Protein kinase C-mediated phospholipase A₂ activation, platelet-activating factor generation and prostacyclin release in spontaneously beating rat cardiomyocytes

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The expression of protein kinase C (PKC) isoenzymes and the effects of PKC activation on myocardial phospholipase A₂ (PLA₂) activity, platelet-activating factor (PAF) generation and eicosanoid release were studied in spontaneously beating cultured rat cardiomyocytes. Western blotting analysis indicated that these cells contain PKC α , β , δ and ζ , but not PKC γ or ϵ . Stimulation of cardiomyocytes with 4 β -phorbol 12-myristate 13-acetate (PMA) led to a rapid increase in particulate-bound PKC activity, a response attributed to the activation of α -, δ - and ζ -type PKCs but not β -type PKC. Translocation of PKC α , δ and ζ was accompanied by simultaneous increases in cellular lysophosphatidylcholine (lyso-PC), PAF, 15(*S*)-hydroxy-5,8,-

11,13-eicosatetraenoic acid (15-HETE), prostaglandin E₂ (PGE₂) and prostacyclin (PGI₂) generation, suggesting that one or more of these isoenzymes directly or indirectly activates a PLA₂ in these cells. Confirming this, 4 β -phorbol 12-monoacetate and 4 α -phorbol had no effect on cellular eicosanoid formation, while the PMA-induced response was fully abolished both in the presence of the PKC inhibitors staurosporine and CGP 41251 and in PKC-down-regulated cells. PKC α , δ and/or ζ therefore appear to play an important role in the PMA-mediated activation of cardiomyocyte PLA₂, an event leading to subsequent production of PGI₂, PGE₂, 15-HETE, lyso-PC and PAF in this tissue.

INTRODUCTION

The mammalian heart synthesizes and secretes an array of cyclooxygenase products, notably prostacyclin (PGI₂) and prostaglandin E₂ (PGE₂), eicosanoids that exhibit potent vasoactive effects (van Bilsen et al., 1989). Endogenous production of these compounds depends primarily on the enzymic release of arachidonate from cellular phospholipids, and various studies suggest that glycerophospholipid and tissue-specific phospholipases (A₂, C and/or D) are implicated in this step (Exton, 1990; Dennis et al., 1991; Currie et al., 1992).

Many stimuli inducing increased phospholipase activity also activate the phospholipid-dependent and calcium-sensitive serine/threonine protein kinase C (PKC) (for review see Kikkawa et al., 1989). Molecular cloning and biochemical analysis have shown that PKC represents a family of at least eight discrete enzymic subspecies exhibiting distinct structures, modes of activation, substrate specificities and tissue distributions (Nishizuka, 1988). PKC isoenzymes are categorized into two groups, according to the presence or the absence of the N-terminal conserved sequence required for the enzyme's activation by calcium. The former group consists of calcium-sensitive PKC α , β I, β II and γ , while the latter comprises calcium-insensitive PKC δ , ϵ , η and ζ (Kikkawa et al., 1989).

Activation of PKC is concomitant with increased phospholipid hydrolysis and/or eicosanoid production in numerous cell types, including vascular smooth muscle cells (Lang and Vallotton, 1989), glomerular mesangial cells (Gronich et al., 1988), renal endothelial cells (Parker et al., 1987) and pinealocytes (Ho and Klein, 1987). Conversely, the inhibition of PKC by various selective inhibitors (Parker et al., 1987; Halenda et al., 1989), as well as the down-regulation of the enzyme by prolonged ac-

tivation with tumour-promoting phorbol diesters, induces a powerful inhibition of arachidonate release (Godson et al., 1990), suggesting that active PKC is required for the normal cellular synthesis of eicosanoids. In view of both the heterogeneity and the specific subcellular distribution of the PKC isoenzyme family (Nishizuka, 1988; Mochly-Rosen et al., 1990), it is likely that certain PKC isoenzymes carry out distinct functions in the modulation of cellular phospholipid hydrolysis (Godson et al., 1990; Huwiler et al., 1991).

