Different patterns of protein kinase C redistribution mediated by α_1 -adrenoceptor stimulation and phorbol ester in rat isolated left ventricular papillary muscle

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1 In rat left ventricular papillary muscle, phenylephrine, an α_1 -adrenoceptor agonist, had a staurosporine-sensitive positive inotropic effect and increased the particulate-associated protein kinase C (PKC) activity without significant changes in total PKC activity or in cytosolic Ca^{2+} /phospholipid-independent kinase (PKI) activity.

2 A PKC stimulant, phorbol 12,13-dibutyrate (PDBu), decreased contractility and slightly increased PKC activity in the particulate fractions, with a marked decrease and increase in total PKC and PKI activities, respectively.

3 The PDBu-induced negative inotropic response was attenuated by two protease inhibitors, leupeptine and a microbial peptide isolated from *Aspergillus japonicus* (E-64), which are known to inhibit the conversion of particulate-associated PKC to PKI.

4 Such differences in the patterns of PKC redistribution, i.e. marked increases in particulate PKC and cytosolic PKI activities caused by phenylephrine and PDBu, respectively, may account for the opposite inotropic effects of PKC stimulation by an α_1 -agonist and a phorbol ester.

Keywords: Protein kinase C (PKC) redistribution; inotropic effect; α_1 -adrenoceptor stimulation; phorbol ester; rat papillary muscle

Introduction

The breakdown of phosphatidylinositol 4,5-bisphosphate (PI-4, 5-P_2), and the resulting generation of inositol trisphosphate (IP_3) and diacylglycerol (DAG) occur during signal transduction induced by a number of neuronal and humoral stimuli (Downes & Michell, 1982; Berridge, 1984). A growing body of evidence indicates that IP_3 mobilizes Ca^{2+} from intracellular Ca^{2+} stores, whereas DAG activates Ca^{2+} /phospholipid-dependent protein kinase (protein kinase C; PKC) (Kikkawa *et al.*, 1983; Somlyo *et al.*, 1985; Nosek *et al.*, 1986). In cardiac muscle, an α_1 -adrenoceptor-mediated positive inotropic effect is associated with the receptor-linked degradation of PI-4, 5-P_2 (Otani *et al.*, 1988; Scholz *et al.*, 1988). For example, α_1 -adrenoceptor stimulation with phenylephrine in the rat isolated left ventricular papillary muscle preparation results in a triphasic inotropic response: a transient positive inotropic effect followed by a transient negative inotropic effect and then a sustained positive inotropic effect (Otani *et al.*, 1988). Our recent studies suggested that the transient response are mediated by IP_3 -induced Ca^{2+} mobilization and that the sustained positive inotropic effect is produced via PKC activation (Otani *et al.*, 1988; 1990).

In contrast, a tumour-promoting phorbol ester, which also activates PKC by substituting for DAG, was shown to decrease the contractility of cardiac muscles (Teutsch *et al.*, 1987; Gwathmey & Hajjar, 1990). Thus, the role of PKC in cardiac contractility is still unclear. Recently, proteolytic degradation of PKC has been proposed as a possible mechanism for the phorbol ester-induced cellular responses observed in a variety of cell types (Tapley & Murray, 1985; Pontremoli *et al.*, 1986; Chida *et al.*, 1986). The cleavage of the membrane-bound PKC by a Ca^{2+} -requiring proteinase such as calpain produces a catalytically active fragment which is then released into the cytosol fraction as a Ca^{2+} /phospholipid-independent kinase (Kishimoto *et al.*, 1983; Melloni *et al.*, 1986; Kishimoto, 1990). Based on these findings, we com-

pared the mode of activation of PKC and the contractility induced by an α_1 -adrenoceptor agonist, phenylephrine, with those induced by a phorbol 12,13-dibutyrate (PDBu) in rat isolated left ventricular papillary muscles. Furthermore, the possibility that proteolytic degradation of PKC has a role in the PDBu-induced negative inotropic effect was examined by use of two calpain inhibitors, leupeptin and a microbial peptide isolated from *Aspergillus japonicus* (E-64).

