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Phorbol Ester-Induced Ventricular Fibrillation in the Langendorff-Perfused Rabbit Heart: Antagonism by Staurosporine and Glibenclamide

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S. C. Black, S. O. Fagbemi, L. Chi, G. S. Friedrichs and B. R. Lucchesi. Phorbol Ester-Induced Ventricular Fibrillation in the Langendorff-Perfused Rabbit Heart: Antagonism by Staurosporine and Glibenclamide. *Journal of Molecular and Cellular Cardiology* (1993) 25, 1427–1438. Using a paced Lagendorff-perfused rabbit heart paradigm, we investigated the role of protein kinase C (PKC) in the development of ventricular fibrillation (VF) in hearts subjected to hypoxia (12 min) and re-oxygenation (40 min). We studied the effect of putative activators and inhibitors of PKC on the incidence of VF. Hearts exposed to 4β-phorbol,12,13-dibutyrate (PDBu), isophorbol or the membrane permeant diacylglycerol analog, 1-oleoyl-2-acetyl-rac-glycerol (OAG), during the prehypoxic phase had an increased incidence of VF during the hypoxic and reoxygenation periods. The incidence of VF was 90%, 83% and 75% in hearts exposed to PDBu, isophorbol and OAG, respectively ($P<0.05$ *vs* control). Perfusion of hearts with PDBu was associated with a significant increase in the membrane fraction of cardiac PKC activity. In the presence of the inactive phorbol ester 4α-phorbol didecanoate, the incidence of VF was 17% ($P>0.05$ *vs* control). PKC activators were profibrillatory at concentrations that did not affect cardiac function: neither left ventricular developed pressure nor coronary perfusion pressure were affected. The effect of PDBu was antagonized by staurosporine: the incidence of VF was 17% in PDBu + staurosporine treated hearts ($P<0.05$ *vs* control). To further study the profibrillatory effect of PDBu, hearts were exposed to PDBu in the presence of the ATP-dependent potassium channel antagonist glibenclamide. The latter prevented PDBu-induced VF. The results show that under the conditions employed, PDBu-induced activation of PKC induces redistribution of PKC activity and is associated with the development of VF.

Key Words: Glibenclamide; Pinacidil; H-7; Phorbol ester; Phorbol 12,13-dibutyrate; Hypoxia; Ventricular fibrillation; Protein kinase C; Staurosporine.

Introduction

The precise role of the Ca^{2+}-activated phospholipid-dependent protein kinase (protein kinase C; PKC) in the regulation of heart function remains to be defined. PKC phosphorylates several myocardial proteins including phospholamban in the sarcoplasmic reticulum (SR) (Movsesian *et al.*, 1984), a 15 kDa protein of the sarcolemma (SL) (Presti *et al.*, 1985) and troponins I and T (Katoh *et al.*, 1983). However, ascribing a functional role to PKC-mediated phosphorylation in the heart has been difficult as incongruous results have shown both PKC-mediated stimulation, and inhibition of SR calcium transport activity (Movsesian *et al.*, 1984; Rogers *et al.*, 1990). Additionally, stimulation of PKC in whole heart did not lead to phosphorylation of phospholamban (Talosi and Kranias, 1992). These disparate results are indicative of the difficulty in determining the role of PKC in the heart under normal physiological conditions.

The observation that PKC is activated during acute myocardial ischemia suggests that PKC may play a role in cardiac arrhythmias, including ventricular fibrillation (VF) (Strasser *et al.*, 1992). In support of this concept, PKC has been shown to affect cardiac transmembrane ion currents. Activation of PKC by 4β-phorbol 12,13-dibutyrate (PDBu, a tumour-promoting phorbol ester and DAG analog known to activate PKC directly) selectively enhanced the normal repolarizing

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current, I_K (the delayed rectifier) in ventricular myocytes (Walsh and Kass, 1988; Tohse *et al.*, 1987). In isolated canine ventricular myocytes, PDBu shortened cardiac action potential duration at 90% repolarization with no significant effect on the plateau potential, indicative of I_K activation (Spinelli *et al.*, 1991). Effects of PDBu-induced activation of PKC on cardiac ion channels are not specific to potassium however, as calcium current (I_{Ca}) (Tohse *et al.*, 1987; Lacerda *et al.*, 1988), sodium current (I_{Na}) (Moorman *et al.*, 1989) and the transient outward current (I_{to}) are influenced by activation of PKC (Apkon and Nerbonne, 1988).

The effects of PKC activation upon transmembrane ion currents and the activation of PKC during ischemia (Strasser *et al.*, 1992), suggests that PKC may influence cellular processes responsible for abnormal cardiac rhythms. To determine the possible role of PKC activation in the genesis of VF, we investigated the effect of PDBu and other putative activators of PKC on the incidence of VF in the Langendorff-perfused rabbit isolated heart subjected to hypoxia and reoxygenation. The effect of PDBu on the distribution of cardiac PKC also was determined. The specificity of phorbol ester-induced responses was determined by studying the effect of staurosporin and 1-(5-isoquinolinesulfonyl)-2-methyl piperazine (H-7), inhibitors of PKC, on the incidence of PDBu-induced VF. Additionally, the inactive phorbol ester 4α-phorbol 12,13-didecanoate (α-PDD) was studied.

