells that were electrically driven at a constant beating rate. As shown in Figure 3, TPA (80 nM) in electrically driven cells still caused a decrease in the amplitude of contraction, velocity of contraction, and velocity of relaxation with a time course and magnitude similar to its effect in spontaneously beating cells. These results demonstrate that the effect of TPA on twitch amplitude is independent of its effect on spontaneous beating frequency and is not simply due to a shift onto the declining phase of the force-frequency curve.

Effects of TPA on Calcium Current

One explanation for the increase in beating rate observed with TPA is that the calcium current, I_Ca, is elevated by the phorbol ester. Therefore, the effects of TPA on I_Ca, using the whole-cell voltage-clamp technique was examined. A sample record of the cadmium-sensitive current at 0 mV is shown in Figure 4A. To limit our measurement of I_Ca to the contribution of only L-type calcium channels, the holding potential was set to −50 mV. As previously reported, current records revealed both transient and steady-state components of I_Ca. TPA (65 nM) increased both components of I_Ca (Figure 4A). TPA does not, however, alter the voltage dependence of the time constant of inactivation (Figure 4B). TPA also does not affect the voltage dependence of either the transient or steady-state components of I_Ca (Figures 4C and 4D). Current-voltage plots in Figures 4C and 4D reveal that the shape of the current-voltage relation of both components...
FIGURE 4. Effect of phorbol ester on cadmium-sensitive $i_{\text{a}}$ in voltage-clamped cultured rat neonatal myocytes. Panel A: $i_{\text{a}}$ elicited by 200-msec depolarizing voltage step from holding potential of $-50$ mV to $0$ mV and is displayed as cadmium-sensitive current. Current records in absence (control) and presence of $65$ nM TPA are displayed. Panel B: Time constant of inactivation ($\tau$) of transient phase of $i_{\text{a}}$ determined as described in "Materials and Methods." Values for $\tau$ in control (•) and TPA-treated cells (A) are shown. Panel C: Effect of TPA (65 nM) on transient component of $i_{\text{a}}$-voltage relation using experimentally determined $\alpha$ and $\beta$ curves (see Figure 2). Panel D: Effect of TPA (65 nM) on steady-state component of $i_{\text{a}}$-voltage relation using experimentally determined $g_r$ and $g_c$ curves (see Figure 5). Panel E: Effect of TPA (65 nM) on $i_{\text{a}}$-voltage relation using Equation 1, with control, $g_r = 24$ nS; 65 nM TPA, $g_r = 30$ nS. Panel F: Effect of TPA (65 nM) on $i_{\text{a}}$-voltage relation using Equation 2, with control, $g_c = 25$ nS; 65 nM TPA, $g_c = 33$ nS.
remains unchanged in the presence of 65 nM TPA, and the reversal potential, 55 mV, is identical under control conditions and in the presence of TPA. However, it is clear that the magnitude of both the transient (I transient) and steady-state (I steady-state) components of I alpha are increased over the entire voltage range.

Previously, we have demonstrated that an appropriate relation to describe the transient and steady-state components of I alpha at a given test potential (V m) is:

\[ I_{\alpha}^{\text{transient}}(V_m) = g_{\alpha} \times (V_m - E_{\text{channel}}) \times d_{\alpha}(V_m) \times (1 - f_{\alpha}(V_m)) \]  

(3)

\[ I_{\alpha}^{\text{steady-state}}(V_m) = g_{\alpha} \times (V_m - E_{\text{channel}}) \times d_{\alpha}(V_m) \times f_{\alpha}(V_m) \]  

(4)

where \( g_{\alpha} \) is a relative conductance term, \( V_m \) is the membrane potential, \( E_{\text{channel}} \) is the reversal potential for the current, \( d_{\alpha}(V_m) \) is the steady-state activation variable, and \( f_{\alpha}(V_m) \) is the steady-state inactivation variable. Since TPA did not affect the shape of either the transient or steady-state I alpha-voltage relation and did not modify \( E_{\text{channel}} \) (Figures 4C and 4D), the observed stimulation in I alpha could be due to changes in \( g_{\alpha} \), \( d_{\alpha} \), or \( f_{\alpha} \), or all of the parameters could be modified. Therefore, the effects of TPA on the steady-state variables (\( d_{\alpha} \) and \( f_{\alpha} \)) were examined using standard voltage protocols (see "Materials and Methods").

