Functional inhibition of protein kinase C-mediated effects in myocardial tissue is due to the phosphatase 2A

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An endogenous protein which inhibits protein kinase C (PKC)-mediated effects has been detected in rat heart ventricular tissue. This functional PKC-inhibitory activity was completely abolished by okadaic acid, making it possible to measure PKC activity in non-purified cell fractions. This suggests that the PKC-inhibitory activity is a type 1 or 2A serine/threonine phosphatase. Confirming this, membrane and cytosolic PKC-inhibitory preparations were found to contain phosphatase activity which was suppressed by okadaic acid, exhibiting an IC_{50} (concn. required for 50% inhibition) of 1.5–2 nM. Furthermore, okadaic acid stimulated prostacyclin production in rat cardiomyocytes and aortic smooth-muscle cells and, like the PKC activator phorbol 12-myristate 13-acetate, it augmented the prostacyclin formation induced by the Ca^{2+} ionophore A23187. Our results strongly suggest that the endogenous PKC ‘inhibitor’ is the cellular phosphatase 2A, which plays an important role in regulating the phosphorylation level of PKC target proteins.

INTRODUCTION

Protein kinase C (PKC), a multifunctional serine/threonine protein kinase, is known to play an important role in cellular signal transduction [1,2]. Various physiological functions have been attributed to PKC, such as involvement in secretion and exocytosis, interaction and down-regulation of receptors, smooth-muscle contraction, gene expression and cell proliferation [2].

The enzyme is Ca^{2+} -sensitive and requires phospholipids, particularly phosphatidylserine, for its activation [1]. Agonist-stimulated phospholipid hydrolysis produces diacylglycerol, which activates PKC by promoting its association with the cell membrane. Tumour-promoting phorbol esters such as phorbol 12-myristate 13-acetate (PMA) and phorbol 12,13-dibutyrate can substitute for diacylglycerol as activators for the enzyme.

Little is known about the physiological inhibition of PKC, as there have been few studies concerning endogenous factors inhibiting PKC activity. Pearson *et al.* [3] have characterized a heat-stable 13.7 kDa inhibitor protein from bovine brain cytosol. Another heat-stable inhibitor of 19 kDa, possibly calmodulin, has been isolated from the same tissue [4], whereas in rat brain a heat-labile 20 kDa PKC inhibitor has been detected [5]. Toker *et al.* [6] have characterized three heat-sensitive PKC inhibitors (29–33 kDa) from sheep brain containing sequence homologies with lipocortins. An endogenous PKC inhibitor has also been found in human neutrophils [7].

The mechanism(s) by which these PKC inhibitors interact with PKC remains to be elucidated. Studies using the supposed phosphatase inhibitor NaF [6,7] indicate that these PKC inhibitors are not associated with phosphatase activity. They also appear not to interact with PKC substrates or cofactors [6,8]. However, Sahyoun *et al.* [9] observed that the cytosolic fraction of rat liver contains phosphatase activity which acts on histones phosphorylated by PKC.

In the present work we describe a functional myocardial PKC-inhibitory activity, which was found to be due to the cellular phosphatase 2A.

EXPERIMENTAL

Preparation of cytosolic and particulate fractions of rat heart ventricular tissue

Heart ventricular tissue from 50-day-old rats was washed and homogenized with 20 mM-Tris/HCl buffer, pH 7.5, containing 2 mM-EDTA, 10 mM-EGTA, 10 mM-dithiothreitol, 0.25 M-sucrose and 30 μ M-leupeptin (buffer A; 1 ml/heart ventricle). The homogenate was centrifuged at 100000 *g* for 1 h at 4 °C, and the supernatant was used as the cytosolic fraction. The pellet was incubated for 30 min at 20 °C with Triton X-100 (1% in buffer A), diluted to the original volume of the homogenate with buffer A (final Triton concn. 0.2%), and centrifuged at 100000 *g* for 1 h. The supernatant obtained was used as the solubilized particulate fraction.

