Depression of the Spontaneous Activity by Phorbol Esters in Young Embryonic Chick Cardiomyocytes

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ABSTRACT—Effects of phorbol esters, 12-O-tetradecanoyl-phorbol-13-acetate (TPA) and 4-β-phorbol-12,13-dibutyrate (PDB), that stimulate protein kinase C (PK-C) on the spontaneous action potential and the ionic currents in cultured embryonic chick ventricular cardiomyocytes were examined using whole-cell voltage-clamp and current-clamp modes. Experiments were performed at room temperature (22°C). The firing rate of spontaneous activity was 61.7±1.6 beats/min (n=12). Phorbol esters (100 nM and 1 μM) caused a negative chronotropic effect, and they inhibited the maximum rate of depolarization and the action potential amplitude. The action potential duration (at 50% repolarization) tended to decrease, and the maximum diastolic potential was unaffected. In whole-cell voltage-clamp experiments, both TPA and PDB inhibited the L-type Ca²⁺ current and the delayed rectifier K⁺ current. The fast time constant of the inactivation phase for the Ca²⁺ current was decreased, but the slow component was unaffected. In addition, PDB (100 nM) enhanced the T-type Ca²⁺ current, accompanied with an increase in its time constant. In contrast, 4-α-phorbol-12,13-didecanoate (PDD), an inactive analogue on PK-C, failed to produce significant changes. These results suggest that the PK-C stimulation induced by phorbol esters might affect the ionic currents and modulate the [Ca]ᵢ, resulting in regulation of the spontaneous activity of embryonic chick heart cells.

Keywords: Phorbol ester, Protein kinase C, Automaticity, Ionic current, Embryonic chick heart cell

Calcium- and phospholipid-dependent protein kinase (PK-C) has been found to play a critical role for regulation of ionic currents and contractility in cardiac muscles (1-4) as well as in smooth muscle cells (5-9) and neurons (10, 11). PK-C can modulate the activity of voltage-dependent Ca²⁺, Cl⁻ and K⁺ conductance (12, 13). Many important functions are mediated through the phosphorylation of some substrates by PK-C (14, 15). However, the effects of PK-C on cardiac functions are still controversial. For instance, an L-type Ca²⁺ current (I_{Ca,L}) is stimulated in rat cardiomyocytes (2) and in rabbit sino-atrial (SA) nodal cells (3), whereas I_{Ca,L} is inhibited in guinea pig ventricular cells (16, 17).

Single ventricular cells from young embryonic chick heart possess spontaneous activity, which decreases during development (18, 19). Many ionic currents would contribute to generation of the spontaneous action potentials. Since the maximum diastolic potential (MDP) of the action potentials is around −60 mV, the I_{Ca,L} and the delayed rectifier K⁺ current (I_K) would play a major role in the regulation of spontaneous action potentials. Automaticity is strongly dependent on intracellular or extracellular Ca²⁺ concentrations ([Ca]ᵢ or [Ca]₀) (3, 20, 21). Since PK-C regulates the [Ca]ᵢ level (17, 22, 23), the [Ca]ᵢ change would modulate the spontaneous activity directly and indirectly. In addition, PK-C activity may be high in young embryonic heart cells (during developing). In the present experiments, therefore, the modulations by PK-C stimulation (induced by phorbol esters) on the spontaneous action potentials and the ionic currents in the spontaneously beating embryonic chick heart cells were investigated.

MATERIALS AND METHODS

Cell culture preparation

Cell cultures were prepared from tissue taken from the ventricle muscle of 3-day-old embryonic chick hearts, using methods similar to those described previously (18, 19). Twelve dozen fertilized White Leghorn chick embryos were incubated for 3 days at 37.5°C and staged to confirm their degree of development. Hearts were re-
moved under sterile conditions and collected in a balanced salt solution (4°C). Tissue dissociation was accomplished by gentle rotation of the tissues in a Mg²⁺-free and Ca²⁺-free Ringer solution containing 0.1-0.4% trypsin (Sigma Chemical, St. Louis, MO, USA). The cell suspensions were harvested at 5-min intervals, pooled, and pelleted by centrifugation (85 x g). The cells were washed three times in tissue culture medium (M199; Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum. The cells were placed onto glass coverslips placed in 35-mm plastic petri dishes (Falcon, Becton Dickinson, NJ, USA) at a concentration of 10⁵-10⁶ cells/ml. The cells were incubated for 1-2 days at 37°C in a humidified atmosphere (5% CO₂ and 95% air) until used for the experiments.