Figure 5A shows that TPA causes a small shift of \( d_{\alpha} \) to more negative voltages (mean shift in \( V_k = -2.9 \pm 1.2 \) mV, \( n = 5 \), \( p < 0.05 \)). Figure 5B shows that TPA causes a small shift of \( f_{\alpha} \) to more positive voltages (mean shift in \( V_k = +2.5 \pm 1.1 \) mV, \( n = 5 \), \( p < 0.05 \)). The shifts in \( d_{\alpha} \) and \( f_{\alpha} \) (Figure 5C) suggest that I alpha should increase following the application of TPA.

The data were further analyzed to determine if these changes in the steady-state variables can account for the increases in I alpha following application of TPA. Small changes in \( d_{\alpha} \) and \( f_{\alpha} \) can cause large changes in I alpha (Equations 3 and 4). The predicted shapes of the current-voltage relation of the transient component of I alpha were calculated using Equation 3 and the experimentally determined \( d_{\alpha}(V_m) \) and \( f_{\alpha}(V_m) \) values (Figure 5), adjusting the value of \( g_{\alpha} \) to fit the data by eye. The smooth curves in Figure 4C are the predicted voltage dependence of the transient component of I alpha in the absence and presence of TPA as determined by this procedure. To fit the data, it was necessary to increase \( g_{\alpha} \) from 23 ± 0.54 nS (control, \( n = 5 \)) to 30 ± 1.2 nS (TPA, \( n = 5 \), \( p < 0.05 \) significantly different from control). Using the same procedure, the voltage dependence of the steady-state component of I alpha was estimated. The smooth curves in Figure 4D were determined using Equation 4 and the experimentally determined \( d_{\alpha} \) and \( f_{\alpha} \) values.
FIGURE 6. Effect of TPA on protein phosphorylation in intact cultured myocytes. Incubation of cells with \([^{32}P]\)orthophosphate, sodium dodecyl sulfate (SDS) gel electrophoresis, and autoradiography were performed as described in "Materials and Methods." Panel A: Autoradiographs corresponding to control cells (lane a) and to cells that have been incubated with 160 nM TPA for 10 minutes (lane b). Numbers at left correspond to molecular weights (in kDa) of bands where increased \([^{32}P]\)-labeling in response to TPA is observed. STD marks 48 kDa radiolabeled band that was used as an internal standard in calculations described in Table 1. Because there was a difference in the degree of labeling between high and low molecular weight regions on gel (see ascending background in Panels B and C), upper 40% of the lanes in this photograph were underexposed relative to lower portion so that entire molecular weight range would be visible on a single image. Panel B: Densitometric scan of autoradiogram corresponding to control cultures. Panel C: This panel shows densitometric scan of cultures treated in identical manner as in Panel B except that cells were exposed to 160 nM TPA for 10 minutes.

determined \(d_a(V_m)\) and \(f_a(V_m)\) values (Figure 5), again adjusting \(g_r\) to fit the data by eye. In this case, \(g_r\) was increased from 25 ± 1.7 nS (control, \(n = 5\)) to 33 ± 0.6 nS (TPA, \(n = 5\), \(p < 0.05\) significantly different from control). The results indicate that the increase in both the transient and steady-state components of \(I_C\) are due to increases in \(g_r\), as well as changes in the steady-state variables, \(d_a\) and \(f_a\). The \(g_r\) values used to fit the transient and steady-state \(I_C\) experimental results are very similar. Taken together, these results indicate that the increase of \(I_C\) produced by TPA addition may be due to alteration of the voltage-dependent properties of \(I_C\) and a possible change in \(g_r\), reflecting a change in the number of active channels, in single channel permeability, or the kinetic properties of the channel (see "Discussion").

