Participation of PKC in Modulation of the Excitation-Contraction Process of Chick Embryo Cardiac Muscle

Junna HATAE and Hiroshi KAWATA

Department of Physiology, School of Medicine, Fukuoka University,
Jonan-ku, Fukuoka, 814-01 Japan

Abstract: Phorbol esters are activators of protein kinase C (PKC) and have been widely used to study the function of this enzyme. We investigated the effect of phorbol ester, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), on tension responses and free cytosolic Ca in small strips of chick embryo ventricle and on the ^{45}Ca influx in primary cultures of chick embryo ventricular cells. TPA (10^{-8}M) decreased twitch tension about 40% when driven at 1 Hz. The free cytosolic Ca measured with fura-PE3 was rapidly increased in 110 mM KCl solution and this high K-induced increase was slightly inhibited by TPA (10^{-8}M), by 12%. The resting ^{45}Ca uptake in

5 mM KCl was slightly increased by a low concentration of TPA (10^{-9}M), but high K-induced ^{45}Ca uptake was decreased by TPA in a dose-dependent manner, but to 40% at the utmost. 4 α -Phorbol, an inactive phorbol ester, did not inhibit ^{45}Ca uptake. These results indicate that TPA decreased the depolarization induced transsarcolemmal Ca uptake and thus the excitation-contraction process was attenuated, but to 40% at most. PKC may participate in the modulation of myocardial Ca homeostasis and contractile state. [Japanese Journal of Physiology, 47, 377–383, 1997]

Key words: protein kinase C, tension, cytosolic free Ca, ^{45}Ca uptake, chick embryo ventricular muscle.

The slow or L-type Ca channel that is sensitive to dihydropyridine (DHP) Ca channel inhibitors is known to be regulated in heart and skeletal muscle by a cyclic-AMP dependent protein kinase (PKA) [1, 2]. It has been recently shown that in addition to PKA, Ca/phospholipid-dependent protein kinase C (PKC) modulates voltage-dependent Ca channels in a variety of cells. Phorbol esters stimulate PKC by acting at the same site as diacylglycerol (DAG) [3], and the effects of phorbol ester were reported by many groups within the last decade and significant species variability has been observed. Some of the phorbol ester effects may not be mediated by PKC [4–6]. The dose ranges of phorbol ester and other distinctions between specific and nonspecific effects have not been clearly established. In cardiac Ca channels, the dual effect of stimulation and inhibition of phorbol ester, 12-O-tetradec-

canoyl phorbol-13-acetate (TPA) [7], and a PKC-independent effect of TPA [6] were reported. In this study, to ascertain the participation of PKC in the regulation of the excitation-contraction process on cardiac muscle, we examined the effects of TPA on contractile state, transmembrane ^{45}Ca uptake, and cytosolic free Ca concentration in chick embryo ventricular muscle. An important point was whether or not phorbol ester acted via PKC. To help determine this, we compared the actions of the active phorbol ester, TPA, with those of its inactive type, 4 α -phorbol.

METHODS

Mechanical responses. The hearts of 12–13-day-old chick embryos were used. Hearts were removed and placed in physiological saline (aerated with 100%

Received on April 24, 1997; accepted on July 7, 1997

Correspondence should be addressed to: Junna Hatae, Department of Physiology, School of Medicine, Fukuoka University, 7-45-1, Nanakuma, Jonan-ku, Fukuoka, 814-01 Japan

O₂) of the following composition (in mM): NaCl, 110; KCl, 5.4; CaCl₂, 1.8; MgCl₂, 1; HEPES, 20; *d*-glucose, 25; and pH 7.4. Ventricles were removed and cut into small strips 5 mm in length and 1 mm in diameter. Each strip was mounted in a small chamber containing 0.2 ml of the saline solution and perfused with normal saline and test solutions. In order to stimulate the muscle, rectangular pulses of 1 Hz and 1 ms in duration were applied from an electronic stimulator through a pair of platinum electrodes placed along the muscle preparation. The isometric tension was measured with a strain gauge (ADTECH TDS 101D, Tokyo, Japan) and recorded on a recticorder (San-ei Recti-Horiz-8K, Tokyo, Japan).