Separation of PKC from its endogenous PKC ‘inhibitor(s)’

PKC activity was almost undetectable in crude cell fractions, but was expressed after DEAE-cellulose chromatography, indicating that crude cell fractions probably contain an inhibitor of PKC and/or a Ca^{2+} -dependent proteinase. The cell fractions were applied to a DEAE-cellulose column (2.5 cm $\times$ 0.9 cm) equilibrated at 4 °C in buffer B (20 mM-Tris/HCl, pH 7.5, 2 mM-EDTA, 2 mM-EGTA and 10 mM-dithiothreitol). The columns were washed with 2 $\times$ 1 ml of buffer B. No enzyme activity was detected in these eluates. The PKC activity and the PKC-inhibitory activity were eluted by using 14 ml of a linear NaCl gradient (0–0.3 M) in buffer B. The flow rate was 350 μ l/min, and 1.5 ml fractions were collected.

Determination of PKC activity and PKC ‘inhibitory activity’

A sample (50 μ l) of each fraction from the column was assayed for PKC activity as previously described [10]. Protein kinase activity was determined by measuring the transfer of 32 P from [γ - 32 P]ATP to histone III-S (Sigma, St. Louis, MO, U.S.A.). The final reaction mixture (240 μ l) contained (0.7–1.2) $\times$ 10⁶ c.p.m. of [γ - 32 P]ATP, 0.15 μ M-ATP, 0.46 mM- $CaCl_2$, 6.9 mM- $MgCl_2$ and 30 μ g of histone III-S. Each fraction was assayed in the absence

Abbreviations used: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; ANP, atrial natriuretic peptide.

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and presence of phosphatidylserine (80 μ g) and diolein (4 μ g). The reaction was carried out at 30 °C for 10 min and was stopped by adding 2 ml of 12% (w/v) trichloroacetic acid in the presence of 600 μ g of γ -globulin added as carrier. After centrifugation (3000 g, 2 min), the pellet was dissolved in 0.5 ml of 1 M-NaOH and precipitated again with 12% trichloroacetic acid. After a second centrifugation the protein precipitate was dissolved in 0.5 ml of 1 M-NaOH, and 32 P incorporation was measured by scintillation counting in Ultima Gold (Packard).

A sample (50 μ l) of each fraction resulting from the DEAE-cellulose chromatography of the ventricular cytosolic or membrane fraction was assayed for its inhibitory activity on the PKC-induced incorporation of 32 P into histone III-S. The pooled PKC-containing fractions depicted in Fig. 1 were used for monitoring PKC-inhibitory effects.

Determination of phosphatase activity

Histone III-S was phosphorylated with partially purified PKC in the presence of [γ - 32 P]ATP at 30 °C for 10 min as described for the PKC assay. Phosphorylation was stopped by incubation for 5 min at 50 °C. Then a sample (50–200 μ l) of the inhibitor, partially purified by DEAE-cellulose chromatography, was added in the presence and in the absence of 1 μ M-okadaic acid. The reaction (dephosphorylation) was carried out at 37 °C for various times and was stopped by adding 2 ml of 12% trichloroacetic acid in the presence of 600 μ g of γ -globulin. The amount of 32 P incorporated into histone III-S was determined as described for the PKC assay.

Cell cultures

Neonatal-rat ventricular cardiomyocytes were obtained from 1–2-day-old Wistar rats by the method of Kem *et al.* [11]. After sequential digestions with trypsin and DNAase, cells were cultured in 5 ml of McCoy's modified 5A medium containing insulin/transferrin/sodium selenite medium supplement (5 μ g/ml, 5 μ g/ml and 5 ng/ml respectively) and 10% (v/v) fetal-calf serum. Cultured cardiomyocytes began to contract spontaneously within 24 h of plating (30–60 beats/min), released atrial natriuretic peptide (ANP; $\approx$ 5 ng/h per 10^6 cells) and exhibited positive staining for pro-ANP, a precursor form of ANP and a specific cardiomyocyte marker [12]. Although the presence of contaminating cell types (e.g. endothelial cells) cannot be excluded, prostacyclin production was found to be strongly correlated with ANP release, suggesting that prostacyclin production is an indicator of cardiomyocytes stimulation. Confluent cells were used on the third day of culture for all experiments described herein.