Stimulation of Protein Phosphorylation by TPA and Angiotensin II

The ability of TPA to activate protein kinase C in intact cultured myocytes was tested. The general approach was to monitor changes in protein phosphorylation in \([^{32}P]\)-labeled myocytes that were exposed to TPA. The pattern of \([^{32}P]\)phosphate incorporation into total cell protein was initially analyzed by SDS gel electrophoresis. As shown in Figure 6A, TPA was effective in stimulating \([^{32}P]\)-incorporation into a number of specific protein bands. Densitometric scanning of autoradiograms revealed that TPA-treated cells had an overall pattern of \([^{32}P]\)-labeling nearly identical to that of untreated cells. However, there were two specific regions, at 32 and 83 kDa, where the increases in radioactivity could be measured on the densitometric scans as shown in Figures 6B and 6C. The phosphorylation reactions stimulated by TPA were studied in more detail. TPA (160 nM) stimulated \([^{32}P]\)-labeling into the two bands relative to untreated cultures over a large number of experiments (Table 1). Stimulation of phosphorylation reactions by TPA were studied in more detail. TPA (160 nM) stimulated \([^{32}P]\)-labeling into the two bands relative to untreated cultures over a large number of experiments (Table 1). Stimulation of phosphorylation was seen at doses as low as 16 nM TPA (data not shown). In contrast, α-PDD (160 nM) did not evoke a significant increase in the phosphorylation state of either band (Table 1) or of any other bands that could be detected on the autoradiograms (data not shown). Since TPA stimulates \(I_C\) in these cells, it is possible that the observed phosphorylation reactions were mediated by increased \(Ca^{2+}\) fluxes rather than from a direct activation of protein kinase C. However, as shown in Table 1, when nifedipine was added to the
cultures (at a concentration that inhibited spontaneous beating), there was no inhibition of the stimulatory response to TPA.

Recently, Albert et al. have reported that an 87-kDa acidic protein is a ubiquitous substrate for protein kinase C. Experiments were done to determine if the 83-kDa protein whose phosphorylation was enhanced in cultured myocytes was a similar protein. Accordingly, cellular proteins were resolved by two-dimensional gel electrophoresis. As shown in Figure 7, TPA did stimulate phosphorylation of an acidic protein of 83 kDa that had an isoelectric point around pH 4.5.

The functional and electrophysiological responses of cultured myocytes to TPA were nearly identical to the responses of the cells exposed to angII. If phorbol esters and this hormone act through a common mechanism, presumably protein kinase C, then angII should also increase phosphorylation of identical proteins. It can be observed from Figure 8 that the response to both agents is very similar; both stimulate $^{32}$P-incorporation into the same set of proteins. When the responses of angII were analyzed from a large number of experiments and cultures, significant phosphorylation of the 83- and 83-kDa bands was observed as shown in Table 1. The results are comparable with those for TPA, although it is clear that TPA is more effective compared with angII in stimulating the phosphorylation of the 83-kDa protein. AngII stimulated phosphorylation in a statistically significant fashion in cultures that were treated with 5 nM hormone for 10 minutes (Table I). However, the effect of the hormone at 5 nM was less consistent from culture to culture compared with its effect at 100 nM. This is most likely due to degradation angII in these experiments where BSA had to be eliminated from the incubation medium to allow for an accurate measurement of cellular protein. Yet, it is essential to include BSA in incubation media to prevent proteolysis of the hormone in these cultures. The specificity of the effects of angII on protein phosphorylation are receptor mediated.