Methods

Preparation and stimulation of rat papillary muscles

Male Sprague Dawley rats weighing 250–300 g were anaesthetized with an intraperitoneal injection of sodium pentobarbitone, and the hearts were quickly removed. Left ventricular papillary muscles, 0.8–1.0 mm in diameter, were suspended in an organ bath containing 20 ml Tyrode solution of the following composition (mM): NaCl 122.5, KCl 5.4, CaCl_2 1.8, MgCl_2 1.1, NaHCO_3 24 and glucose 10 (pH 7.4), aerated with 95% O_2 and 5% CO_2 at 32°C. The muscle was initially loaded with 500 mg and driven electrically by rectangular pulses with a frequency of 1 Hz, a duration of 10 ms and a voltage ranging from 2–4 V which was twice threshold. The isometric tension was measured by a force displacement transducer (Shinko-Tsusin, Japan. UL-2) and recorded on a pen-writing recorder (Seconic, Japan. SS-250F) through an amplifier (Shinko-Tsusin, DS-601B). The preparation was allowed to equilibrate for 50 min and was then treated with 10 μM phenylephrine or 0.1 μM phorbol 12,13-dibutyrate (PDBu) for the indicated periods. When phenylephrine was used, 0.3 μM propranolol was always present to inhibit β -adrenoceptor stimulation at this phenylephrine concentration. We used PDBu because of its lower hydrophobicity (compared to other potent phorbol esters) that could facilitate association of the phorbol ester with the inner leaflet of the sarcolemmal membrane of intact tissues (Kikkawa *et al.*, 1983). A calpain inhibitor (1 mM), either

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leupeptin or E-64 (microbial peptide isolated from *Aspergillus japonicus*), was added to the buffer 60 min before the addition of PDBu. Changes in the contractility after the administration of PDBu were measured at the indicated times and were expressed as a % of the contractility just before the addition of PDBu.

Determination of protein kinase C

At the end of each experiment, the single papillary muscle was quickly blotted and frozen in liquid nitrogen. The frozen muscles were weighed and homogenized in a glass homogenizer to which 1.0 ml of ice-cold buffer A (20 mM Tris/HCl, pH 7.4, 250 mM sucrose, 5 mM diNa-EDTA 1, 1 mM EGTA) containing 0.005% leupeptin, 100 units ml^{-1} aprotinin and 0.4 mM phenylmethylsulphonylfluoride (PMSF) was added. The homogenates were centrifuged at 3,000 g for 20 min. The supernatants were centrifuged again at 105,000 g for 90 min. The resulting supernatants were saved as cytosolic fractions. The pellets resulting from the 105,000 g centrifugation were suspended in 0.5 ml of buffer A containing 0.1% Triton X-100. After 15 min of incubation, this suspension was centrifuged again at 105,000 g for 60 min, and the resulting supernatant was saved as a solubilized particulate fraction. Both fractions were applied to a 0.2 ml column of DE-52 (Whatman Inc., NJ, U.S.A.) equilibrated with buffer B (buffer A minus sucrose). After being washed with 10 ml of buffer B, PKC was eluted by the addition of buffer B containing 0.1 M NaCl (PKC fraction). In the case of the cytosolic fraction, the column was further eluted with buffer B containing 0.4 M NaCl to determine Ca^{2+} /phospholipid-independent protein kinase activity (PKI fraction) (Chambers & Eilon, 1988).

PKC activity was determined by measuring the incorporation of ^{32}P from [$\gamma\text{-}^{32}\text{P}$]-ATP into histone H1 as described by Kishimoto *et al.* (1983), with a slight modification. The standard reaction mixture of 0.25 ml contained 20 mM Tris-HCl (pH 7.4), 10 mM $\text{Mg}(\text{SO}_4)_2$, 0.2 mg ml^{-1} histone H1, 50 μM [$\gamma\text{-}^{32}\text{P}$]-ATP (40–60 c.p.m. pmol^{-1}), 3 mM CaCl_2 , 20 $\mu\text{g ml}^{-1}$ phosphatidylserine (PS), 2 $\mu\text{g ml}^{-1}$ diolein and 20–80 μl of PKC fraction. After 20 min of incubation at 30°C, the reaction was terminated by the addition of 3 ml of 25% trichloroacetic acid (TCA). Acid-precipitable materials

were collected on a cellulose nitrate membrane filter with 0.45 μm pores (Toyo Roshi, Tokyo, Japan), washed with 25% TCA and transferred to a vial to count the radioactivity. PKC activity was defined as the difference between ^{32}P incorporation into histone in the presence and absence of calcium and phospholipid. Ca^{2+} /phospholipid-independent kinase activity was measured in the PKI fraction and was defined as the ^{32}P incorporation in the presence of EGTA instead of Ca^{2+} and phospholipid. Under these conditions, the ^{32}P incorporation was linear with time up to 30 min. Protein concentration was determined by Lowry's method (Lowry *et al.*, 1951), with bovine albumin used as standard.