Vascular smooth-muscle cells were isolated from thoracic aortas of female Wistar rats (40–50 days old) as previously described [10]. After enzymic dispersion with collagenase and elastase, cells were plated in 90 mm plastic Petri dishes or six-well culture plates and cultured in Dulbecco's modified Eagle's medium containing 10% fetal-calf serum. Confluent monolayers were obtained after 6–7 days of plating. Aortic smooth-muscle cells of passages 2–5 were used for the experiments described herein.

Functional tests

Six-well tissue-culture plates containing aortic smooth-muscle cells or rhythmically beating cardiomyocytes were washed and incubated for 20 min at 37 °C with 1 ml of a Krebs–Ringer buffer [10], containing 0.2% BSA and 0.2% glucose. The supernatant was then replaced by fresh buffer, and the cells were incubated at 37 °C with air/CO₂ (19:1), in the presence of various stimulating agents. At the end of the incubation period, the prostacyclin content of the media was determined by a specific radio-

immunoassay of its stable metabolite 6-oxo-prostaglandin F_{1 α} as described previously [10,13], by using a specific rabbit antiserum which was kindly provided by Dr M. J. Dunn (Division of Nephrology, Case Western Reserve University, Cleveland, OH, U.S.A.).

Statistical analysis

ANOVA using the Scheffe F-test criterion for unbalanced groups was applied for statistical comparison.

RESULTS AND DISCUSSION

Fig. 1 shows the elution patterns of PKC activity and PKC-inhibitory activities from DEAE-cellulose columns with a linear gradient of 0–0.3 M-NaCl. One peak of PKC-inhibitory activity was found in the membrane fractions of heart ventricular tissue, eluted after PKC, between 110 mM- and 240 mM-NaCl. In contrast, two peaks of PKC-inhibitory activity were eluted from the cytosol; a first, smaller one, eluted before PKC at approx. 14 mM-NaCl, and a second, much larger one, eluted after PKC between 110 mM- and 250 mM-NaCl (Fig. 1). In this study we examined the PKC-inhibitory activity eluted at the higher

Fig. 1. Elution of PKC and PKC-inhibitory activities from DEAE-cellulose chromatography

Cytosolic and membrane fractions were prepared from rat heart ventricular tissue and applied to a DEAE-cellulose column as described in the Experimental section. Elution was carried out with a linear (0–0.3 M) NaCl gradient. Samples of each fraction were assayed for PKC activity and for PKC-inhibitory activity (see the Experimental section). PKC activity ($\square$) is expressed as c.p.m. of 32 P incorporated into histone III-S/10 min per 100 μ l sample. PKC-inhibitory activity ($\blacksquare$) was tested by adding 50 μ l of each fraction to PKC samples of previously determined activity (defined as 0% inhibition). NaCl concentration (||||), determined by conductivity measurements, was found to increase from 0.01 M (fractions 1–3) to 0.25 M (fraction 11). The Figure represents a typical example from a series of seven experiments that consistently gave the same type of results.

Table 1. Influence of cytosolic and membrane PKC-inhibitor preparations on PKC activity

Cytosolic and membrane PKC activity eluted from DEAE-cellulose columns was measured without (control) and with membranous (Memb) or cytosolic (Cyt) PKC-inhibitor preparations (IP) (18 μ g of protein/50 μ l), in the presence and in the absence of 1 μ M okadaic acid (OA). PKC activity is expressed as c.p.m. of 32 P incorporated into histone III-S/10 min per 50 μ l sample. Each value is the mean $\pm$ S.E.M. of four determinations.