Little is known about the intracellular signalling mechanisms controlling myocardial prostaglandin production. A 40 kDa glycerophospholipid-specific myocardial phospholipase A₂ (PLA₂) has been purified and characterized (Hazen et al., 1990, 1991). While this enzyme appears to be mainly calcium-insensitive, it is activated by hypoxia, a condition known to activate PKC in other tissues (Louis et al., 1988; Cardell et al., 1991). It is therefore possible that myocardial eicosanoid generation is controlled by a specific PKC isoenzyme that directly or indirectly activates myocardial PLA₂. We tested this hypothesis by incubating cultured spontaneously beating rat cardiomyocytes with the PKC activator 4 β -phorbol 12-myristate 13-acetate (PMA) and by measuring the effect in terms of PKC and PLA₂ activation, cellular eicosanoid production, platelet-activating factor (PAF) generation and translocation of PKC isoenzymes from the cytosolic compartment to the cellular particulate fraction.

MATERIALS AND METHODS

Materials

[³H]Choline, [³H]PGE₂, 6-oxo-[³H]PGF_{1 α} and radioimmunoassay kits for the assessment of PGD₂, PGF_{2 α} , thromboxane B₂,

Abbreviations used: PKC, protein kinase C; PKM, degradation product of PKC; PMA, 4 β -phorbol 12-myristate 13-acetate; PDBu, 4 β -phorbol 12,13-dibutyrate; PLA₂, phospholipase A₂; lyso-PC, lysophosphatidylcholine; PAF, platelet-activating factor; PGI₂, prostacyclin (prostaglandin I₂); LTC₄ etc., leukotriene C₄ etc.; 15-HETE, 15(*S*)-hydroxy-5,8,11,13-eicosatetraenoic acid; ANP, atrial natriuretic factor; HRP, horseradish peroxidase.

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peptidoleukotriene (LTC₄, LTD₄ and LTE₄), 15(*S*)-hydroxy-5,8,11,13-eicosatetraenoic acid (15-HETE) and PAF formation were obtained from Amersham International (Amersham, Bucks., U.K.). Phorbol diesters and derivatives, staurosporine, quinacrine and phospholipids were from Sigma (St. Louis, MO, U.S.A.). McCoy's modified 5A medium, fetal calf serum and rabbit polyclonal antibodies against PKC δ , ϵ and ζ were from Gibco (Basel, Switzerland). Monoclonal antibodies directed against PKC α , β (β I and β II) and γ peptide sequences were obtained from Seikagaku America Inc. (Rockville, MD, U.S.A.). ¹²⁵I-labelled sheep anti-mouse and anti-rabbit antibodies were obtained from NEN. Horseradish peroxidase (HRP)-conjugated goat anti-rabbit and acid phosphatase-conjugated goat anti-mouse antibodies were purchased from Bio-Rad (Brussels, Belgium). The PKC inhibitor CGP 41251 was a gift from Ciba-Geigy (Basel, Switzerland). Anti-PGE₂ and anti-6-oxo-PGF_{1 α} antisera were generously donated by the Institut Pasteur (Paris, France) and Dr. M. J. Dunn (Division of Nephrology, Case Western Reserve University, Cleveland, OH, U.S.A.) respectively.

Cell culture

Neonatal rat cardiomyocytes were obtained from 1–2-day-old Wistar rats according to Kem et al. (1991). The majority of cultured cells (i.e. > 90%) began to spontaneously contract within 24 h of plating (40–80 beats/min), released atrial natriuretic peptide (ANP; ~ 5 ng/h per 10⁶ cells) and exhibited positive staining for pro-ANP, a precursor form of α ANP and a specific cardiomyocyte marker. PGI₂ production co-purified with cellular ANP secretion when mesenchymal cells were eliminated from culture according to Blondel et al. (1971), indicating that PGI₂ production originates from myoblasts and not from contaminating cell types. Substantiating this, cultured myocytes did not contain ϵ -type PKC (a rat fibroblast marker; Borner et al., 1992) or bind acetylated low-density lipoprotein (endothelial cell marker; Stein and Stein, 1980), while phorbol diester-induced PGI₂ production was strongly correlated with ANP release ($r = 0.93$, $P < 0.0005$, $n = 9$). Confluent cells were used on the third day of culture for all experiments described herein.