Whole-cell voltage-clamp and current-clamp experiments

Whole-cell voltage-clamp and current-clamp recordings were made with an Axopatch patch-clamp amplifier (Axon Instruments, Burlingame, CA, USA) and standard techniques. Patch pipettes were fabricated with a two-stage puller, and they had a resistance of 3-5 MΩ. The series resistance error was less than 3-7 mV, and no compensation was used. Experiments were carried out at room temperature (22°C). The data were stored and analyzed on an IBM-AT microcomputer, using the PCLAMP analysis program (Axon Instruments). Current traces were filtered, using a cut-off frequency of 1 kHz, for plotting. The amplitude of the I_Ca,l was determined as the difference between the peak current and the current level measured at 100 msec after the onset of the step. The magnitude of the I_K was determined by taking the difference between the value of the current at the end of a long clamp pulse and zero current level. All values are given as the mean±S.E.M. The differences of the mean values were analyzed by Student's t-test for paired data, and a P value less than 0.05 was considered significant.

Experimental solutions

The glass coverslips containing the cells were placed in the bath-chamber and were superfused with a modified Tyrode's solution. The composition of the modified Tyrode's solution was: 137 mM NaCl, 5.4 mM KCl, 1.8 mM CaCl₂, 1.0 mM MgCl₂, 0.3 mM NaH₂PO₄, 5.0 mM glucose and 5.0 mM HEPES. The pH was adjusted to 7.4 with NaOH. In voltage-clamp experiments, to avoid the interference of other currents, 10 μM tetrodotoxin (TTX) was added to the bath solution to block the fast Na⁺ current. 12-O-Tetradecanoyl-phorbol-13-acetate (TPA), 4-β-phorbol-12,13-dibutyrate (PDB) and 4-α-phorbol-12,13-didecanoate (PDD) (Sigma Chemical Co.) were dissolved with DMSO and were then adjusted to the desired concentrations directly in the bath solution. The pipette solution (intracellular) contained: 110 mM K-aspartate, 20 mM KCl, 2 mM MgCl₂, 10 mM EGTA, 5 mM Mg-ATP, 5 mM creatine phosphate and 5 mM HEPES (pH 7.2). The pCa was adjusted to 8.

![Fig. 1. Effects of PDB on the ionic currents in an embryonic chick heart cell. The current-clamp experiment was performed at room temperature. PDB (100 nM) was added to the bath solution. A: Spontaneous action potentials. B: The maximum rate of depolarization. C: High-speed traces of the action potentials at dots (a and b) above the action potential recordings in panel A. The short line at the left of the action potential recordings in panel C represents the zero mV.](image-url)
RESULTS

Effects on the spontaneous action potentials

Current-clamp experiments were performed to examine the effects on the action potentials of spontaneously beating embryonic chick cardiomyocytes. In normal Tyrode’s solution, the cycle length (CL) in the spontaneously beating cells was 974±12 msec (n=12). Application of PDB (100 nM) caused a negative chronotropic effect (Fig. 1). The amplitude of action potentials (APA) and the maximum rate of depolarization ($V_{max}$) were decreased by 13.9±2.0% (n=6, P<0.05) and 46.7±2.9% (n=6, P<0.01). The maximum diastolic potential (MDP) was unaffected. The action potential duration at 50% repolarization (APD) was shortened, but not significantly. The changes in the action potential parameters are summarized in Table 1. Lower concentrations did not affect them significantly. PDB in this cell caused an arrest approximately 1.2 min after its application. The incidence of arrest was 25% at 100 nM and 33.3% at 1 μM of PDB. As shown in Fig. 2, TPA (1 μM) also caused the negative chronotropic effect, and the effects of TPA on the action potentials were similar to those of PDB. The incidence of arrest was 8.3% at 100 nM and 16.7% at 1 μM of TPA. The effects of PDB were more potent than those of TPA. These responses were irreversible even after the 30-min wash-out. In contrast, PDD, a phorbol ester that does not activate PK-C, did not affect the action potentials. Pretreatment with H-7 (10 μM), an inhibitor of PK-C, caused by itself the negative chronotropic effect (by 15.3±3.1%, n=4, P<0.05), and it did not antagonize the responses induced by phorbol esters.