Treatment	PKC activity (c.p.m.)	
	Cytosol	Membrane
Control	3094 $\pm$ 75	1951 $\pm$ 47
Control + OA	3496 $\pm$ 95	2127 $\pm$ 55
Cyt IP	876 $\pm$ 104	430 $\pm$ 62
Memb IP	814 $\pm$ 89	397 $\pm$ 59
Cyt IP + OA	3214 $\pm$ 166	2012 $\pm$ 114
Memb IP + OA	3312 $\pm$ 281	2106 $\pm$ 161

concentrations of NaCl. The characterization of the first small peak of PKC-inhibitory activity eluted from cytosolic preparations and its relationship to the second large peak require further study.

The fractions containing the cytosolic and the membrane PKC 'inhibitor', eluted after the PKC, were pooled and were compared for their inhibitory action on cytosolic and membrane PKC activity. As shown in Table 1, these two 'inhibitors' decreased both cytosolic and membrane PKC activity to the same extent. Both inhibitors were heat-sensitive; inhibitory activity was fully retained after 5 min at 50 $^{\circ}$ C, but was completely abolished after heating for 5 min at 70 $^{\circ}$ C or 100 $^{\circ}$ C.

Exposure of cytosolic and membrane PKC inhibitor to trypsin (0.2 mg/ml) for 2 h at 37 $^{\circ}$ C also completely suppressed PKC-inhibitory activity, suggesting that the PKC inhibitor is a polypeptide. In the presence of the alleged phosphatase inhibitor NaF (10 mM), the heart ventricular PKC inhibitor retained its full activity. These results are in agreement with the findings by Toker *et al.* [6], who observed that sheep brain contains PKC-inhibitory activity which is eluted after PKC from DEAE-cellulose chromatography and which is also insensitive to the presence of NaF. Toker *et al.* [6] concluded that the PKC-inhibitory activity isolated from brain homogenate contains no phosphatase activity.

In the present study we have taken advantage of the recent availability of okadaic acid, a potent and specific inhibitor of serine/threonine protein phosphatases [14,15], to characterize the nature of the endogenous PKC-inhibitory activity in heart ventricular tissue. Okadaic acid, a polyether derivative of C₃₈ fatty acid [16], has a highly specific inhibitory action of type 1 and type 2A phosphatases [14,15]. It has been shown to cause a general stimulation of protein phosphorylation in intact cells without affecting any of the relevant protein kinases [17], and it has also been reported to be a potent tumour promoter that is not an activator of PKC [18].

Addition of 1 μ M-okadaic acid abolished all PKC-inhibitory activity eluted from DEAE-cellulose chromatography from cytosolic and membrane fractions of heart ventricular tissue, as shown in Table 1. This observation indicates that the heart ventricular PKC 'inhibitor' is a serine/threonine phosphatase (phosphatase-1 and/or -2A).

As mentioned previously, PKC activity was not measurable in non-purified cell fractions. However, addition of 1 μ M-okadaic acid made it possible to measure PKC activity in small samples of non-purified cytosolic and membrane fractions from heart ven-

Table 2. Influence of okadaic acid on kinase activity in non-purified subcellular fractions of heart ventricular tissue

Kinase activity was measured in non-purified cytosolic and membrane fractions with or without 1 μ M-okadaic acid (OA). The difference between kinase activities in the presence and in the absence of 80 μ g of phosphatidylserine (PS) and 4 μ g of diolein (D) represents PKC activity. Protein kinase activity is expressed as c.p.m. of 32 P incorporated into histone III-S/10 min per 5 μ l sample. Values are means $\pm$ S.E.M. of six determinations.

Cell fraction	Kinase activity (c.p.m.)	
	Without PS/D	With PS/D
Cytosol		
Without OA	3884 $\pm$ 613	3641 $\pm$ 496
With OA	4240 $\pm$ 384	10824 $\pm$ 129
Membranes		
Without OA	2892 $\pm$ 461	2165 $\pm$ 320
With OA	2803 $\pm$ 285	6768 $\pm$ 488

tricular tissue, as shown in Table 2. The values obtained by this method were slightly but not significantly higher than those obtained by using DEAE-cellulose chromatography. In four different preparations the values of cytosolic and membrane PKC activity were found to be 116 $\pm$ 12% and 121 $\pm$ 16%, respectively, of the PKC activities measured in DEAE-cellulose-purified subcellular fractions. Similarly, addition of 0.1–1 μ M-okadaic acid made it also possible to measure PKC activity in small samples of crude cell fractions from cultured neonatal rat cardiomyocytes and aortic smooth-muscle cells (results not shown). Thus, in the presence of an adequate okadaic acid concentration for the amount of phosphatase(s) present in the sample, it is possible to measure PKC activity without prior chromatographic purification.