Determination of eicosanoid formation

Six-well tissue culture plates containing spontaneously beating confluent cardiomyocyte monolayers (~ 0.4 mg of cell protein/well) were washed and incubated for 10 min at 37 °C with 2 ml of Krebs–Ringer buffer containing 0.2% (w/v) BSA and 0.2% (w/v) glucose. After replacing the supernatant with 1 ml of fresh buffer, the cells were incubated at 37 °C for the indicated times in the presence of the various pharmacological agents. Eicosanoid release was determined according to radioimmunological methods previously described (Wuthrich et al., 1986), or as described in the various kits (Amersham). As a control, qualitative t.l.c. analysis of cardiomyocyte eicosanoid production was performed by extracting eicosanoids from 2 litres of PMA-stimulated culture medium, followed by separation using the ethyl acetate-based solvent system described by Hurst et al. (1987). T.l.c. results paralleled those obtained by radioimmunoassay.

Subcellular fractionation and determination of PKC activity

PMA-stimulated cells were subfractionated as previously described (Lang and Vallotton, 1989). Semi-purification of PKC was carried out by DEAE-Sepharose chromatography according to the method of Godson et al. (1990). Eluates were assayed for PKC activity by measuring phosphorylation of histone III-S in

the presence and absence of lipid activators, as described by Lang and Vallotton (1989).

Electrophoresis and immunoblotting

Protein extracts were separated by SDS/PAGE on a 10% slab gel and transferred to nitrocellulose membranes according to the method of Towbin et al. (1979). Anti-PKC antibody binding was carried out according to Borner et al. (1992), using monoclonal antibodies specifically directed against PKC isoforms α , β (β I and β II) and γ , or polyclonal rabbit antibodies specifically directed against PKC δ , ϵ and ζ peptide segments. Following incubation with ¹²⁵I-labelled sheep antibody, membranes were washed and autoradiographed at –70 °C for either 3–4 days (PKC α , β , δ and ζ) or > 30 days (PKC γ and ϵ). Alternatively, anti-PKC antibody binding was detected by incubating membranes with either HRP-conjugated anti-rabbit IgG (for PKC δ , ϵ and ζ) or alkaline phosphatase-conjugated anti-mouse IgG (for PKC α , β and γ), followed by colorimetric detection (Bio-Rad).

Lysophosphatidylcholine (lyso-PC) and PAF determination

Lyso-PC and PAF determinations were carried out according to the method of Ho and Klein (1987), modified for lyso-PC determination in cultured cells. Preconfluent monolayers (90 mm Petri dishes; second day of culture) were labelled for 24 h with 5 ml of McCoy's modified 5A medium containing [³H]choline (10 μ Ci/ml) and stimulated for the indicated times with 0.1 μ M PMA. Cellular phospholipids were extracted in chloroform/methanol (2:1, v/v), and PC, lyso-PC and PAF were separated by t.l.c. using a chloroform/methanol/acetic acid/water (55:43:4:3, by vol.) solvent system. While adequate separation of authentic bovine heart PC, lyso-PC and PAF was achieved (R_F values of 0.33, 0.05 and 0.2 respectively), lyso-PAF co-migrated with lyso-PC in this system. Plasmalogen contaminants present in the PC standard could not be resolved from authentic PC (results not shown). Results were analysed by means of a Berthold linear t.l.c. analyser, as well as by scraping and scintillation counting. As a control, radioimmunological determination of PMA-induced PAF formation was carried out by assaying nitrogen-dried chloroform/methanol extracts resuspended in 100 μ l of Tris/HCl buffer (pH 7.4).

Statistical analysis

Student–Fisher unpaired bilateral t tests and/or ANOVA using the Scheffe F -test criterion for unbalanced groups were used as applicable. A value of $P < 0.05$ was accepted as statistically significant. Results represent the means $\pm$ S.E.M. of at least three experiments performed in duplicate or triplicate.