Effects on the ionic currents

Whole-cell voltage-clamp experiments were performed to examine the underlying ionic currents of the spontaneous activity. Test pulses were applied to −30 to +40 mV from a holding potential of −40 mV, within the voltage range of the action potential (Fig. 3A). The amplitude of $I_{Ca,L}$ current reached a maximum at 0 mV: −238±2.9 pA (n=14). The $I_K$ current at +50 mV was 340±3.3 pA (n=14). The average capacitance was 10.8±2.2 pF (n=8). These control values were similar to recent results (18, 19). Administration of PDB (100 nM) inhibited the $I_{Ca,L}$ (at 0 mV) by 15.9% and $I_K$ (at +50 mV) by 28.6%. Figure 3B shows the current-voltage (I-V) curves for the $I_{Ca,L}$ and $I_K$ currents of this cell. The average values of

**Table 1. Effects of phorbol esters on the spontaneous action potentials in young embryonic chick cardiomyocytes**

|        | n | APA (mV) | APD (msec) | MDP (mV) | $V_{max}$ (V/sec) | CL (msec) |
|--------|---|----------|------------|----------|------------------|-----------|
| Control| 6 | 86±2     | 246±8      | 60±1     | 15±3             | 960±11    |
| PDB 30 nM | 5 | 85±2    | 246±7      | 60±2     | 14±2             | 987±12    |
| 100 nM | 6 | 80±2    | 248±3      | 58±2     | 11±4**           | 1020±10   |
| 1 μM   | 6 | 74±3*   | 226±3      | 56±1     | 8±3**            | 1200±9**  |
| Control| 6 | 83±2     | 248±5      | 60±2     | 14±3             | 980±11    |
| TPA 30 nM | 5 | 83±2    | 240±4      | 60±3     | 14±2             | 989±12    |
| 100 nM | 6 | 80±4    | 234±6      | 59±2     | 14±1             | 998±8     |
| 1 μM   | 6 | 74±2*   | 230±6      | 57±2     | 10±3**           | 1095±7*   |
| Control| 5 | 85±2    | 246±6      | 60±2     | 14±2             | 978±10    |
| PDD 1 μM | 5 | 83±2    | 245±4      | 60±2     | 13±2             | 961±11    |

The reported values are mean±S.E.M. PDB: 4,13-phorbol-12,13-dibutyrate, TPA: 12-O-tetradecanoyl-phorbol-13-acetate, PDD: 4-α-phorbol-12,13-didecanoate. APA: Action potential amplitude, APD: Action potential duration at 50% repolarization, MDP: Maximum diastolic potential, $V_{max}$: Maximum rate of depolarization, CL: Cycle length. *P<0.05, **P<0.01, ***P<0.001, with respect to the control value.

**Fig. 2. Effects of phorbol ester on a spontaneously beating embryonic chick ventricular myocyte.** The current-clamp experiment was performed at room temperature. TPA (1 μM) was added to the bath solution. A: Action potentials. B: The maximum rate of depolarization.
Fig. 3. Current-voltage relationship for the ionic currents in an embryonic chick heart cell. A: Current traces in the absence and presence of PDB (100 nM). A test pulse was applied to -30 to +40 mV from a holding potential of -40 mV. B: Current-voltage curves for I_{Ca,1} and I_K. Symbols used are I_{Ca,1} (open circles) and I_K (open triangles) in the control and I_{Ca,1} (filled circles) and I_K (filled triangles) in the presence of 100 nM PDB.