Table 1. Stimulation of Protein Phosphorylation in Intact Myocytes by Angiotensin II and Phorbol Esters

<table>
<thead>
<tr>
<th></th>
<th>Normalized peak heights</th>
<th>Percent increase</th>
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<tbody>
<tr>
<td></td>
<td>32 kDa</td>
<td>83 kDa</td>
</tr>
<tr>
<td>Control</td>
<td>0.50±0.04</td>
<td>0.55±0.04</td>
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<tr>
<td>TPA (160 nM)</td>
<td>0.71±0.06</td>
<td>1.06±0.07</td>
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<td>Control</td>
<td>0.54±0.02</td>
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<td>TPA (160 nM) + nifedipine (10 μM)</td>
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<td>α-PDD (160 nM)</td>
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<td>0.60±0.03</td>
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<td>AngII (5 nM)</td>
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<td>0.65±0.07</td>
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<tr>
<td>AngII (5 nM) + Sar1, Ala8-angll (500 nM)</td>
<td>0.65±0.04</td>
<td>0.44±0.05</td>
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Cells were labeled with $^{32}$P-phosphate and incubated for another 10 minutes with angiotensin (AngII), 12-O-tetradecanoylphorbol-13-acetate (TPA), or 4-2-phorbol-12,13-didecanoate (α-PDD) as indicated. Sarcosine, alanine8-angll and nifedipine were added 1 minute prior to addition of angII and TPA, respectively. Levels of protein phosphorylation were determined using sodium dodecyl sulphate gel electrophoresis and autoradiography as described in "Materials and Methods." Normalized peak height values in each experiment were obtained by dividing peak heights of 32 kDa and 83 kDa bands by peak height of 48 kDa protein, measured in same lane of gel.

Phosphorylation state of 48 kDa protein was not altered by any agents used here. Values in the n column represent number of trials for each experimental condition. Mean ± SEM.

*p<0.05 significantly different from control; NS, not significantly different from control.

*p<0.01 significantly different from control.

$t<0.001 significantly different from control.

FIGURE 7. Separation of 83 kDa protein by two-dimensional electrophoresis. Proteins from $^{32}$P-labeled cells were resolved using two-dimensional electrophoresis as described in "Materials and Methods." Autoradiographs correspond to acidic half of isoelectric focusing gels: A, control cultures; B, cultures treated with TPA (1 μg/ml) for 10 minutes. Arrows indicate position of 83 kDa protein.
Second, the effects are specific since kinase C for several reasons. First, TPA is very potent effects on cardiocytes in stimulating the cells, with threshold doses in the range of 10 nM. Second, the effects are specific since protein phosphorylation in intact myocytes. Incubation of cells with [32P]orthophosphate, SDS gel electrophoresis, and autoradiography were performed as described in “Materials and Methods.” Figure displays portions of densitometric scans of autoradiographs corresponding to control cultures and cultures that have been treated for 10 minutes with either 100 nM angiotensin II or 150 nM TPA. Molecular weights of bands are labeled as in Figure 6.

Discussion

In recent studies, angII was found to stimulate contractile frequency and cadmium-sensitive calcium current (I_{Ca}) in cultured neonatal rat ventricular myocytes.\(^{12}\) While these responses were found to be independent of cAMP, the hormone did activate a sustained phosphoinositide hydrolysis in these cells, as evidenced by increases in inositol monophosphate (IP) and inositol bisphosphate (IP\(_{2}\)) in [\(^{3}H\)]inositol-labeled cells.\(^{12}\) The present study was initiated to examine the physiological significance of one of the metabolites of the phosphoinositide pathway in mediating the observed alterations in cardiac function. The approach has been to examine the effects of a phorbol ester, TPA, which mimics the effects of DAG by activating protein kinase C. The major findings are that TPA stimulates contractile frequency, decreases contractile tension, and increases cadmium-sensitive I_{Ca} in cultured myocytes. A comparison of the physiological and biochemical responses of cardiac cells to TPA and angII indicates that they act through a common mechanism.