Statistical analysis

The data are expressed as mean $\pm$ s.e.mean. Student's *t* test was used for statistical analysis. The differences between two groups with *P* values less than 0.05 were considered to be significant.

Results

In rat left ventricular papillary muscles treated with 0.3 μM propranolol, 10 μM phenylephrine produced a triphasic inotropic response: a transient positive inotropic effect followed by a transient negative inotropic effect and then a sustained positive inotropic effect (Figure 1a, left). Changes in the transient positive, the transient negative or the sustained positive inotropic phase following the administration of phenylephrine were 106 ± 2 , 85 ± 2 , or $151 \pm 6\%$ (mean $\pm$ s.e.mean; $n = 6$) of that observed before the treatment, respectively, as has been shown in a previous study (Otani *et al.*, 1990). The sustained positive inotropic effect was reduced by staurosporine, a PKC inhibitor, to $118 \pm 7\%$ ($n = 6$, $P < 0.01$ vs phenylephrine only) of the basal contractility, without changes in the transient positive and the transient negative inotropic effects (Figure 1a, right). In contrast, 0.1 μM PDBu gradually decreased the contractile force, reaching $43 \pm 7\%$ ($n = 6$) of that observed before the treatment with PDBu, as shown in Figure 1b, left. A biologically inactive phorbol ester, 4 α -phorbol, had no effect at the same concentration (Figure 1b, right). Staurosporine had no effect

Figure 1 Representative traces of the effects of phenylephrine and phorbol esters on contractile force. Rat left ventricular papillary muscles pretreated with vehicle (0.01% dimethylsulphoxide, a, left) or 100 nM staurosporine (a, right) for 20 min were exposed to 10 μM phenylephrine (PE) in the presence of 0.03 μM propranolol. (b) The preparations were treated with 0.1 μM phorbol 12,13-dibutyrate PDBu (left), or 0.1 μM 4 α -phorbol (right) for 10 min.

on this PDBu-induced negative inotropic effect (data not shown).

PKC activities in cytosolic and particulate fractions of rat isolated left ventricular papillary muscles were measured before and after the addition of phenylephrine or phorbol esters (Figure 2). Phenylephrine ($10\ \mu\text{M}$) increased the PKC activity in the particulate fractions in a time-dependent manner, and the maximum level ($14 \pm 4\ \text{pmol min}^{-1}\ \text{mg}^{-1}$ protein, $n = 6$) was reached after 5 min (Figure 2a). Thereafter, the maximum activity in the particulate fraction remained unchanged or declined only slightly. The change in PKC activity in the cytosol fraction showed an inverse relationship to that in the membrane fraction. The minimum activity in the cytosolic fraction was $23 \pm 6\ \text{pmol min}^{-1}\ \text{mg}^{-1}$ protein ($n = 6$) at 5 min and remained unchanged thereafter.

We next examined the effect of the phorbol ester on the distribution of PKC (Figure 2b). PDBu at $0.1\ \mu\text{M}$ increased the PKC activity in the particulate fraction to $6.3 \pm 0.2\ \text{pmol min}^{-1}\ \text{mg}^{-1}$ protein ($n = 6$), which was almost half of the maximum activity induced by phenylephrine. This was achieved within 1 min and remained unchanged thereafter. In contrast with the action of phenylephrine, PKC activity in the cytosolic fraction continued to decrease significantly for 20 min. 4 α -Phorbol ($0.1\ \mu\text{M}$) had no effect on the PKC activities in either fraction (Figure 2b).