RESULTS

Phorbol diester-induced activation of cardiomyocyte PKC

Incubation of cultured neonatal rat cardiomyocytes with PMA (0.1 μ M) led to a rapid increase in particulate-bound PKC activity (Figure 1a). The response was biphasic, with maximal activation of PKC at 15 min ($+58 \pm 8.3\%$, $n = 8$) being followed by the rapid disappearance of membranous PKC activity at 60 min ($-70 \pm 7.4\%$, $t_{1/2} = 12$ min, $n = 4$). Overall, the increase in particulate-bound PKC activity was accompanied by a corresponding decrease in cytosolic PKC activity ($-64.4 \pm 6.8\%$ at 15 min, $n = 8$).

Immunoblotting experiments indicated that cultured cells contain PKC isoforms α , β , δ and ζ (~ 80 kDa bands; Figure 1b). PKC γ and ϵ were not detected in particulate or cytosolic

Figure 1 Phorbol diester-induced PKC activation in rat cardiomyocytes

(a) Cultured cardiomyocytes were stimulated with 0.1 μ M PMA for the indicated times, subfractionated and assayed for PKC activity as described in the Materials and methods section. Membranous (●) and cytosolic (○) PKC activity is expressed as a percentage of control (basal) values. (b) PMA-induced translocation of α -, δ -, and ζ -type PKCs. Cells were stimulated with 0.1 μ M PMA for 15 min, subfractionated and Western blotting analysis was carried out as described in the Materials and methods section. Upper row, membranous cell fractions; lower row, cytosolic cell fractions. The indicated isoenzyme-specific anti-PKC antisera were used. Lanes 1, controls; lanes 2, PMA-treated cells. Blots for α -, β -, δ - and ζ -type PKCs were autoradiographed for 3–4 days, while those blotted for PKC γ and PKC ϵ were exposed for 30 days. Results represent the means of 4–8 experiments performed in duplicate (a) or are representative results of 4–8 identical experiments (b).

cardiomyocyte preparations, despite prolonged autoradiography, massive overloading of PAGE wells and attempts at colorimetric detection. While the α , δ and ζ isoenzymes were detected in both the cytosolic and particulate cell fractions, localization of the β isoenzyme was restricted to the cytosol, where it was mainly detected in the proteolytically degraded 68 kDa form termed 'PKM' (Girard et al., 1986). Degraded forms were also detected for the α , δ and ζ PKC isoenzymes, with their apparent molecular masses varying from 68 kDa for α -type PKM to 40 and 50 kDa for δ and ζ PKMs. Treatment with 0.1 μ M PMA for 15 min resulted in a marked increase in immunodetectable PKC α , δ and ζ in the particulate fraction (Figure 1b, upper row), a response accompanied by a decrease in PKC α , δ and ζ in the cytosolic fraction (Figure 1b, lower row). Stimulation with PMA had no effect on the amount of β -type PKC detected in the particulate fraction (none), nor on the levels of cytosolic α -, β -, δ - or ζ -type PKMs.

Phorbol diester-induced PLA₂ activation, PAF production and eicosanoid formation

Stimulation of [³H]choline-labelled cardiomyocyte cultures

Figure 2 Phorbol diester-induced PAF production

[³H]Choline-labelled cardiomyocyte cultures were exposed to 0.1 μ M PMA for the indicated times and PAF production was determined as described in the Materials and methods section. ●, PMA-stimulated cells; ○, controls. Results represent the means $\pm$ S.E.M. of 4–6 experiments performed in duplicate.

with 0.1 μ M PMA for 30 min led to a 41% increase in lyso-PC and/or lyso-PAF production (from 25522 ± 1210 to 35970 ± 1870 c.p.m./Petri dish; $n = 3$). This response was accompanied by a sustained increase in PAF production, as ascertained both by t.l.c. ($+59 \pm 9.4\%$ at 30 min; $n = 4$; see Figure 2) and in a scintillation proximity assay (from 22.0 ± 5.3 to 85.1 ± 10.6 pg/h per mg of cell protein; $n = 3$).