Effect of TPA on Contraction

TPA increases the contractile frequency of the spontaneously beating myocytes and causes negative inotropic effects as evidenced by decreases in shortening amplitude, shortening velocity, and relaxation velocity (Figure 1). The effects of TPA on cardiocytes observed here are most likely mediated by protein kinase C for several reasons. First, TPA is very potent in stimulating the cells, with threshold doses in the range of 10 nM. Second, the effects are specific since a-PDD, a phorbol ester that does not stimulate protein kinase C, was inactive even at doses 80-fold higher.

Several observations indicate that the negative inotropic responses are not artifacts of the increase in beating frequency. In electrically stimulated cells, the force-frequency curve remains relatively flat within the frequency range of the TPA response seen in spontaneously beating cells.\(^{12}\) Also, TPA evokes negative inotropic effects in cells that are electrically driven at a constant rate. Our results are consistent with two recent reports in which TPA evokes negative inotropic responses in isolated rat ventricular cardiac myocytes and in rat papillary muscle preparations.\(^{29,30}\)

It is generally accepted that the increase in the frequency of contraction elicited by \(\beta\)-adrenergic agonists is due to an activation of the cAMP-dependent protein kinase.\(^{31,32}\) A recent report indicates that TPA can increase adenylate cyclase in erythrocytes.\(^{33}\) However, there is biochemical and physiological evidence that cAMP is not involved in the TPA-evoked effects in cultured cardiac cells reported here. Stimulation of cAMP-dependent protein kinase with isoproterenol resulted in the phosphorylation of a group of proteins clearly distinct from those observed with TPA treatment (data not shown). Further, in contrast with TPA application, elevation of cAMP results in both positive chronotropic and inotropic responses in cultured neonatal rat myocytes.\(^{12}\) Thus, taken together, the data presented in this study indicate that other distinct classes of chronotropic agents may use the protein kinase C pathway to elicit frequency changes in the heart.

Effect of TPA on \(I_{Ca}\)

Single-cell voltage-clamp studies have demonstrated that phorbol esters activate calcium current carried by \(L\)-type channels in cardiac cells. TPA causes an increase in both the transient and steady-state components of \(I_{Ca}\) without altering the reversal potential. In theory, an enhancement in calcium current can be the result of either modification of the properties of preexisting channels or the recruitment of additional channels. The increase in \(I_{Ca}\) reported here can be attributed to both alterations of the steady-state activation and inactivation variables (\(d_{a}\) and \(f_{s}\)) (Figure 5) and changes in the relative conductance term, \(g_{s}\). Presumably, \(g_{s} = W \times \gamma_{\text{channel}} \times n\), where \(W\) is a probability-weighting coefficient reflecting the probability that the channel is open (independent of \(d_{a}\) and \(f_{s}\)), \(\gamma_{\text{channel}}\) is the single channel conductance, and \(n\) is the number of functional channels.\(^{23}\) Thus, an increase in \(g_{s}\) can be achieved by an increase in the conductance of a single channel, recruitment of previously inactive channels, and/or alterations in channel kinetics. Future studies using patch-clamp techniques should distinguish among these possibilities.

To our knowledge, this is the first demonstration that phorbol esters increase cadmium-sensitive calcium channel activity in cardiac tissue. Recently, protein kinase C has been implicated in the stimulation of calcium current in \(Aplysia\) neurons\(^{34,35}\) and in the...
inhibition of calcium influx or current in two secretory cell lines, snail neurons, and aortic smooth muscle cells. It is interesting to note that patch-clamp studies have revealed that TPA stimulates the appearance of covert Ca2+ channels in Aplysia neurons. As further evidence for an interaction between protein kinase C and Ca2+ channels, Navarro has reported that phorbol esters increase the number of dihydropyridine receptors, presumably Ca2+ channels, in cultured chick myotubes. Thus, the current observations in cardiac cells are consistent with studies with noncardiac tissues indicating that protein kinase C can modify Ca2+ channels. From the accumulating evidence, it appears that the L-type Ca2+ channel is regulated differently in various tissues by this kinase. The observation that TPA increases whole-cell cadmium-sensitive Ca2+ current when application of the agent results in negative effects on tension in spontaneously beating cells was unexpected. The mechanisms that could result in a decline in tension when ICa increases are not known. It may be that TPA application results in a sufficiently large decrease in resting calcium levels such that the magnitude of the calcium transient is less despite the increases in ICa. Such an explanation is compatible with the reduction in resting [Ca2+]i evoked by phorbol ester reported by Uglesity et al in adult heart cells. A second possibility is reduced sensitivity of the myofilaments for [Ca2+]i following application of TPA. One report has provided evidence that some cardiac myofilibrillar proteins may be substrates for protein kinase C.40