Free cytosolic Ca. Intracellular free Ca concentration was measured using the Ca-sensitive fluorescent dye fura-PE3. Fura-PE3 is similar to fura-2 both in use and fluorescent properties, but because it is protonated in the cytosol, it cannot easily diffuse out of the cytosol [8]. The cells loaded with fura-PE3 remain brightly loaded and responsive to changes in the concentration of free cytosolic Ca for several hours. For the measurement of the spectrum of fura-2-Ca complex in various pCa solutions and that of chick embryo ventricle, the excitation wavelength used was between 300–425 nm and the emission spectra were collected at 500 nm as reported in a previous paper [9]. The concentration of free Ca was controlled by EGTA buffers assuming the logarithm of the apparent stability constant (*pK*) for the Ca-EGTA complex as 6.36 at pH 7.0 and ionic strength of 0.16 M. The small strips of 12–13-d-old chick embryo ventricle were loaded with 50 μ M fura-PE3 acetoxymethyl ester (AM) for 1 h. The muscle was then plated into a small chamber and washed for 15 min in normal Ringer solution to remove extracellular and bound dye. Fura-PE3 fluorescence was measured at 340 and 380 nm (excitation) and 500 nm (emission) in a quiescent muscle. At the end of an experiment, Triton-X100 (0.02%) was added to the chamber to allow equilibration of the intracellular and extracellular Ca. EGTA (5 mM) was subsequently added to remove all free Ca and the fluorescence ratio (340/380) was continuously monitored. Fluorescence signals were measured with a JASCO CAF 110 spectrofluorometer (Tokyo, Japan). The UV illumination was obtained from a 75 W light source with a xenon arc lamp. The fluorescence light was guided to a photomultiplier tube and displayed on a pen recorder and input to the computer (NEC PC98, Tokyo, Japan) via an analog-to-digital (A-D) converter.

Cell culture. Primary monolayer cultures of chick embryo ventricular cells were prepared. Hearts

were removed from 12-d-old chick embryos and ventricular cells were isolated by an enzyme solution (0.05% trypsin type IV and 0.01% collagenase type I) at 37°C for 7 min. Cell suspensions were centrifuged, and the pellet was resuspended in D-MEM/F-12 culture medium. The cell suspension was diluted to 5×10^5 cells/ml, plated onto 35 mm plastic culture dishes, and grown in a humidified atmosphere of 5% CO₂ and 95% O₂ at 37°C.

Calcium flux measurement. Ventricular cells were cultured for 3 d prior to ⁴⁵Ca influx experiments. TPA or nitrendipine were applied in each culture dish for 5 min, and as a control, solvents were used instead of TPA or nitrendipine. The culture media were aspirated off and replaced by 1 ml of an EGTA solution containing solvent, TPA, or nitrendipine. The composition of the EGTA solution was as follows (mM): NaCl, 110; KCl, 5.4; EGTA, 0.2; HEPES, 20; and glucose, 25 (pH 7.4). After 5 min, this solution was aspirated off and replaced by 1 ml of a standard saline solution or a high-K solution. The composition of the standard solution was as follows (in mM): NaCl, 110; KCl, 5.4; CaCl₂, 1.8; MgCl₂, 1.0; HEPES, 20; and glucose, 25 (pH 7.4). A high-K solution having 80 mM KCl in equimolar substitution for NaCl and both solutions was supplemented with 3.3 μ Ci of ⁴⁵Ca per ml. After 10 s, the ⁴⁵Ca-containing solutions were quickly aspirated and the cells washed rapidly 3 times with 1.5 ml aliquots of ice-cold EGTA solution. TPA, nitrendipine, or solvents were included throughout the experiment except during the washout phase. After ⁴⁵Ca uptake, cells were digested with 0.5% NaOH and radioactivity was quantitated using a liquid scintillation analyzer (TRI-CARB 2500, Packard Instrument Company, USA). Proteins were determined by the methods of Lowry *et al.* [10] using crystalline bovine serum albumin as the standard. Experiments were carried out at room temperature and the cells were not driven through the experiment.

⁴⁵CaCl₂ was purchased from Amersham International plc (USA). Nitrendipine (ethyl, methyl-1,4-dihydro-2,6-dimethyl-4-(3-nitrophenyl) pyridine-3,5-diester) was a gift from Yoshitomi Pharmaceutical Ltd. (Osaka, Japan). TPA and 4 α -phorbol were purchased from Sigma (St Louis, USA). Nitrendipine stock solution was made in ethanol, and TPA and 4 α -phorbol stock solutions were made in dimethylsulfoxide.