As shown in Figure 3(a), PMA-induced eicosanoid generation exhibited kinetics identical to those observed for PMA-induced PAF production. PMA-induced PGI₂ generation was sustained throughout and beyond 1 h incubations ($+198 \pm 38\%$ at 1 h; $n = 8$), as cardiomyocytes continued to produce PGI₂ for up to 3 h after the initial stimulation ($+243 \pm 65\%$ at 3 h; $n = 4$; results not shown). The response was found to be concentration-dependent (Figure 3b), with the EC₅₀ for PMA-induced 6-oxo-PGF_{1 α} production being estimated at 2 ± 0.5 nM ($n = 6$).

PMA-stimulated rat cardiomyocytes also exhibited increases in the synthesis and secretion of PGE₂ (from 33.2 ± 15.6 to 242.2 ± 36.2 pg/h per mg of cell protein; $n = 4$) and 15-HETE (from 55.0 ± 12.4 to 132.2 ± 15.6 pg/h per mg of cell protein; $n = 3$), and to a lesser extent of thromboxane B₂ (from 2.1 ± 1.8 to 4.6 ± 2.0 pg/h per mg of cell protein; increase insignificant at $n = 3$). While PGD₂, PGF_{2 α} and the peptidoleukotrienes LTC₄, LTD₄ and LTE₄ were not detected by direct radioimmunoassay of the stimulation media, qualitative t.l.c. analysis indicated that trace amounts of PGF_{2 α} were released into the media of PMA-stimulated cells (results not shown).

Mechanism of phorbol diester-induced PGI₂ production

We further investigated the specificity of PMA-induced cardiomyocyte PGI₂ production by incubating cells with 4 β -phorbol 12-monoacetate and 4 α -phorbol, which are inactive PMA analogues lacking the diester configuration and/or the 4 β stereochemistry required for specific PKC binding and activation (Nishizuka, 1988). As expected, 1 μ M concentrations of both 4 β -phorbol 12-monoacetate and 4 α -phorbol were ineffective in promoting 6-oxo-PGF_{1 α} production (Table 1). Conversely, the active PMA analogue 4 β -phorbol 12,13-dibutyrate (PDBu;

Figure 3 Phorbol diester-induced 6-oxo-PGF_{1α} production

Cardiomyocytes were incubated with 0.1 μM PMA for the indicated times (a) or with various concentrations of PMA for 1 h (b) and 6-oxo-PGF_{1α} determinations were carried out as described in the Materials and methods section. (a) Time course of PMA-induced PGI₂ production: ●, PMA-stimulated cells; ○, controls. (b) Concentration-dependence of PMA-induced PGI₂ production. Results shown represent the means ± S.E.M. of 6–8 experiments performed in triplicate determinations.

0.1 μM) mimicked PMA-induced PGI₂ release (238 ± 57%; *n* = 4), while the PKC inhibitors CGP 41251 and staurosporine totally abolished PDBu- and/or PMA-induced 6-oxo-PGF_{1α} production in these cells (Table 1). Finally, PMA-induced PGI₂ production was also inhibited in the presence of the purported PLA₂ inhibitor quinacrine (10 μM; 94.2% inhibition, see Table 1), a compound which failed to inhibit semi-purified PKC when tested in the activity assay used in establishing the time course of PKC activation shown in Figure 1 (activities: semi-purified PKC, 13862 ± 2405 c.p.m./10 min per mg of protein; semi-purified PKC + 10 μM quinacrine, 14989 ± 2604 c.p.m./10 min per mg of protein; *n* = 3).