Effects of TPA and Angiotensin II on Protein Phosphorylation

We have shown that TPA stimulated phosphate incorporation into a discrete set of proteins in intact cardiocytes. Phosphorylation was reproducibly enhanced into two bands of 32 and 83 kDa. This pattern of protein phosphorylation was distinct from that observed under conditions where cAMP production was stimulated with the β-agonist, isoproterenol (data not shown). Several results indicate that the observed phosphorylation changes are directly mediated by protein kinase C: 1) α-PDD, the inactive phorbol ester, failed to stimulate phosphorylation, 2) the labeling was not influenced by Ca2+ channel activation since nifedipine did not attenuate the responses to TPA, and 3) the 83-kDa acidic protein that was phosphorylated is almost certainly the well-characterized protein kinase C substrate.40 A recent study indicates that the phosphorylation state of this protein is a useful marker for protein kinase C activity in intact cells. Thus, while the functional significance of these phosphorylation reactions remains to be defined, the results provide evidence at the molecular level that protein kinase C is activated in intact cardiocytes.

One of the striking results from this study was that TPA evoked an identical set of functional and electrophysiological modifications as previously observed for angII. Table 2 summarizes the similarity in the effects evoked by the two agents in cultured cardiac cells. Both compounds stimulate beating frequency with concomitant decreases in shortening amplitude and velocity as well as increase calcium current by altering the steady-state parameters (d0 and f0) and the relative conductance parameter, g. If the two agents act through a common mechanism, presumably protein kinase C, then angII should stimulate phosphorylation of the same proteins. This hypothesis was confirmed in experiments reported here. The response of angII to enhance phosphorylation of the 32- and 83-kDa proteins was blocked by a known angII antagonist, Sar1,Ala2-angII, in the cardiocyte system. Taken together, these results support the view that protein kinase C is an intracellular mediator of the effects of angII on cultured cardiocytes. In summary, by the use of electrophysiological and biochemical techniques, we have demonstrated that activation of protein kinase C can lead to significant alterations in mechanical and electrical properties of cardiac cells. Comparative studies with angII suggest that protein kinase C is an important factor mediating hormonal regulation of cardiac function.

Table 2. Comparison of Effects of Phorbol Ester and Angiotensin II on Contractility and Calcium Current Properties in Cultured Cardiac Myocytes

<table>
<thead>
<tr>
<th></th>
<th>Contractility</th>
<th>Ca2+ Current</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Beating frequency</td>
<td>Shortening amplitude</td>
</tr>
<tr>
<td>TPA</td>
<td>+†</td>
<td>-†</td>
</tr>
<tr>
<td>Angiotensin II*</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

*Data obtained from Allen et al12 using cultured cells identical to those described in this report.
†Indicates a positive effect on these parameters.
‡Indicates a negative effect on these parameters.

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Key Words • isolated ventricular myocytes • quinidine • action potential • ionic currents • calcium current • potassium current
Phorbol ester increases calcium current and simulates the effects of angiotensin II on cultured neonatal rat heart myocytes.

A Dösemeci, R S Dhallan, N M Cohen, W J Lederer and T B Rogers

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