RESULTS

Effects of TPA on the tension response

To assess the influence of TPA on the myocardial inotropic state of 12–13-d-old chick embryonic heart,

Fig. 1. Effects of TPA (10^{-8} M) on electrically stimulated tension responses in chick embryonic heart. Ventricular strips of 12–13-d-old chick embryonic heart were stimulated with rectangular pulses of 1 Hz and 1 ms duration by a pair of platinum electrodes. TPA was applied after steady-state tension responses around 30 min.

the contractile response of ventricular muscle was studied. Figure 1 shows the time course of the negative inotropic effect of TPA in chick embryo ventricle electrically driven at 1 Hz. The twitch tension was gradually decreased by 40% within 10 min after the application of 10^{-8} M TPA (59.4 ± 9.24 at 10 min; $n=3$).

Effects of TPA on cytosolic Ca

To test the hypothesis that the negative inotropic effect of TPA is associated with Ca handling by muscle cells, we examined the effect of TPA on ^{45}Ca uptake and cytosolic Ca content. The effect of TPA on the free cytosolic Ca was investigated next. Small strips of 12–13-d-old chick embryo ventricular muscle were incubated in $50 \mu\text{M}$ fura-PE3 AM for 1 h. Figure 2B shows the fluorescence excitation spectrum of chick embryo ventricle loaded with fura-PE3 (Curve a). The fluorescence at saturation $[\text{Ca}]$ by treatment with 0.2% Triton X-100 and that of zero $[\text{Ca}]$ are shown in curves b and c, respectively. Since clear wavelength dependencies and Ca^{2+} concentration dependencies were observed in the spectrum of the fura-2–Ca complex (Fig. 2A), the cytosolic Ca in chick ventricle containing dye can be estimated from the peak wavelength of spectrum in Curve a of Fig. 2B and cytosolic Ca^{2+} concentration was speculated to be around pCa 8.0.

Using these fura-PE3 AM-loaded muscles, the fluorescence ratio was measured. When the concentration of KCl was raised from 5 to 110 mM, the fluorescence signal at 340 nm was increased and that at 380 nm was decreased; consequently, the fluorescence ratio (340 nm/380 nm) was increased markedly (Fig. 3A).

Fig. 2. Changes in fura-2 fluorescence. The range of excitation spectra used was 300–425 nm and the emission spectrum was 500 nm. **A:** Fura-2 ($1 \mu\text{M}$) fluorescence was measured as a function of excitation wavelength in intracellular buffer containing free Ca concentrations ranging from 0 to pCa 4.5. **B:** Fura-2 fluorescence spectra recorded from a chick embryo ventricular muscle strip loaded with fura-PE3 AM for 1 h. Spectrum a shows results from a fura-PE3 AM loaded muscle, b is the fluorescence at $[\text{Ca}]$ saturation in 0.2% Triton-X100, and c is the fluorescence at zero $[\text{Ca}]$ in 5 mM EGTA solution.

This marked increase reflects an increase of $[\text{Ca}]_i$. The high K-induced rise of free cytosolic Ca was reduced by 12% when the muscle was pretreated with TPA (10^{-8} M) for 10 min (Fig. 3B). This K induced rise of free cytosolic Ca recovered after the muscle was washed with normal Ringer's solution for 40 min (Fig. 3C).

DHP receptor of chick embryonic heart

We next examined ^{45}Ca uptake in primary cultures of 12-d-old chick embryo ventricular cells. The relation between ^{45}Ca uptake and K concentration is shown in Fig. 4. Cells were incubated in HEPES-buffered medium containing ^{45}Ca in various concentrations of KCl (from 5 to 110 mM). Solutions of various K concentrations containing ^{45}Ca were applied for 10 s. The amount of ^{45}Ca uptake was increased as the K concentration increased reaching a maximum at around 80 mM KCl.