Materials and Methods

Guidelines for animal research

The procedures followed in this study were in accordance with the guidelines of the University of Michigan Committee on the Use and Care of Animals. Veterinary care was provided by the University of Michigan Unit for Laboratory Animal Medicine. The University of Michigan is accredited by the American Association of Accreditation of Laboratory Animal Medicine, and the animal care and use program conforms to the standards in "The Guide for the Care and Use of Laboratory Animals", DHEW Publ. No. (NIH) 86-23.

Isolated heart preparation

Isolated hearts were obtained from male New Zealand white rabbits (2.0–2.2 kg). Animals were killed by cervical dislocation and the heart excised rapidly via a medial sternotomy and placed in heparinized perfusion buffer to remove residual blood. The aorta was dissected free of extraneous tissue and cannulated to a Langendorff perfusion apparatus. Hearts were perfused retrogradely with a modified Krebs–Henseleit buffer at 37°C, at an initial mean perfusion pressure of 50 ± 5 mmHg, and at a flow rate of 18–22 ml/min. The pulmonary vein and vena cava were ligated and the pulmonary artery was cannulated. The left ventricle was vented with a cannula passed through the left atrium so as to produce a close system. The buffer was composed of (in mM): NaCl 117; KCl 1.41; $MgCl_2$-$6H_2O$ 1.2; KH_2PO_4 1.1; $CaCl_2$-$2H_2O$ 2.4; NaH CO_3 25; glucose 5.0; sodium salt of L-glutamate 5.0; sodium pyruvate 2.0; (pH 7.4). The final K^+ concentration was 2.5 mM. This concentration was chosen to increase the sensitivity of the assay to VF as reflected in the incidence of VF when hearts were exposed to profibrillatory agents (Lubbe *et al.*, 1978) under hypoxic conditions. The solution was passed through a gas permeable medical grade tubing (0.05" I.D. and 0.77" O.D., Silastic™) connected in series with the perfusion line as described by Hamilton *et al.* (1974), and was gassed with 95% O_2 and 5% CO_2. The oxygen tension in the perfusion medium entering the aorta equilibrated with the respective gas phases within 1 min of changing the gas mixture in the chamber surrounding the gas permeable tubing. Oxygen tension was recorded continuously with an in line Clark type oxygen electrode (Instech Dual Oxygen Electrode Amplifier Model 203, Instech Laboratories, Plymouth Meeting, PA).

For the determination of cardiac function a latex balloon was inserted through the left atrium into the left ventricle. The balloon was expanded with distilled water to achieve an end-diastolic pressure of 2.5 to 5.0 mmHg. The intraventricular balloon was attached via a rigid cannula to a pressure transducer set at the level of the heart and permitted the continuous recording of left ventricular systolic

and end-diastolic pressures. Hearts were paced electrically with two platinum pin electrodes attached to the right atrium. Square wave pulses (2.5–3.0 Hz, twice threshold current and 3 ms duration) were delivered with an S-88 Grass Stimulator (Grass Instruments Company, Quincy, MA). An electrocardiogram was recorded with electrodes attached at the aorta and the apex of the heart. All recorded parameters were made using a Grass Model 7D Polygraph (Grass Instruments Company, Quincy, MA).

Protocol

Hearts were equilibrated with 95% O_2/5% CO_2 for a period of 10–15 min before commencement of the experiment. After the stabilization period, hearts were subjected to 12 min of hypoxia by gassing the perfusion fluid with 95% N_2 and 5% CO_2. At the end of the hypoxic period, the buffer was gassed with 95% O_2/5% CO_2 and the hearts continuously monitored for 40 min after return to normoxic conditions. Phorbol esters (active and inactive) and OAG were administered (via the perfusion buffer reservoir) 5 min before the induction of hypoxia and remained present in the perfusion buffer throughout the remainder of the experimental protocol. PKC inhibitors were added to the perfusion buffer and equilibrated with the heart for a period of 10 min before exposing the hearts to the PKC activators. For the purpose of this study, VF was defined as the appearance of a rapid, sustained irregular ECG waveform (typical of VF), occurring simultaneously with the complete loss of ventricular function as evidenced by the complete cessation of pressure development. Spontaneous recovery of coordinated contractions were never observed in any of the hearts that developed VF.