Inhibition are summarized in Table 2. On the other hand, TPA (100 nM) also decreased I_{Ca,1} and I_K (Fig. 4, A and B). The average inhibitions for I_{Ca,1} and I_K are also shown in Table 2.

Since PK-C affects the time constants for I_{Ca,1} in rabbit SA nodal cells (3, 17), the effects of phorbol esters on the time course of the inactivation for I_{Ca,1} in embryonic chick cells were examined (Fig. 5A). The I_{Ca,1} current in embryonic chick cardiomyocytes exhibits two exponentials. The fast and slow time constants (t_f and t_s) were 8.9±0.6 msec and 22.1±1.1 msec (n=7) in normal Tyrode's solution, respectively. PDB decreased the t_f value to 6.6±0.7 msec (n=4, P<0.05) at 100 nM and to 5.3±0.5 msec (n=4, P<0.01) at 1 µM (Fig. 5B). However, PDB did not decrease t_s to any significant extent (Fig. 5C). TPA also decreased t_f by 18.7±2.6% (n=4, P<0.05) at 100 nM and by 31.8±2.4% (n=4, P<0.01) at 1 µM, but did not affect t_s.

As shown in Fig. 6, A and B, PDB (100 nM) enhanced the T-type Ca^{2+} current (I_{Ca,T}) at 0 mV by 14.2%. The I_{Ca,T} current is low threshold and transient. The holding potential was -80 mV. To avoid the influence of the fast Na"^+ current, TTX (10 µM) was added to the bath solution. The average enhancement was 15.3±3.1% (n=3, P<0.05) at 100 nM and 18.8±2.4% (n=3, P<0.01) at 1 µM of PDB. The fast and slow time constants were 2.82 msec and 11.48 msec in the control, and they were 3.47 msec and 18.10 msec in the presence of 100 nM PDB.

In contrast, PDD never produced any effects. These responses to phorbol esters were not reversible even after the 30-min washout.

DISCUSSION

Isolated ventricular myocytes from young embryonic chick heart possess spontaneous activity (18, 19). The activity was approximately 62 beats/min, and the MDP was around -60 mV. The present experiments were designed to examine the effects of PK-C stimulation by phorbol esters on the spontaneous activity in isolated single embryonic (3-day) chick heart cells. The results were as follows: a) PDB and TPA caused a negative

Table 2. Inhibitions by phorbol esters of ionic currents in young embryonic ventricular cardiomyocytes

|          | n  | I_{Ca,1}   | I_K    |
|----------|----|-----------|--------|
| PDB      | 6  | -32.9±2.4* | -30.7±3.1** |
| 1 µM     | 6  | -68.5±3.0*** | -56.3±3.2*** |
| TPA      | 5  | -24.1±2.2*  | -15.8±3.6*  |
| 1 µM     | 5  | -51.4±2.3*** | -30.3±3.0**  |

Values (%) represent the mean±S.E.M. I_{Ca,1}: L-type Ca^{2+} current, I_K: delayed rectifier K"^+ current, PDB: 4-β-phorbol-12,13-dibutyrate, TPA: 12-O-tetradecanoyl-phorbol-13-acetate. *P<0.05, **P<0.01, ***P<0.001, with respect to the control value.
Fig. 4. Current-voltage relationship for the ionic currents in an embryonic chick heart cell. A: Current traces in the absence and presence of TPA (100 nM). A test pulse was applied to $+10 \text{ mV}$ from a holding potential of $-40 \text{ mV}$. B: Current-voltage curves for $I_{\text{Ca,L}}$ and $I_K$. Symbols used are $I_{\text{Ca,L}}$ (open circles) and $I_K$ (open triangles) in the control and $I_{\text{Ca,L}}$ (filled circles) and $I_K$ (filled triangles) in the presence of 100 nM TPA.