Fig. 3. Effects of TPA on high-K-induced increase of the fluorescence ratio (340 nm/380 nm) in a fura-PE3 loaded chick embryo ventricular muscle strip. **A** shows the control record. The fluorescence ratio was rapidly increased by the application of 110 mM KCl. **B** shows the high-K-induced increase of fluorescence ratio in a pretreatment of 10^{-8} M TPA for 10 min. **C** shows the record after washout with normal saline solution for 40 min. The high-K-induced increase of fluorescence ratio almost recovered to the control level.

Fig. 4. The K concentration-response curve for ^{45}Ca uptake in 12-d-old chick embryonic ventricular cells after 3 d of culturing. Each point represents the mean $\pm$ SEM of three experiments. Cells were incubated in HEPES-buffered medium containing ^{45}Ca in various concentrations, of KCl (from 5 to 110 mM) for 10 s.

Figure 5 shows the effects of the DHP Ca channel antagonist nitrendipine on the K-stimulated ^{45}Ca uptake. Cells were preincubated in a solvent of nitrendipine (ethanol) or various concentrations of nitrendipine (10^{-9} – 10^{-6} M) for 10 min, and each preparation was incubated in HEPES-buffered medium containing ^{45}Ca in the absence or presence of nitrendipine for 10 s. The black bars in Fig. 5A show ^{45}Ca uptake in 80 mM KCl and gray bars show the resting ^{45}Ca uptake in 5 mM KCl solution. The ^{45}Ca uptake in 80 mM

Fig. 5. Inhibition of Ca uptake by nitrendipine in chick embryonic ventricular cell cultures. **A:** Black bars show the ^{45}Ca uptake in 80 mM KCl solution for 10 s and gray bars show resting ^{45}Ca uptake (5 mM KCl solution). **B:** Concentration-response curves for nitrendipine effects on stimulated Ca uptake (Ca uptake in 80 mM K solution minus Ca uptake in 5 mM K solution). $\text{EC}_{50} = 4 \times 10^{-9}$ M. Vertical bars, standard errors (three or four experiments).

KCl was 4.92 ± 0.4 nmol/mg protein (control) and was decreased by nitrendipine in a concentration-dependent manner, whereas that of resting ^{45}Ca uptake was not altered by nitrendipine. Stimulated Ca uptake was obtained by subtracting the resting ^{45}Ca uptake from that observed in the 80 mM KCl solution. Figure 5B shows the percentage inhibition of stimulated Ca uptake by nitrendipine. The inhibition by nitrendipine was concentration-dependent. Half maximum inhibition occurred at a concentration 4 nM.

Effect of TPA on Ca uptake

To investigate the contribution of PKC in the regulation of DHP sensitive Ca channel, TPA, a PKC activator, was used. Figure 6 shows the effect of TPA on Ca uptake. The black bars show the uptake in 80 mM K and gray bars show that in 5 mM K. The control Ca uptake in the high-K solution was 4.36 ± 0.1 nmol/mg protein and was decreased by TPA in a concentration-dependent manner (3.77 ± 0.21 nmol/mg protein at

DISCUSSION

Fig. 6. Effects of TPA on Ca uptake in the monolayer of chick embryonic ventricular cell cultures. Black columns indicate uptake in 80 mM K^+ solution and gray columns indicate uptake in 5 mM K^+ solution. Vertical bars show standard errors.

Fig. 7. Effects of TPA and 4α-phorbol on Ca uptake in chick embryonic ventricular cell cultures. These data were obtained from the same series of preparation. The concentrations of TPA, 4α-phorbol, and nitrendipine were 10^{-6} M.

10^{-9} M, 3.38 ± 0.14 nmol/mg protein at 10^{-8} M, and 3.22 ± 0.14 nmol/mg protein at 10^{-6} M). But in the 5 mM K solution, Ca uptake was slightly increased by the low concentration of TPA (1.5 ± 0.18 nmol/mg protein at 10^{-9} M) as compared to that of the control (1.29 ± 0.01 nmol/mg protein).

To investigate whether these effects were mediated by PKC or directly by phorbol ester itself, we examined the effects of 4α-phorbol on ^{45}Ca uptake and show the results in Fig. 7. Although, Ca uptake in a high-K solution was 3.47 ± 0.46 nmol/mg protein and decreased by 40% using 10^{-6} M TPA (2.15 ± 0.15 nmol/mg protein), there was no significant decrease using 10^{-6} M 4α-phorbol (3.12 ± 0.02 nmol/mg protein). In addition, nitrendipine 10^{-6} M abolished the Ca uptake increased by high K in the presence of both TPA and 4α-phorbol (0.84 ± 0.07 and 0.80 ± 0.16 nmol/mg protein, respectively).