Next, we compared the effects of phenylephrine and PDBu on the total PKC activity (particulate + cytosol) and Ca^{2+} /phospholipid-independent kinase activities (PKI) in the cytosolic fraction. Phenylephrine did not significantly affect either of the kinase activities (Figure 3a), while PDBu

decreased the total PKC activity and increased the PKI activity in the cytosolic fraction (Figure 3b). These findings suggest that a decrease in total PKC activity during PDBu treatment is partly due to conversion of PKC into a Ca^{2+} /phospholipid-independent form of the enzyme, probably by Ca^{2+} -dependent proteinase (calpain).

If the phorbol ester-induced negative inotropic effect is mediated by the loss of PKC and the formation of PKI, pretreatment with a calpain inhibitor could attenuate this negative inotropic effect by reducing the proteolytic degradation of PKC. The following study was undertaken to test this possibility. The papillary muscles were first treated with 1 mM of one of the following two proteinase inhibitors (Melloni *et al.*, 1986; Wang, 1990), leupeptin or E-64, for 60 min and exposed to $0.1\ \mu\text{M}$ PDBu (Figure 4). These inhibitors attenuated the PDBu-induced negative inotropic effect, indicating that this negative inotropic effect occurred, at least in part, via stimulated formation of PKI.

Discussion

There is very little information regarding the role of PKC in the regulation of the cardiac contractility. α_1 -Agonists and phorbol esters are known to have different PKC-mediated effects on cardiac contractility: the former have positive inotropic effects (Otani *et al.*, 1990; Scholz *et al.*, 1988), while the latter have negative inotropic effects (Teutsch *et al.*, 1987; Gwathmey & Hajjar, 1990). This may be due to a difference in the way that these agents stimulate PKC. Indeed, the

Figure 2 Effects of phenylephrine and phorbol esters on protein kinase C (PKC) activity. Rat left ventricular papillary muscles were treated with $10\ \mu\text{M}$ phenylephrine (a) or $0.1\ \mu\text{M}$ phorbol esters (b) for the indicated periods. PKC activity was determined in the cytosolic (open symbols) and particulate (closed symbols) fractions, which were prepared as described in Methods. ○ ●, Phenylephrine (a); □ ■, PDBu (b); △ ▲, 4 α -phorbol (b). Each point represents the mean of 6 preparations; s.e.mean shown by vertical lines. * $P < 0.05$, ** $P < 0.01$ compared with the basal value obtained before the addition of the agents.

Figure 3 Effects of phenylephrine and phorbol 12,13-dibutyrate (PDBu) on the total protein kinase C (PKC) activity and Ca^{2+} /phospholipid-independent kinase (PKI) activity. Rat left ventricular papillary muscles were treated with $10\ \mu\text{M}$ phenylephrine (a) or $0.1\ \mu\text{M}$ PDBu (b) for the indicated periods. Total PKC activity (cytosol plus particulate PKC activity; ○) and PKI activity in cytosol (●) were measured as described in Methods. Each point represents the mean of 6 preparations; vertical lines show s.e.mean; * $P < 0.05$; ** $P < 0.01$ compared with the basal value.

Figure 4 Effects of leupeptin and E-64 on the phorbol 12,13-dibutyrate (PDBu)-induced negative inotropic response. Rat left ventricular papillary muscles were exposed to $0.1 \mu\text{M}$ PDBu with or without (control; ●) pretreatment with 1 mM of either leupeptin (○) or E-64 (△) for 60 min. Contractile force at the indicated time is expressed as a percentage of that obtained just before the addition of PDBu. Each point represents the mean of 6 preparations; vertical lines show s.e.mean. * $P < 0.05$; ** $P < 0.01$ compared with the corresponding control value at each time.

present study shows that these agents cause different redistribution patterns of PKC with opposite inotropic effects.