Fig. 5. Time course of the inactivation phase of $I_{\text{Ca,L}}$ in embryonic chick cardiomyocytes. A: Current traces in the control and in 1 $\mu$M PDB. B: Changes in the fast time constants ($\tau_f$) for $I_{\text{Ca,L}}$ by PDB (0.1 and 1 $\mu$M). C: Changes in the slow time constants ($\tau_s$) for $I_{\text{Ca,L}}$ by PDB (0.1 and 1 $\mu$M). *$P<0.05$, **$P<0.01$, with respect to the control value.

Fig. 6. Enhancement of the T-type Ca$^{2+}$ current in an embryonic chick heart cell. A test pulse was applied to $0 \text{ mV}$ from a holding potential of $-80 \text{ mV}$. TTX (10 $\mu$M) was added to avoid the influence of the fast Na$^+$ current. A: Current traces in the absence and presence of 100 nM PDB. B: Current-voltage relationship for the T-type Ca$^{2+}$ current. Symbols used are the control (open circles) and 100 nM PDB (filled circles).
The I_{Ca,L} current is activated during pacemaker depolarization: a) an I_{Ca,L} current (24, 25), b) a decaying conductance I_k (26, 27) and c) a hyperpolarization-activated inward current (I_h) (28). The I_h mainly contributes to the pacemaker current in cardiac Purkinje fibers (29, 30). In sino-atrial (SA) nodal cells, however, the pacemaker depolarization to the threshold potential would not be generated by only one current, but by a combination of these three currents and others (3, 30, 31).

The I_{Ca,L} current is activated during pacemaker depolarization and promotes further depolarization, finally leading to the upstroke of the following action potential. The threshold for activation of the I_{Ca,L} current is around \(-40\) mV. The pacemaker depolarization during diastole is generated from around \(-60\) mV. Thus, it is unlikely that I_{Ca,L} makes a major contribution to the pacemaker potential. Secondly, the I_k current is activated by the preceding action potential, and its decaying during diastole generates and develops the pacemaker depolarization. Finally, in 3-day-old embryonic chick heart cells, the I_h current is also present, but it makes only a minor contribution to generation of the pacemaker potential (18, 19), which is consistent with the I_h current in SA nodal cells (3, 30, 31), because the MDP was around \(-60\) mV, and the threshold potential for activation of I_h is approximately \(-60\) mV. Furthermore, the I_h requires long (for over 3 sec) and more negative voltage (\(-120\) mV) of hyperpolarizing pulses for its activation. Thus, the I_h could not be activated fully during diastole.

In the present experiments, PK-C stimulation inhibited both I_{Ca,L} and I_k, consistent with the previous reports in spontaneously beating rabbit SA nodal cells (3), in guinea pig ventricular myocytes (16), and in aortic smooth muscle cells (9). The inhibitions of both currents depressed the APA and V_{max} and would slow the rate of the pacemaker depolarization. However, the I_{Ca,T} was enhanced by PK-C stimulation. It is suggested that I_{Ca,T} contributes to the early phase during diastole, and I_{Ca,L} contributes to the last phase (30, 32, 33). The I_{Ca,L} generates the depolarization of the last part of the pacemaker potential because a dihydropyridine Ca^{2+} antagonist prohibited only the last part of the pacemaker potential of SA nodal cells (34–36). However, the I_{Ca,T} current makes a minor contribution to the pacemaker potential since the inhibitors of I_{Ca,T} current did not produce any effects on the initial phase of the pacemaker potential (31, 37). Therefore, the negative chronotropic effect would be due to inhibitions of I_{Ca,L} and I_k and also due to interactions with other ionic currents.

**Contribution to pacemaker potential**

Application of phorbol esters caused a negative chronotropic effect and inhibited I_{Ca,L} and I_k. These modulations of the ionic currents would result in the negative chronotropic effect. There are at least three currents known to contribute to the pacemaker potential (or phase 4 depolarization): a) an I_{Ca,L} current (24, 25), b) a decaying conductance I_k (26, 27) and c) a hyperpolarization-activated inward current (I_h) (28). The I_h mainly contributes to the pacemaker current in cardiac Purkinje fibers (29, 30). In sino-atrial (SA) nodal cells, however, the pacemaker depolarization to the threshold potential would not be generated by only one current, but by a combination of these three currents and others (3, 30, 31).