Preparation of cytosolic and particulate fractions

Isolated Langendorff-perfused hearts were frozen quickly using aluminium clamps precooled to the temperature of liquid nitrogen. Hearts were stored at −70°C until prepared for the PKC assay (all heart tissue was assayed within 24 h). To obtain the cytosolic and membrane fractions containing the inactive and active fractions of PKC, respectively, the procedure of Yuan *et al.* (1987) was used. Heart tissue or its homogenate were kept on ice during all phases of the preparation. Hearts were pulverized under liquid nitrogen and thawed in eight volumes of buffer containing 20 mM Tris-Cl, pH 7.4; 250 mM sucrose; 5 mM EDTA; 0.005% (w/v) leupeptin; 5 mM benzamidine, 0.3% (v/v) β-mercaptoethanol in deionized, distilled water (buffer I). Pulverized heart tissue was homogenized in buffer I for a period of 5 s using a Tissumizer (Tekmar Company, Cincinnati, Ohio) tissue homogenizer at 55% maximal speed. The homogenate was centrifuged for a period of 20 min at 14 000 × **g**. The supernatant was isolated and centrifuged at 105 000 × **g** for a period of 90 min. The supernatant from this centrifugation step contained the cytosolic fraction. The pellet from the initial 14 000 × **g** centrifugation step was resuspended in eight volumes of buffer I, and homogenized three times for a period of 10 s each, with a 5 s interval. Re-homogenized samples were centrifuged at 14 000 × **g** for 20 min, with the supernatant centrifuged for a period of 90 min at 105 000 × **g**. The pellet from the 105 000 × **g** centrifugation step was resuspended in 3.0 ml of buffer I and contained the membrane fraction.

Assay of protein kinase C activity

Protein kinase C activity of cytosolic and membrane fractions was determined using a PKC enzyme assay system (Amersham, Arlington Heights, IL). PKC activity was defined as the specific calcium- and phospholipid-dependent activity (i.e. [activity in the presence of calcium and phospholipid]-[activity in the absence of calcium and phospholipid]).

Protein assay

Protein content of the membrane and cytosolic fractions was determined using a Bradford Protein Assay kit (Bio-Rad, Richmond, CA) (using bovine gamma globulin as the protein standard).

Drugs used

All chemicals used in this study were purchased from Sigma Chemical Company, St.

Louis, MO, unless otherwise specified. The following chemicals were used: glibenclamide, phorbol 12,13-dibutyrate (PDBu); 4α-phorbol 12,13-didecanoate (αPDD); 1-(5-isoquinolinyl-sulfonyl)-2-methyl-piperazine dihydrochloride (H-7), 4α-phorbol (isophorbol), staurosporine, 1-oleoyl-2-acetyl-rac-glycerol (OAG) (Sigma Chemical Co.). Phorbol esters and staurosporine initially were dissolved in dimethyl sulphoxide. Stock solutions of the phorbol esters were made each day and protected from light. Physiological buffer was used for dilution to the required concentrations. In experiments involving phorbol esters, the perfusion apparatus was covered with aluminium foil to prevent photo-degradation of the esters. Radiolabelled adenosine-5′-triphosphate (γ-^{32}P-ATP) was obtained from Amersham (Arlington Heights, IL).

Statistics

Data are presented as mean ± S.E.M. Analysis of variance was used to compare differences between mean values among different groups. The incidence of VF was analysed using Fisher's exact test. For all comparisons, $P < 0.05$ was the criterion for statistical significance.

Results

Preliminary experiments were conducted to determine the effect of hypoxia alone in the presence of 2.5 mM K^+. Hypoxia was induced by changing the gas in the membrane lung from 95% O_2/5% CO_2 to 95% N_2/5% CO_2. The pO_2 of the normoxic perfusion buffer was 507 ± 20 mmHg ($n = 10$). Upon changing to the nitrogen/carbon dioxide gas mixture, the pO_2 of the perfusion buffer decreased rapidly, and reached a new steady state within 3 min. The pO_2 of the perfusion buffer during hypoxic perfusion was 56 ± 8 mmHg ($n = 10$, $P < 0.05$ *vs* pre-hypoxia). During the hypoxic period, hearts were unable to follow the pacing stimulus and at the end of hypoxic period heart rate decreased to approximately 20 beats/min despite continuous atrial pacing. Upon re-oxygenation, heart rate returned to the pacing rate after an intervening period of arrhythmias. VF did not occur during the 12 min period of hypoxic perfusion or during the re-oxygenation phase (40 min) in the absence of phorbol esters or diacylglycerol analogs.

TABLE 1. Effect of protein kinase C activators and 4-α phorbol 12,13-didecanoate on the incidence of ventricular fibrillation in the isolated rabbit heart

Group	Percent incidence of ventricular fibrillation	*n*
Control	0	10
PDBu (30 nM)	90[1]	10
Isophorbol (30 nM)	83[1]	6
OAG (500 nM)	75[1]	4
4αPDD (100 nM)	17	6

[1] $P < 0.05$ *vs* control.