The elution pattern of PKC activity after DEAE-cellulose chromatography (Fig. 1) was not significantly changed when PKC activity was measured in the presence of 1 μ M-okadaic acid (results not shown), whereas all PKC-inhibitory activity was abolished, suggesting that DEAE-cellulose chromatography efficiently separates PKC from cellular phosphatase(s).

The conclusion that endogenous PKC-inhibitory activity is due to the presence of cellular phosphatase(s) was further confirmed by the fact that PKC-inhibitory preparations showed a pronounced phosphatase activity which was concentration- and time-dependent (Fig. 2). However, this phosphatase did not dephosphorylate entirely histone III-S that had been phosphorylated with partially purified PKC and [γ - 32 P]ATP in the presence of phospholipids. Interestingly, the remaining 8410 $\pm$ 644 c.p.m. of 32 P-labelled histone III-S corresponded to the 8559 $\pm$ 591 c.p.m. of 32 P incorporated in the absence of phospholipids. Thus the phosphatase activity of the PKC-inhibitory preparation appears to affect mainly PKC-specific phosphorylation. The same phosphatase activity was completely abolished by addition of 1 μ M-okadaic acid, as illustrated in Fig. 2.

Fig. 3 shows that okadaic acid inhibited this phosphatase activity in a concentration-dependent manner, exhibiting for the membrane and the cytosolic phosphatase a similar IC₅₀ of 1.5 nM and 2 nM respectively. These results suggest that in both cell fractions the endogenous PKC 'inhibitor' is the phosphatase 2A, since it has been reported that the IC₅₀ for phosphatase 2A ranges from 1 to 2 nM [14,15], whereas those for phosphatases 1 and 2B are in the ranges 270–500 nM and 3–10 μ M respectively, and phosphatase 2C is not affected by okadaic acid [14,15].

In order to study the cellular influence of serine/threonine

Fig. 2. Determination of phosphatase activity in PKC-inhibitory preparation

Histone III-S was phosphorylated with partially purified PKC in the presence of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and phospholipids at 30°C for 10 min (see the Experimental section). Dephosphorylation was carried out by adding $200\ \mu\text{l}$ of $20\ \text{mM}$ -Tris/HCl buffer, pH 7.4, containing PKC-inhibitory preparation from DEAE-cellulose chromatography, in the absence and in the presence of $1\ \mu\text{M}$ -okadaic acid. Control, $\square$ — $\square$; addition of PKC-inhibitory activity (62 and $124\ \mu\text{g}$ of protein, $\circ$ — $\circ$ and $\circ$ — $\circ$; addition of okadaic acid and PKC-inhibitory activity ($124\ \mu\text{g}$ of protein), $\bullet$ — $\bullet$. The reaction mixture was incubated at 37°C for the indicated times, and the phosphorylation level of histone III-S was determined as described in the Experimental section. Each value is the mean $\pm$ S.E.M. of 3–5 determinations.

Fig. 3. Concentration-dependence of the inhibitory effect of okadaic acid on phosphatase activity

Histone III-S was phosphorylated with partially purified PKC and $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the presence and absence of phospholipids at 30°C for 10 min (see the Experimental section). PKC-specific ^{32}P incorporation into histone III-S was calculated by subtracting the phospholipid-independent ^{32}P incorporation from the phospholipid-dependent ^{32}P incorporation. Phosphatase activity was investigated by adding $200\ \mu\text{l}$ of $20\ \text{mM}$ -Tris/HCl buffer, pH 7.4 (control), or $200\ \mu\text{l}$ of Tris/HCl buffer containing membrane ($\bullet$) or cytosolic ($\circ$) PKC-inhibitory preparation from DEAE-cellulose chromatography, in the presence of the indicated concentration of okadaic acid. The reaction mixture was incubated at 37°C for 40 min. The amount of ^{32}P incorporated into histone III-S was determined as described in the Experimental section and is expressed as % of control. Each value is the mean $\pm$ S.E.M. of 3–6 determinations.