This study shows that the tumor promoter TPA has a negative inotropic effect on the contractile state of chick embryo ventricle, so we speculate the PKC activation results in diminished contractile amplitude in cardiac muscle. The decrease in contractile state produced by TPA is likely to be due to a reduction in the Ca influx via the voltage-dependent L-type Ca channel. ^{45}Ca uptake studies indicate that the decrease in $[Ca]_i$ results from diminished Ca entry presumably via slow Ca channels. It seems that this K-stimulated Ca uptake was induced through DHP-sensitive Ca channel, since it was inhibited by the L-type Ca channel antagonist, nitrendipine, in a concentration-dependent manner ($IC_{50} = 4$ nM).

Leatherman [11] reported that 1 μM TPA produced a concentration and time-dependent decrease in the amplitude of cell motion. These results indicate that TPA reduced transsarcolemmal Ca uptake and the size of the rapid exchangeable Ca pool by 11%, and thus the excitation-contraction process. However, they observed Ca uptake and the cytosolic Ca in a resting state. In our reports, neither the resting Ca uptake nor the cytosolic Ca in resting state were decreased by TPA, but both the high-K-induced Ca uptake and high-K-induced cytosolic Ca measured with fura-PE3 were reduced by TPA. Inhibition of the tension responses, Ca uptake and $[Ca]_i$ by the application of TPA was not so large, at most 40%. Marinissen and co-workers [12] also reported results that the resting Ca uptake was reduced by TPA and that staurosporine and H7, a PKC inhibitor, increased Ca uptake in a concentration-dependent manner, thus they concluded that PKC activation induced the reduction of cytosolic Ca. We also observed that the decrease of ^{45}Ca uptake was antagonized by a pretreatment (5 min) of PKC inhibitor, 20 μM H7 (Fig. 8). This supports the assumption that PKC activation may take part in the regulation of Ca channel.

In rat myocardium, the negative inotropic effect of TPA has been associated with a reduction of cytosolic Ca [13]. However, Lacerda showed the dual effect of TPA on voltage-dependent Ca channels, (i.e., TPA stimulates DHP-sensitive ^{45}Ca influx in primary cultured rat ventricular myocytes within 5 s, but, after a 20 min pre-incubation period, the phorbol ester markedly inhibits ^{45}Ca influx). In our experiments, no significant differences in Ca uptake were observed between short (10 s) and long (10 min) periods of incubation with TPA. These discrepancies may be partly explained by species differences (rat vs. chicken). Recently, TPA decreased L-type Ca channel, an indepen-

Fig. 8. Effects of TPA in the presence of PKC inhibitor H7. Cells were incubated 10 min without (control) or with TPA (10^{-8} , 10^{-6} M) and $20 \mu\text{M}$ H7. Black bars show ^{45}Ca uptake in 80 mM KCl solution and open bars shown that of resting ^{45}Ca uptake. Each bar represents the mean $\pm$ SE of three experiments.

dent effect on PKC, in guinea pig ventricular cardiomyocyte [6] and chick sensory neurons [4]. But, in our experiment, although TPA reduced K-stimulated Ca uptake, 4α -phorbol, an inactive phorbol did not affect K-induced Ca uptake. From these results it was concluded that TPA reduced voltage-dependent L-type Ca channel via PKC activation, thus the excitation-contraction process.

At present it is unclear what mechanism accounts for the inhibition of Ca channel activity by TPA in chick heart cells. The possibility of the inhibition of K-induced Ca influx by TPA is due to stimulation of the Na-Ca exchange mechanism. Since the high-K solution was made by the replacement of KCl with equimolar NaCl, the lowering of the external Na resulted in a more rapid increase in $[\text{Ca}]_i$ than can be explained by the Na-Ca exchange bringing Ca into the cell [14]. In the resting state, since Ca influx was larger than the decrease of $[\text{Ca}]_i$ via the stimulation of the Na-Ca exchanger by TPA, total Ca uptake was slightly increased. Another possibility is that inhibition could be due to direct phosphorylation of the channel by TPA [4, 6]. Alternatively, there is cross-talk between PKA and PKC. Marinissen *et al.* [12] reported that PKC may play a negative regulatory action on the PKA pathway, and Houslay [15] reported that PKC-dependent phosphorylation may affect the activity of the adenylate cyclase pathway and/or cyclic AMP phosphodiesterases. Furthermore, it has been reported that the α -subunit of G_i , the inhibitory guanine nucleotide regulatory protein, is a substrate for protein kinase C [16]. Such events could secondarily lead to the inhibition of voltage-dependent Ca channel activity in the heart.