Effect of PKC activators and inhibitors on the incidence of ventricular fibrillation

The incidence of VF in control hearts (in the absence of PKC agonists or antagonists), hearts perfused with activators of PKC and also the inactive analog of PDBu, 4αPDD, is summarized in Table 1. PDBu (30 nM), isophorbol (30 nM) and OAG (500 nM) each significantly increased the incidence of VF compared to control hearts. The effect of the inactive PDBu analog, αPDD was studied to determine if the onset of VF was due to activation of PKC or to a non-specific effect of either the phorbols or the DAG analog studied. At a concentration of 100 nM, αPDD did not increase the incidence of VF. Since different activators of PKC were shown to be associated with a significant increase in the incidence of VF, and because the inactive phorbol ester αPDD was without a profibrillatory effect, the effect of compounds known to inhibit PKC were studied. The data are presented in Table 2. The PKC inhibitor staurosporine (10 nM) prevented PDBu-induced VF. Although the incidence of PDBu-induced VF in the presence of H-7 (10 μM) was reduced to 50%, the decrease was not significant. Because previous studies with this model have implicated the ATP-dependent potassium channel (K_{ATP}) to play a role in the genesis of VF (Chi *et al.*, 1993), the effect

TABLE 2. The effect of protein kinase C inhibitors and glibenclamide on the incidence of ventricular fibrillation induced by phorbol 12,13-dibutyrate in the isolated rabbit heart

Group	Percent incidence of ventricular fibrillation	n
Control	0	10
PDBu (30 nM)	90[1]	10
PDBu (30 nM) + Staurosporine (10 nM)	17[2]	6
PDBu (30 nM) + H7 (10 μM)	50	6
PDBu (30 nM) + Glibenclamide (1.0 μM)	0[2]	6

[1] $P < 0.05$ *vs* control. [2] $P < 0.05$ *vs* PDBu alone. PDBu = phorbol 12,13-dibutyrate.

of the ATP-dependent potassium channel blocker, glibenclamide, was studied. The aim of these experiments was to determine if PKC-induced VF was associated with an effect on the ATP-dependent potassium channel. As shown in Table 2, glibenclamide at a concentration of 1 μM prevented PDBu-induced VF in the hypoxic-reoxygenated heart model used.

Effect of protein kinase C activators and inhibitors on contractile function in the isolated rabbit heart

To determine if the profibrillatory effect of the phorbol esters and DAG analogs studied was related to altered cardiac dynamics, two indices of cardiac function were studied: left ventricular developed pressure (LVDP) and coronary perfusion pressure. LVDP during the pre-hypoxic, hypoxic and re-oxygenation periods of control hearts (in the absence of PKC agonists or antagonists) and hearts perfused with PDBu (30 nM), isophorbol (30 nM), OAG (500 nM) or αPDD (100 nM) is represented in Figure 1(a). Initially, left ventricular developed pressures were in excess of 80 mmHg in each group studied. During the 12 min hypoxic period all hearts exhibited a decrease in left ventricular developed pressure. In control hearts, LVDP recovered to 60 ± 6 mmHg, after 10 min of re-oxygenation. Left ventricular developed pressure increased progressively to 69 ± 9 mmHg by 40 min of re-oxygenation in control hearts. Compared to controls, hearts exposed to PDBu, isophorbol or OAG demonstrated no differences in the decrement of LVDP during hypoxia or in the recovery of LVDP during the re-oxygenation phase. Because the majority of hearts exposed to PDBu fibrillated within the first 19 min of re-oxygenation (9/10), further statistical comparison between control and PDBu-treated hearts could not be made. In hearts exposed to the inactive phorbol ester, αPDD, the rate and extent of recovery tended to be lower compared to controls, although the difference in function was not significant.

The data of Figure 1(b) show changes in LVDP during the pre-hypoxic, hypoxic and re-oxygenation periods of control hearts [the data are identical to those represented in the control group of Fig. 1(a)] and hearts perfused with PDBu (as with the control data, these data are identical to those represented in Fig. 1(a) for the PDBu group), PDBu + staurosporine (10 nM) and PDBu + H7 (10 μM). There were no significant differences in LVDP in response to hypoxia and re-oxygenation in hearts exposed to PDBu, PDBu + staurosporine or PDBu + H7. Similarly, there was no difference in the LVDP of hearts exposed to PDBu + glibenclamide during the experimental protocol (data not shown).

The data of Figure 2 illustrate the time course of the change in coronary perfusion pressure during the experimental period in the PKC activator and PKC inhibitor groups studied, respectively. Coronary perfusion pressure decreased within the first 5 min of hypoxia, followed by a steady rise to and above that of the pre-hypoxic value after 12 min of hypoxia. During the re-oxygenation period, there was a progressive increase in coronary perfusion pressure in control hearts, reaching 76 ± 6 mmHg after 40 min of re-