As a final control, we tested the effect of PKC down-regulation on PMA-induced PGI₂ production. Figure 4 shows that pre-treatment of cells for 15 min or 1, 3, or 24 h with phorbol diester severely impaired PMA-induced PGI₂ formation (84–95% inhibition). This result was in agreement with the 15 min time

Table 1 Effect of PMA analogues and PKC and PLA₂ inhibition on 6-oxo-PGF_{1α} production

Cultured cardiomyocytes were incubated with PMA (0.1 μM), PDBu (0.1 μM), 4β-phorbol 12-monoacetate (12-PA, 1 μM) or 4α-phorbol (1 μM) in the presence or absence of staurosporine (1 μM), CGP 41251 (1 μM) or quinacrine (10 μM) for 1 h, and 6-oxo-PGF_{1α} was determined as described in the Materials and methods section. Results represent the means ± S.E.M. of the indicated numbers of experiments (in parentheses) performed in triplicate.

Treatment	6-Oxo-PGF _{1α} production (pg/h per mg of cell protein)			
	None	Staurosporine (1 μM)	CGP 41251 (1 μM)	Quinacrine (10 μM)
Control	120 ± 10 (14)	86 ± 10 (8)	77 ± 17 (4)	86 ± 8 (4)
PMA (0.1 μM)	379 ± 48 (14)	94 ± 11 (8)	86 ± 24 (4)	101 ± 20 (4)
PDBu (0.1 μM)	406 ± 69 (4)	112 ± 16 (3)	—	—
12-PA (1 μM)	164 ± 22 (4)	99 ± 26 (3)	—	—
4α-Phorbol (1 μM)	136 ± 24 (4)	75 ± 25 (3)	—	—

Figure 4 Effect of PKC down-regulation on PMA-induced 6-oxo-PGF_{1α} production

Cultured cardiomyocytes were pretreated with PMA (0.1 μM) for the indicated times, washed twice in Krebs–Ringer buffer and further stimulated with 0.1 μM PMA for 1 h. PGI₂ release was determined as described in the Materials and methods section. Results represent the means ± S.E.M. of 4–5 experiments performed in triplicate determinations.

course of PMA-induced PKC activation depicted in Figure 1, and suggests that PMA-induced PGI₂ generation is inversely correlated to the amount of cytosolic PKC which is available for translocation to the membranous fraction.

DISCUSSION

Our results strongly suggest that phorbol diester-induced PGI₂ production in cultured neonatal rat cardiomyocytes is a PKC α-, δ- and/or ζ-dependent, PLA₂-mediated process. This is supported by evidence that activation of these isoenzymes occurs simultaneously with increases in lyso-PC, PAF, PGI₂, PGE₂ and 15-HETE formation. Indeed, PMA-induced PGI₂ generation and PAF production exhibit strikingly similar kinetic profiles, suggesting that a PLA₂ is involved in PMA-induced eicosanoid generation in these cells. Furthermore, PMA-induced PGI₂

generation displays an EC₅₀ consistent with the K_d for phorbol diester binding in this tissue (~2 nM; see Limas and Limas, 1986), while the inactive phorbol ester analogues 4 β -phorbol 12-monoacetate and 4 α phorbol had no effect on PGI₂ release. Finally, PMA-induced PGI₂ production was massively inhibited by the PKC inhibitors CGP 41251 and staurosporine, by the PLA₂ inhibitor quinacrine and by down-regulation of cellular PKC activity.

Our findings are consistent with previous reports indicating that phorbol diester-induced activation of calcium-sensitive PKC α is concomitant with increased arachidonic acid release, prostaglandin formation and/or lysophospholipid production in both canine kidney endothelial cells and rat mesangial cells (Godson et al., 1990; Huwiler et al., 1991). However, our results further indicate that the calcium-insensitive δ and ζ PKC isoenzymes may also play a role in PKC-mediated PLA₂ activation. Interestingly, we have found that simultaneous activation of PKC isoforms α , δ and ζ also occurs in phorbol diester-stimulated rat aortic smooth muscle and transformed Chinese hamster ovary cells (D. J. Church and U. Lang, unpublished work). In this light, it is likely that one of these isoforms is at the basis of PMA-induced PLA₂ activation, as all three isoenzymes are either widely or universally expressed (Kikkawa et al., 1989; Mizuno et al., 1991), and a voluminous literature attests to the multitude of tissues generating lysophospholipids upon activation of PKC.