It has been shown that α_1 -adrenoceptor-stimulation of cardiac muscle causes receptor-linked degradation of PI-4, 5-P₂ and the resulting formation of IP₃ and DAG (Otani *et al.*, 1988; Scholz *et al.*, 1988). In the present study, α_1 -adrenoceptor stimulation with phenylephrine had a positive inotropic effect accompanied by an increase in the particulate PKC activity and a decrease in the cytosolic PKC activity, but PKI activity was unaffected by this treatment. A similar α_1 -adrenoceptor-mediated translocation of PKC after exposure to noradrenaline has been reported to occur in cultured cardiac myocytes and in brain neurones (Henrich & Simpson, 1988; Kalberg & Sumners, 1990). The present study and others suggest that an increase in the membrane-bound PKC activity via translocation from the cytosol in the presence of a PKC activator, DAG, is responsible for the α_1 -adrenoceptor-mediated cellular responses. This hypothesis is also supported by the finding that staurosporine inhibits the phenylephrine-induced positive inotropic effect (Figure 1), since staurosporine is reported to inhibit membrane-bound PKC activity at the concentration used in the present study ($0.1 \mu\text{M}$) by interacting with the catalytic subunit of PKC (Nakadate *et al.*, 1988). It has been suggested that an increase in the membrane-bound PKC in cardiac myocytes elicits sustained positive inotropic effect via Ca²⁺-channel activation (Dösemeci *et al.*, 1988) and/or Na⁺/H⁺ exchange activation (Ikeda *et al.*, 1988). The latter possibility is strongly supported by our recent paper which showed that the α_1 -adrenoceptor-mediated positive inotropic effect can be reduced by inhibitors of Na⁺/H⁺ exchange as well as by PKC inhibitors (Otani *et al.*, 1990).

On the other hand, PDBu increased only slightly particulate-associated PKC activity. In marked contrast to the change in particulate-associated PKC, PDBu decreased the total PKC activity and increased the PKI activity. These data are in good agreement with those obtained from cultured cardiac myocytes and neutrophils treated with phorbol myristate acetate (PMA) (Henrich & Simpson, 1988; Melloni *et al.*, 1986). The biologically inactive phorbol ester, 4 α -phorbol, did not cause such a redistribution of PKC, excluding the possibility that there was a nonspecific interaction of PKC with PDBu. When PKC is stimulated by a phorbol ester, membrane-bound PKC is cleaved by a Ca²⁺-dependent neutral protease such as calpain, to produce a catalytically active fragment, which does not require Ca²⁺ and phospholipid, and which acts as an irreversibly activated cytosolic kinase, i.e. PKI (Kishimoto *et al.*, 1983; Kishimoto, 1990). Such a protease-mediated down-regulation of PKC and the occurrence of PKI, has been implicated as an alternate mechanism of PKC activation and of the resulting cellular response by phorbol ester (Pontremoli *et al.*, 1986; Chida *et al.*, 1986; Melloni *et al.*, 1986). The present study shows that leupeptin and E-64, which are reported to be specific inhibitors of thiol proteinase (calpain) (Wang, 1990), attenuated the PDBu-induced negative inotropic effect, which was relatively insensitive to staurosporine. These two calpain inhibitors have been shown to prevent the loss of total PKC activity and the conversion of membrane-bound PKC to PKI at concentrations similar to or lower than those used in this study (Tapley & Murray, 1985; Pontremoli *et al.*, 1986; Melloni *et al.*, 1986). Furthermore, in neutrophils treated with phorbol 12-myristate 13-acetate (PMA), exocytosis of granule contents such as lysosomal enzymes and vitamin B₁₂ binding protein can be inhibited by leupeptin and E-64 (Pontremoli *et al.*, 1986), suggesting that these PMA-induced events occur via the conversion of membrane-bound PKC to cytosolic PKI. When these data are taken together, it is reasonable to conclude that increased PKI activity may be involved in the PDBu-induced negative inotropic effect. There is, however, controversial evidence that leupeptin does not affect the PKC-mediated responses in platelets treated with PMA (Alonso *et al.*, 1989). This may indicate that the roles of PKI may differ, depending on the cell and tissue type. Furthermore, although the mechanism by which PKI caused the negative inotropic effect was not addressed in this study, there are at least the following possibilities: (1) inhibition of Ca²⁺ accumulation in sarcoplasmic reticulum (Rogers *et al.*, 1990) and (2) decrease in myofilament Ca²⁺ sensitivity (Gwathmey & Hajjar, 1990). Either mechanism could result in a decrease in the force of contraction.

In conclusion, α_1 -adrenoceptor stimulation with phenylephrine and direct stimulation of PKC by PDBu accelerated the translocation of PKC from the cytosolic to the particulate fractions. Furthermore, the modes of redistribution were different; phenylephrine changed neither the total PKC activity nor PKI activity, while PDBu decreased the total PKC activity and increased PKI in the cytosol. Such a difference in the patterns of PKC distribution may account for the opposite inotropic effects after PKC stimulation.

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