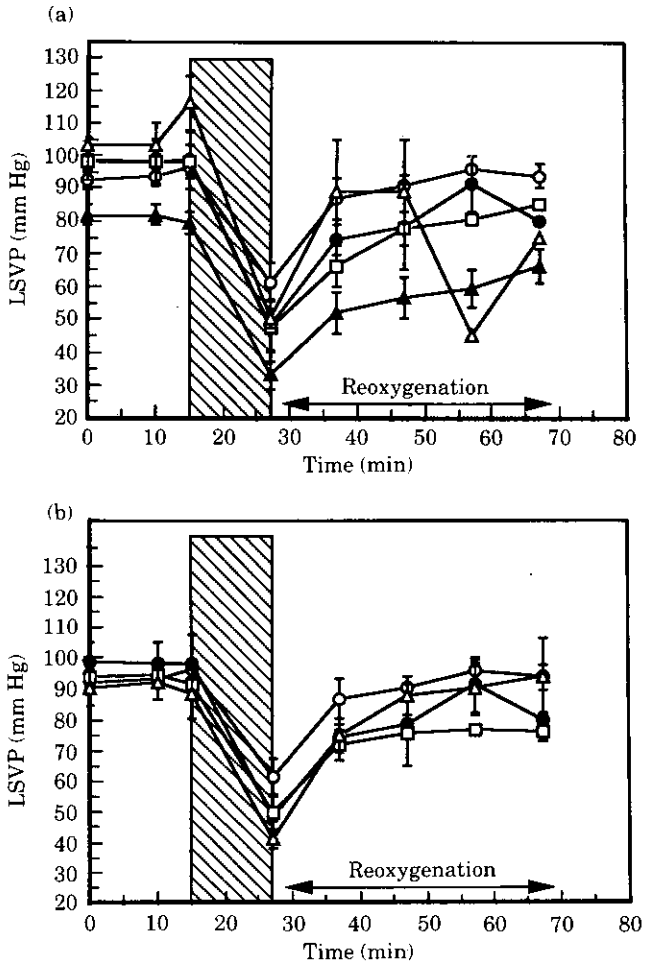

FIGURE 1. (a) Data show the effect of 12 min of hypoxia and 40 min (maximum) of re-oxygenation upon left ventricular developed pressure (LVDP) in Langendorff-perfused isolated rabbit hearts exposed to buffer alone (control), PDBu (30 nM), Isophorbol (30 nM), OAG (500 nM) or αPDD (100 nM). (b) Data show the effect of 12 min of hypoxia and 40 min (maximum) of re-oxygenation in Langendorff-perfused isolated rabbit hearts exposed to buffer alone (control), PDBu (30 nM), PDBu (30 nM) + staurosporine (10 nM) and PDBu (30 nM) + H7 (10 μM). For both (a) and (b) the bar with diagonal lines represents the period of hypoxia, and the period before the bar represents the equilibration period.

oxygenation. Pre-hypoxic exposure of hearts to PDBu (30 mM) increased coronary perfusion pressure. Other activators of PKC, and αPDD, had no significant effect on pre-hypoxic perfusion pressure or the steady rise in perfusion pressure after re-oxygenation [Fig. 2(a)]. As represented in Figure 2(b), pretreatment of the isolated rabbit hearts with PKC inhibitors (staurosporine or H-7) did not significantly change the steady rise in perfusion pressure observed during and after the hypoxic periods.

Effect of 4β-phorbol,12,13-dibutyrate on the distribution of myocardial protein kinase C activity

The effect of PDBu on the cellular distribution of PKC activity was determined. Hearts

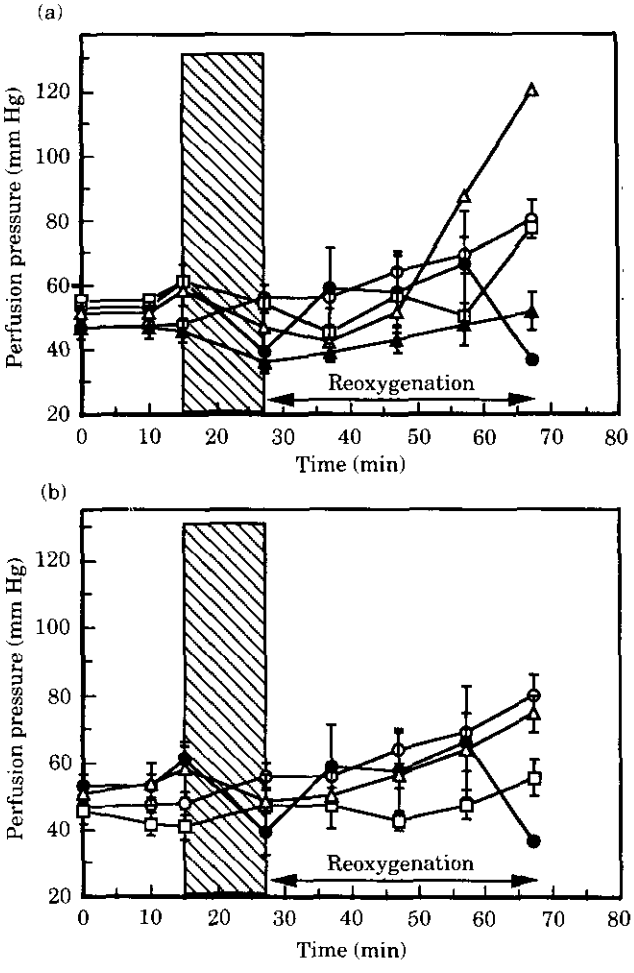

FIGURE 2. (a) Data show the effect of 12 min of hypoxia and 40 min (maximum) of re-oxygenation upon coronary perfusion pressure in Langendorff-perfused isolated rabbit hearts exposed to buffer alone (control), PDBu (30 nM), Isophorbol (30 nM), OAG (500 nM) or αPDD (10 nM). —○—, Control; —●—, PDBu; —□—, Isophorbol; —△—, OAG; —▲—, αPDD. (b) Data show the effect of 12 min of hypoxia and 40 min (maximum) of re-oxygenation upon coronary perfusion pressure in Langendorff-perfused isolated rabbit hearts exposed to buffer alone (control), PDBu (30 nM), PDBu (30 nM) + saturosporine (10 nM) and PDBu (30 nM) + H7 (10 μM). For both (a) and (b) the bar with diagonal lines represents the period of hypoxia, and the period before the bar represents the equilibration period. —○—, Control; —●—, PDBu; —□—, PDBu + staurosporine; —△—, PDDu + H7.