Short-term (10–20 min) phorbol diester-induced myocardial PKC activation has previously been described in both beating rat heart and cultured cardiomyocytes (Yuan et al., 1987; Capogrossi et al., 1990). To the best of our knowledge, the present study constitutes the first full description of the rapid kinetics of PKC down-regulation in cultured spontaneously beating rat cardiomyocytes. While PMA induced a biphasic response in terms of membranous PKC activation in these cells, the disappearance of membranous cardiomyocyte PKC activity occurs more rapidly than that observed in other tissues (Lang and Vallotton, 1989). Furthermore, the finding that cultured cardiomyocytes contain α , δ and ζ 80 kDa PKCs, as well as relatively large amounts of various isoenzymic forms of PKM, is in agreement with previous immunoblotting, chromatographic and immunocytochemical studies performed with rat and bovine heart tissue (Girard et al., 1986; Mochly-Rosen et al., 1990; Allen and Katz, 1991; Mizuno et al., 1991). While the existence of myocardial calcium-insensitive PKC ϵ has previously been inferred by Northern blotting analysis of rat myocardium (Schaap et al., 1989), our immunoblotting results indicate that 3-day-old cultured neonatal rat cardiomyocytes do not express this isoenzyme, and further suggest that if PKC ϵ is indeed present in rat heart tissue, its expression occurs either in non-myocyte cells or during more advanced stages of myocardial development.

It has been shown that micromolar concentrations of non-esterified fatty acids and lysophospholipids activate PKC in cell-free systems *in vitro* (Oishi et al., 1988; Shinomura et al., 1991). In this context, our results clearly indicate that PMA-induced cardiomyocyte eicosanoid generation is a PKC-mediated event. Indeed, the presence of 80 kDa cytosolic PKC appears to be an absolute prerequisite for PMA-induced PGI₂ production in these cells, since PKC down-regulation massively inhibited PMA-induced eicosanoid formation. It can be surmised that a certain amount of cytosolic PKC must be readily available in the basal (i.e. non-stimulated) state in order for PMA to induce PKC translocation and subsequent PLA₂ activation.

The hypothesis that PKC activation leads to increased PLA₂ activity in cultured cardiomyocytes is re-inforced by the finding that PMA-induced eicosanoid production occurs simultaneously with cellular lyso-PC and PAF formation. While the inhibitory

effects of both staurosporine and quinacrine support this, both compounds are noted for their relative lack of specificity. However, CGP 41251 exhibits high specificity for PKC (Meyer et al., 1989), while quinacrine (10 μ M) failed to inhibit purified PKC when tested in the *in vitro* PKC assay system used in this study. Taken together, it appears that PKC is indeed directly or indirectly activating a PLA₂ in cultured cardiomyocytes.

There is reason to believe that the cardiac PLA₂ involved in PMA-induced lysophospholipid and eicosanoid production is considerably different from the cellular PLA₂s that have been identified, purified and characterized from other sources (Gronich et al., 1988; Halenda et al., 1989; Clark et al., 1991). Although the subcellular distribution of the 40 kDa cardiac PLA₂ appears to be calcium-sensitive (Hazen et al., 1991), the activity of this enzyme is unaffected by calcium concentration, making it one of the few calcium-insensitive cellular PLA₂s discovered to date. In this context, the calcium ionophore A23187 is a remarkably poor stimulator of eicosanoid production in these cells (Church et al., 1991).

Clearly, further studies need to be performed in order to determine whether the PKC-activated myocardial PLA₂ inferred by our studies bears a relationship to any of the receptors for activated PKC recently detected in neonatal rat heart tissue (Mochly-Rosen et al., 1991). In view of both the subcellular localization of activated 40 kDa myocardial PLA₂ (Hazen et al., 1990) and the fact that PKC activation leads to the phosphorylation of a 40–45 kDa sarcolemmal protein in both canine and avian cardiac tissue (Iwasa and Hosey, 1984; Yuan and Sen, 1986), myocardial PLA₂ may indeed be a substrate for α -, δ - or ζ -type PKC.

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