**PK-C stimulation on I_k and APD**

APD is one of the factors that regulate the spontaneous activity. At plateau, the inward and outward currents are practically in balance, and a small change in one of the currents will greatly affect the course of the potential (38). From a theoretical point of view, the APD would be regulated mainly by the time-dependent I_k current. In this study, PK-C inhibited the I_k. The I_k decrease should prolong the APD. However, the APD was shortened (but not significantly). In canine Purkinje fibers, the APD was also shortened (4). The discrepancy shows that the regulation of APD may be independent of only the I_k.

Since the I_k current in cardiac cells is dependent on [Ca^2+], and/or [Ca^2+], the Ca^{2+}-activated I_k would be indirectly stimulated through the enhancement of I_{Ca,L} when [Ca^2+] is low. In this study, however, the [Ca^2+] level was pCa 8 (10 mM EGTA in the pipette). Therefore, it is unlikely that the responses may be due to the modulation of Ca^{2+}-activated K^+ current. The shortening of APD may be regulated by the magnitude and inactivation of the inward currents (the fast Na^+ current, I_Na, and I_{Ca,L}). The inactivation process of I_{Ca,L} is composed of two exponentials (3, 16, 41). PK-C stimulation decreased only the fast time constant (Ca^{2+}-sensitive component) for the
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I\textsubscript{Ca,L} inactivation. I\textsubscript{ca} would not contribute to the AP configuration of the embryonic heart cells since the MDP is \(-60\) mV. Thus, the decrease in the fast time constant for inactivation of I\textsubscript{Ca,L} might result in the shortening of APD. Therefore, these results suggest that the PK-C-induced APD modulation is due to a balance between the decrease in the fast inactivation of I\textsubscript{Ca,L} and the I\textsubscript{K} inhibition. PK-C might activate other ionic currents (i.e., Cl\textsuperscript{-} current, ATP-sensitive K\textsuperscript{+} current and intracellular Ca\textsuperscript{2+} currents) of embryonic chick heart cells.

Modulation of \([\text{Ca}]_i\) level

The spontaneous firing rate is dependent on the \([\text{Ca}]_i\) and/or \([\text{Ca}]_o\). Stimulation of PK-C inhibited the \(I_{\text{Ca,L}}\). However, the fast component of the inactivation phase was stimulated and its time constant was decreased (but the slow component was unaffected). The fast component is dependent on the \([\text{Ca}]_i\) level (17, 42, 43), and the decrease in the time constant may result from elevation of the \([\text{Ca}]_i\) level. Also, the inhibition of peak of \(I_{\text{Ca,L}}\) may be due to the \([\text{Ca}]_i\) elevation. The results are consistent with those of rabbit SA nodal cells (17) and guinea pig cardiomyocytes (16). In this study, however, these responses to phorbol esters would be due to their direct actions, because \([\text{Ca}]_i\) level was buffered. The automaticity in the SA nodal cells is initially stimulated by the \([\text{Ca}]_i\) elevation, and it is strongly depressed under the calcium overload (20, 21). Actually the \([\text{Ca}]_i\) level was elevated by PK-C stimulation (17, 22, 23). In addition, PK-C elicited the arrhythmias in rabbit SA nodal cells (3), and it caused a negative inotropic effect and reduced the post-rest potentiation in canine Purkinje fibers (4). In embryonic chick cardiomyocytes, thus, the modulation of automaticity would also be related with changes in \([\text{Ca}]_i\).

Among the membrane transport system that serve Ca\textsuperscript{2+} homeostasis, voltage-dependent Ca\textsuperscript{2+} channels (T- and L-types) have been implicated as one target of PK-C regulation. The cardiac functions are also modulated not only by these direct actions on the ionic channels, but also by the indirect actions on exchange systems across the membrane and internal stores. Therefore, these results suggest that PK-C stimulation may regulate the physiological functions by modulating the mechanisms to normalize \([\text{Ca}]_i\) (5, 8). Further experiments are required to elucidate this mechanism.

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