were perfused with 30 nM PDBu (or vehicle) for a period of 10 min under normoxic conditions, followed by 12 min of hypoxic perfusion and either 5 or 20 min of re-oxygenation. At the 5 and 20 min time points of re-oxygenation, PKC activity was determined. The 5 and 20 min time points were chosen to bracket the period of re-oxygenation where the incidence of PDBu-associated VF was highest. The effect of vehicle and PDBu on the distribution of PKC activity after 5 and 20 min of re-oxygenation is shown in Figures 3(a) and 3(b), respectively. There was a significant shift in the myocardial distribution of PKC activity at 5 and 20 min of re-oxygenation in hearts exposed to 30 nM PDBu. Cytosolic PKC activity was reduced by approximately 80% in hearts exposed to PDBu. The membrane PKC activity of PDBu-treated hearts increased by 177% above the control level. The total (cytosolic and membrane) specific activities of the control and PDBu-

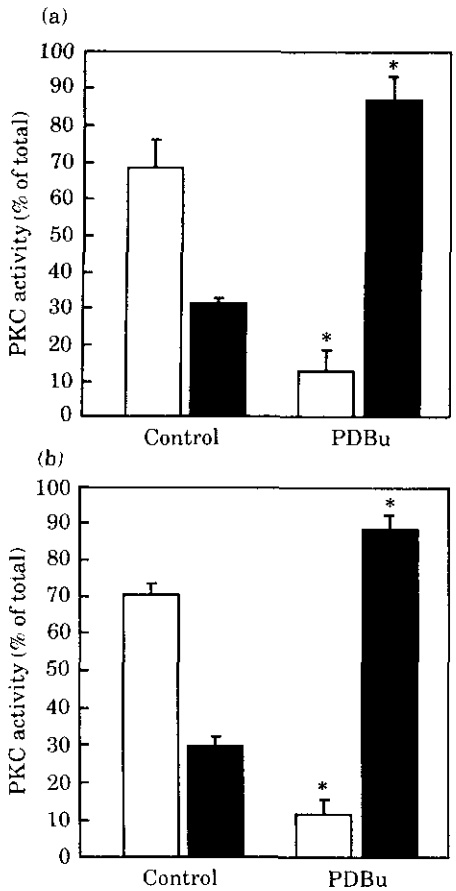

FIGURE 3. Distribution of protein kinase C activity between the cytosolic and membrane fractions of isolated Langendorff-perfused rabbit hearts exposed to 30 nM PDBu or vehicle (control). Hearts were equilibrated with PDBu or vehicle, subjected to a hypoxic period, and PKC activity was determined after 5 min (a) and 20 min (b) of re-oxygenation. Open bars represent PKC activity from the cytosolic fraction and closed bars represent PKC activity from the membrane fraction. *Significantly different from respective control. Data shown are mean ±S.E.M., control $n=5$, PDBu-treated $n=6$.

treated hearts was not different at either the 5 or 20 min time points (5 min control and PDBu-treated totals were 971 ± 442 pmoles P_i transferred/mg protein/min and 1387 ± 284 pmoles P_i transferred/mg protein/min, respectively; 20 min control and PDBu-treated totals were 1042 ± 490 pmol P_i transferred/mg protein/min and 1551 ± 215 pmol P_i transferred/mg protein/min, respectively).

Discussion

The present report focuses on the study of phorbol-ester-induced VF in the Langendorff-perfused rabbit isolated heart. To our knowledge this study is the first to indicate that PKC activation participates in the genesis of VF. This suggestion is supported by our data showing that the tumour-promoting phorbol ester PDBu, which was shown to activate PKC, induced VF in the perfused rabbit heart subjected to hypoxia and re-oxygenation. Hearts subjected to hypoxia and re-oxygenation alone did not develop VF, and PKC was not redistributed. The profibrillatory effect of PDBu was demonstrated at a concentration of 30 nM, which is identical to that shown to reduce the action potential duration in isolated ventricular myocytes (Spinelli *et al.*, 1991). Other studies have used similar concentrations of PDBu to demonstrate functional effects of PKC activation. In the rat isolated heart, threshold effects of PDBu on heart rate, coronary flow and force of contraction ascribed to PKC activation, were observed at concentrations of PDBu between 10 and 100 μM (Yuan *et al.*, 1987). Yuan *et al.* demonstrated translocation of PKC from the cytosolic to particulate compartments at a PDBu concentration of 1 μM. An important methodological difference between the Yuan *et al.* investigation and our study is that hypoxia was not involved in the former evaluation. The profibrillatory effect of PDBu, occurring through PKC activation, also was observed with the cell membrane permeant analog of DAG (1-oleoyl-2-acetyl-rac glycerol, OAG) and isophorbol.