We thank Dr. D. Rampe for critical reading of the manuscript, valuable comments, and helpful suggestions. We are also grateful to Dr. Y. Ishida and Dr. T. Isayama for valuable advice and discussions, to Ms. M. Yasukouchi for technical assistance and to Ms. K. Ogata for preparing the manuscript.

REFERENCES

1. Trautwein W and Hescheler S: Regulation of cardiac L-type calcium current by phosphorylation and G proteins. *Annu Rev Physiol* 52: 257–274, 1990
2. Hosey MM and Lazdunski M: Calcium channels: molecular pharmacology, structure and regulation. *J Membr Biol* 104: 81–105, 1988
3. Blumberg PM, Jaken S, König B, Sharkey NA, Leach KL, Jeng AY, and Yeh E: Mechanism of action of the phorbol ester tumor promoters: specific receptors for lipophilic ligands. *Biochem Pharmacol* 33: 933–940, 1984
4. Hockberger P, Toselli M, Swandulla D, and Lux HD: A diacylglycerol analogue reduces neuronal calcium currents independently of protein kinase C activation. *Nature* 338: 340–342, 1989
5. Doerner D, Abdel-Latif M, Rogers TB, and Alger BE: Protein kinase C-dependent and -independent effects of phorbol esters on hippocampal calcium channel current. *J Neurosci* 10: 1699–1706, 1990
6. Asai T, Pelzer S, McDonald TF, and Pelzer DJ: A phorbol ester reduces L-type calcium channel current in isolated guinea-pig ventricular cardiomyocytes independently of protein kinase C activation. *J Physiol (Lond)* 438: 226P, 1991
7. Lacerda AE, Rampe D, and Brown AM: Effects of protein kinase C activators on cardiac Ca^{2+} channels. *Nature* 335: 249–251, 1988
8. Vorndran C, Minta A, and Poenie M: New fluorescent calcium indicators designed for cytosolic retention or measuring calcium near membranes. *Biophys J* 69: 2112–2124, 1995
9. Hatae J, Fujishiro N, and Kawata H: Spectroscopic properties of fluorescence dye fura-2 with various divalent cations. *Jpn J Physiol* 46: 423–429, 1996
10. Lowry OH, Rosebrough NJ, Farr AL, and Randall RJ: Protein measurement with the Folin phenol reagent. *J Biol Chem* 193: 265–275, 1951
11. Leatherman GF, Kim D, and Smith TW: Effect of phorbol esters on contractile state and calcium flux in cultured chick heart cells. *Am J Physiol* 253: H205–H209, 1987
12. Marinissen MJ, Selles J, and Boland R: Involvement of protein kinase C in $1,25(\text{OH})_2$ -vitamin D_3 regulation of calcium uptake by cultured myocytes. *Cell Signal* 6: 531–538, 1994
13. Uglesity A, Sharma VK, and Sheu S-S: Effect of protein kinase C activation on the inotropic response induced by α -adrenoceptor stimulation in rat myocardium. *Biophys J* 51: 264a, 1987
14. Crespo LM, Grantham CJ, and Cannel MB: Kinetics, stoichiometry and role of the Na-Ca exchange mechanism in isolated cardiac myocytes. *Nature* 345: 618–621, 1990

15. Houslay M D: 'Crosstalk': a pivotal role for protein kinase C in modulating relationships between signal transduction pathways. *Eur J Biochem* 195: 9-27, 1991
16. Katada T, Gilman AG, Watanabe Y, Bauer S, and Jakobs KH: Protein kinase C phosphorylates the inhibitory guanine-nucleotide-binding regulatory component and apparently suppresses its function in hormonal inhibition of adenylate cyclase. *Eur J Biochem* 151: 431-437, 1985