Fig. 4. Effect of okadaic acid on prostacyclin production in rat cardiomyocytes and aortic smooth-muscle cells

Neonatal-rat ventricular cardiomyocytes and aortic smooth-muscle cells were isolated and cultured as described in the Experimental section. Confluent cultured cardiomyocytes (a) and aortic smooth-muscle cells (b) were stimulated with $0.1\ \mu\text{M}$ -PMA and/or $1\ \mu\text{M}$ -A23187 for 30 min in the absence ($\square$) and in the presence ($\boxplus$) of $1\ \mu\text{M}$ -okadaic acid. The incubation medium was removed and assayed for 6-oxo-prostaglandin $\text{F}_{1\alpha}$ release. Values represent means ($\pm$ S.E.M.) of 3–4 separate experiments performed in triplicate determinations.

phosphatase(s), we examined the effect of okadaic acid on cardiomyocytes and aortic smooth-muscle cells. In both cell types PMA has been shown to induce PKC activation, leading to prostacyclin production [10,19]. PMA has also been found to augment the prostacyclin response induced by increased cytosolic free Ca^{2+} concentrations [13,19]. As shown in Fig. 4, exposure to $1\ \mu\text{M}$ -okadaic acid for 30 min increased prostacyclin production from 0.15 ± 0.02 to $0.47 \pm 0.04\ \text{ng/mg}$ of cell protein in cardiomyocytes and from 0.14 ± 0.02 to $2.21 \pm 0.29\ \text{ng/mg}$ of cell protein in aortic smooth-muscle cells. Like PMA, okadaic acid augmented the A23187-induced prostacyclin response in both cell types. Since protein phosphatase-1 and -2A are likely to be the main enzymes that reverse the effects of PKC [20], it appears that a constant serine/threonine phosphatase activity is responsible for maintaining low levels of PKC-dependent phosphorylation in resting cells. Our results obtained with okadaic acid on prostacyclin production in cardiomyocytes and aortic smooth-muscle cells indeed resemble those observed with PMA. Similar observations have been made by Tanti *et al.* [21], studying the influence of PMA and okadaic acid on the uptake of 2-deoxyglucose by skeletal muscle. However, inhibition of phosphatase 2A by okadaic acid does not appear to mimic entirely the activation of PKC-dependent protein phosphorylation, since in aortic smooth-muscle cells stimulation of prostacyclin formation is greater with okadaic acid than with PMA ($P < 0.01$), whereas in cardiomyocytes the okadaic acid-induced response is weaker than that stimulated by PMA ($P < 0.01$) (Fig. 4).

In summary, our results suggest that the endogenous PKC-inhibitory activity detected in the cytosolic and membrane fractions of heart ventricular tissue is due to the cellular

phosphatase 2A playing an important role in regulating the phosphorylation level of PKC target proteins.

We thank Ms. C. Gerber-Wicht, Mrs. M. Rey and Mrs. M. Klein for their excellent technical assistance, Mrs. C. Gerber-Wicht and Mrs. M. Rey for skilful secretarial help and Dr. M. F. Rossier and Dr. A. M. Capponi for helpful discussions. This study was supported by Grants from the Swiss National Science Foundation (31.27727.89), from the Swiss Foundation of Cardiology, from the Geneva Cancer League and from the Sandoz Foundation for Advancement of Medical and Biological Sciences. D. J. C. was partially supported by a post-graduate scholarship from the Glaxo Institute of Molecular Biology, Geneva, Switzerland.

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Received 29 January 1992/23 March 1992; accepted 1 April 1992