That the observed profibrillatory effect of PDBu was related to the activation of PKC is supported by the observation that the inactive stereoisomer of PDBu, αPDD (100 nM), which does not activate PKC (Castagna *et al.*, 1982), did not exhibit a profibrillatory effect. Others have used αPDD as a negative control at similar concentrations to demonstrate specificity of putative phorbol-induced responses. For example, in cultured neonatal myocytes αPDD, at a concentration of 160 nM did not

influence protein phosphorylation profiles shown to be affected by the PKC activator 12-*O*-tetradecanoylphorbol-13-myristate (160 nM) (Dosemeci *et al.*, 1988). Support for a role of PKC in the genesis of VF in our paradigm was the failure of hypoxia and re-oxygenation alone to cause a change in the distribution of PKC activity to the same extent as PDBu + hypoxia and re-oxygenation. The 17-fold greater ratio of the proportion of membrane to cytosolic PKC activity of PDBu treated hearts indicates that hypoxia/re-oxygenation alone was an ineffective stimulus for PKC redistribution. The lack of PKC activation correlated with the absence of VF in control hearts. Additional pharmacological support for the involvement of PKC in the genesis of PDBu-induced VF using the current experimental paradigm was the observation that staurosporine reduced the incidence of PDBu-induced VF. This observation is in keeping with the ability of staurosporine to inhibit the catalytic domain of PKC. The PKC inhibitor H-7 reduced the incidence of PDBu-induced VF to 50%. The explanation for the attenuated reduction in the incidence of PDBu-induced VF by H-7 is not known. However, H-7, compared to staurosporine, is a relatively weak inhibitor of PKC activity. This is supported by reports showing a reduced or even a lack of effect of H-7 on PKC activity in Swiss 3T3 fibroblasts (Burch *et al.*, 1988) and mouse epidermal cells (Nakadate *et al.*, 1989). H-7 is also less effective than staurosporine in antagonizing PDBu-induced increases in femoral artery tension (Merkel *et al.*, 1991). However, the PKC inhibitory effect of H-7 may be dose related and it therefore remains to be determined if a higher concentration of H-7 would have greater antifibrillatory activity in our model of PDBu-induced VF. Alternatively the greater efficacy of staurosporine against PDBu-induced VF may relate to its broader spectrum of kinase inhibitory activity (including cAMP-dependent protein kinase) Hidaka and Kobayashi, 1992), since elevated myocardial cAMP has been associated with an increased susceptibility to VF (Worthington and Opie, 1992).

PKC-activating phorbol esters and OAG did not induce VF during the normoxic equilibration period. Under conditions in which the heart is subjected to ischemia or hypoxia activation of an outward potassium current via the $I_{K,ATP}$ channel may be observed. Hearts subjected to a hypoxic period may become more susceptible to profibrillatory agonists under conditions in which cellular ATP is reduced (Arena and Kass, 1989). In Langendorff-perfused hearts, tissue ATP content is reduced after 12 min of hypoxia and remains depressed throughout 40 min of re-oxygenation (Chi *et al.*, 1993). At cellular ATP concentrations less than 500 μM the ATP-dependent potassium channel ($I_{K,ATP}$) agonist pinacidil, increased the activity of the $I_{K,ATP}$ (i.e. enhanced potassium efflux). In contrast, at ATP concentrations above 3.0 mM the action of pinacidil on the channel was inhibited (Arena and Kass, 1989; Tseng and Hoffman, 1990). The current results show that the profibrillatory effect of PKC activation is manifest under conditions in which myocardial ATP concentration is known to be reduced. The similarity in the profibrillatory effect of pinacidil (Chi *et al.*, 1993) and PDBu suggest that each may be acting through a mechanism involving $I_{K,ATP}$. Blockade of PDBu-induced VF by glibenclamide suggests that the $I_{K,ATP}$ may be a common site of action. It is known that stimulation of PKC activates the $I_{K,ATP}$ channel in other, non-cardiac, cell types (DeWeille *et al.*, 1989; Ribalet *et al.*, 1989). Although a direct relationship between PKC and the $I_{K,ATP}$ remains to be shown in cardiac cells, such evidence provides support for the possibility that PKC modulation of the $I_{K,ATP}$ contributed to the development of VF in our model. However, in view of the number and complexity of factors considered to underlie the genesis of VF and the number of possible different sites of action of phorbol esters (PKC-dependent and PKC-independent actions), a common mechanism remains speculative at this time.

The profibrillatory effect of PDBu may be related to PKC-induced increases in intracellular calcium $[Ca^{2+}]_i$. During hypoxia, $[Ca^{2+}]_i$ is known to be elevated by an increase in Ca^{2+} influx via Ca^{2+} channels, Na^{2+}/Ca^{2+} exchange and an increase in the sarcolemma permeability (Shen and Jennings, 1972). Further, it has been shown that PKC activation by phorbol esters results in the phosphorylation of L-type cardiac Ca^{2+} channels (Hosey *et al.*, 1989), an effect associated with

an increase in the Ca^{2+} inward current. PKC has also been suggested to stimulate cardiac Na^{2+}/Ca^{2+} exchange (Watson and Karmazyn, 1991). In isolated myocardial cells, PKC activation has been shown to aggravate hypoxic myocardial injury, manifest as increased creatine kinase loss, via an increase in $[Ca^{2+}]_i$ (Ikeda *et al.*, 1988). Therefore phorbol ester-induced activation of PKC may act synergistically (or at least additively) with hypoxia to increase intracellular calcium. An augmentation in intracellular calcium can lead to increased after depolarizations, altered membrane ion conductance, triggered activity (Eisner *et al.*, 1984; Kass *et al.*, 1982), and VF. In agreement with this suggestion are data showing that $[Ca^{2+}]_i$ oscillation plays a crucial role in triggering VF in the isolated, isovolumic ferret heart (Kihara and Morgan, 1991).

Although we have presented data to support a role of phorbol ester-induced activation of PKC as a mechanism for the increased incidence of VF in PDBu-treated hearts, the effects of phorbol esters on the heart are complex, hence other possible PDBu effects may contribute to the genesis of VF. The study of Watson and Karmazyn (1991) showed that the non-PKC activating phorbol ester α-PDD had a negative inotropic effect, increased resting tension and stimulated the transient release of 6-keto $PGF_{2\alpha}$ from rat isolated hearts, suggesting that phorbol esters influence cardiac function independent of PKC activation. Additionally, it may be relevant that phorbol esters modulate the release of atrial naturetic factor and norepinephrine (Musgrave and Majewski, 1989a, 1989b) from myocardial cells. Further, it remains possible that PDBu-induced VF occurred in a PKC-dependent manner, but independent of effects on cellular ion homeostasis.

Spinelli *et al.* (1991) demonstrated an increase in the outward K^+ current in canine ventricular muscle exposed to pinacidil or to the protein kinase C activator, PDBu (30 nM). The membrane effects of pinacidil or PDBu were blocked by glibenclamide and thus presumably reflected changes in the ATP-regulated potassium current. The effects of pinacidil and PDBu upon the outward K^+ current are consistent with our previously reported observations (Chi *et al.*, 1993) as well as those reported in the present study in that both pinacidil and PDBu increased the incidence of VF in the perfused rabbit heart subjected to hypoxia followed by re-oxygenation. Furthermore, pretreatment with glibenclamide provided protection against the development of VF. The observations support the concept that the mechanism for initiation of VF can be antagonized by a decrease in the outward current and suggest that selective inhibition of a repolarizing current, possibly involving inhibition of PKC, may provide a novel approach to developing an intervention capable of preventing VF.

In summary, the results of this study demonstrate that the Langendorff-perfused rabbit isolated heart, exposed to well characterized activators of PKC, PDBu, isophorbol or OAG, during hypoxia and re-oxygenation is prone to develop VF. The profibrillatory effect of PDBu was not observed with αPDD, and was blocked by the PKC antagonist staurosporine. The precise mechanism by which phorbol esters and OAG lead to VF remains to be determined. It is possible that subsequent to PKC activation under hypoxic conditions, cellular protein phosphorylation results in altered cellular ionic homeostasis, the functional manifestation of which is VF. The specific membrane channel(s) or regulatory protein putatively phosphorylated by PKC under our experimental conditions remains to be determined. PDBu has been reported to enhance I_K (Walsh and Kass, 1988), and can regulate I_{Ca} (Tohse *et al.*, 1987; Lacerda *et al.*, 1988) and I_{to} (Apkon and Nerbonne, 1988) and in non-cardiac cells modulate the activity of the ATP-dependent potassium channel (DeWeille *et al.*, 1989; Ribalet *et al.*, 1989). In our studies the prevention of PDBu-induced VF by glibenclamide, however, provides evidence for the participation of the ATP-dependent potassium channel in the genesis of PDBu's proarrhythmic effect in the hypoxic/re-oxygenated isolated heart. Evidence obtained from patch-clamp electrophysiological studies and the demonstration that sulphonylureas block the effects of potassium channel openers supports this conclusion (Quast and Cook, 1989; Findlay *et al.*, 1989; Findlay, 1992). Our laboratory is currently studying the effect of analogs of PDBu and inhibitors of

PKC translocation and/or kinase activity to determine if PKC is activated during the immediate onset of VF. Whether or not hypoxia and ischemia induced VF is associated with activation of PKC is likewise under study in order to differentiate PKC-dependent and PKC-independent actions of phorbol esters. Identification of myocardial PKC activation in the genesis of cardiac rhythm disturbances suggests a unique arrhythmogenic mechanism and provides an opportunity to design antiarrhythmic and/or antifibrillatory agents that possess a greater degree of specificity over that of currently available drugs. Prevention of the decrease in membrane refractoriness, due to myocardial hypoxia and or ischemia, by inhibition of possible PKC-dependent actions on the ATP-dependent potassium channel may provide a useful approach to the prevention of reentrant arrhythmias. Additional studies are warranted to explore this concept.

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