Characterization of a specific form of protein kinase C overproduced by a C3H 10T $\frac{1}{2}$ cell line

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Murine embryo fibroblasts (C3H 10T $\frac{1}{2}$) which were genetically engineered to overproduce the β_1 isoform of protein kinase C (PKC- β_1) were used to obtain homogeneous preparations of PKC- β_1 for the purpose of characterizing the specific structural and functional properties of this isoform. Fractionation of PKC activity from these cells by hydroxyapatite chromatography produced one major peak, which represented 93 % of the total cellular PKC activity and was not detected in control cells. This major peak of activity was shown by Western-blotting analysis with a β_1 -specific antiserum to be the overproduced β_1 -isoform, and exhibited a band at 77 kDa. The functional properties of the overproduced PKC- β_1 were established with regard to phospholipid-dependence, Ca $^{2+}$ -dependence, responsiveness to a phorbol ester tumour promoter, activation by arachidonic acid (plus Ca $^{2+}$), and inhibition by known PKC inhibitors. From these studies we conclude that PKC- β_1 overproduced by C3H 10T $\frac{1}{2}$ cells exhibits the structural and functional properties previously ascribed to native PKC. Furthermore, these data provide the first definitive biochemical characteristics of this isoform of PKC.

INTRODUCTION

Protein kinase C (PKC) is a Ca $^{2+}$ - and phospholipid-dependent protein kinase which participates in the transduction of growth signals generated at the cell surface by various hormones and growth factors [1,2]. During regulated cell growth, stimulation of PKC is thought to occur via the agonist-induced generation of diacylglycerol, a product of phosphatidylinositol turnover [3]. PKC also functions as the major cellular receptor for the phorbol ester tumour promoters [1], a finding that has defined for this enzyme an instrumental role in carcinogenesis [4]. The diversity of processes in which PKC has been implicated [2,5–7] has led to continued interest in its structure, function and regulation.

Over the past few years, the use of PKC-specific oligonucleotide probes has demonstrated multiple PKC-encoding genes in cDNA libraries from rat brain [6–9]. Such findings have indicated that PKC activity is expressed by a family of at least seven functionally and structurally related isoenzymes. Preparation and identification of some of these isoforms from tissue homogenates have been facilitated by the use of hydroxyapatite chromatography [10–12]. The elution pattern of the PKC isoenzymes from rat brain describes three major peaks designated, in order of their elution, types I, II and III, which by immunochemical analysis correspond to the γ , β_1/β_2 and α isoforms respectively [13]. Because the three peaks often overlap and the positions in this profile of the other PKC isoforms are not known, there exists the possibility that each PKC type prepared by elution from hydroxyapatite is contaminated by other PKC isoforms. Nevertheless, studies

to examine these peak activities for their cofactor requirements, their responsiveness to tumour promoters [14,15] and to unsaturated fatty acids [16] have revealed subtle differences among the three PKC subtypes. Western-blotting analysis and immunofluorescent techniques using antisera directed against specific isoforms of PKC have provided evidence that the individual isoforms are distributed variously in many mammalian tissues and cell types (reviewed in ref. [6]). It is not yet known whether each isoform responds to specific external stimuli and/or selects unique cellular substrates. Until now, efforts to study *in vivo* a single PKC isoenzyme with regard to agonist-dependence and substrate specificity have been hampered by lack of a suitable system [6].

For the purpose of studying an individual PKC isoform both *in vivo* and *in vitro*, our laboratory has constructed two PKC- β_1 -overproducing cell lines, Rat 6 fibroblasts and C3H 10T $\frac{1}{2}$ murine embryo fibroblasts, each of which stably expresses the enzyme 50- and 11-fold higher respectively than their control cells. The method by which this was accomplished has been described elsewhere [17]. Briefly, the full-length PKC- β_1 cDNA was inserted into a pMV7 plasmid [18], and then packaged into defective retroviral particles by use of the ψ 2 cell system [19]. The viral particles were used to infect the recipient fibroblast cell lines, which consequently overproduced the PKC- β_1 protein. The growth-distorting effects engendered in Rat 6 fibroblasts by the overexpression of PKC- β_1 have been described [17], as well as a study examining the phenotypic alterations in the C3H 10T $\frac{1}{2}$ PKC-overexpression system [20].

The present paper describes the biochemical

Abbreviations used: PKC, protein kinase C; TPA, tetradecanoylphorbol acetate; H-7, 1-(5-isoquinolinesulphonyl)-2-methylpiperazine dihydrochloride; PS, phosphatidylserine; EGF, epidermal growth factor.

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characterization of the PKC- β_1 protein overproduced by C3H 10T $\frac{1}{2}$ cells. Our results demonstrate the homogeneity and identity of the PKC- β_1 activity by its chromatographic behaviour on hydroxyapatite, and by immunochemical assay. The functional character of the overproduced isoform is shown by its responsiveness to Ca^{2+} , phospholipid, arachidonic acid and phorbol ester, and by its sensitivity to known PKC inhibitors. Significantly, these studies provide the first definitive biochemical properties of a stably expressed and homogeneous population of a PKC isoform.

MATERIALS AND METHODS

Materials

Tissue-culture medium and calf serum were purchased from Flow Laboratories, 150 mm-diam. plates were from Nunc, Neomycin sulphate (G418) was from Gibco, and nitrocellulose paper was from Millipore (0.45 μm pore size). DEAE-Sephacel, histone III-S, tamoxifen and reagents for the determination of inorganic phosphorus were from Sigma. Phosphatidylserine (PS) was acquired from Avanti Polar Lipids (Pelham, AL, U.S.A.), arachidonic acid was from Nu-Chek-Prep (Elysian, MN, U.S.A.), phenyl-Sepharose was purchased from Pharmacia, hydroxyapatite (Bio-Gel HT) was from Bio-Rad, and [γ - ^{32}P]ATP (0.5–3 Ci/mmol) was a product of Amersham. The EGF-receptor synthetic peptide substrate (R-K-R-T-L-R-R-L) was synthesized by J. Wideman (Columbia University) and leupeptin was a gift from W. Troll (New York University). The inhibitors H-7 and staurosporine were purchased from Seikagaku Kogyo Co. (Tokyo, Japan). TPA was a product of LC Services (Woburn, MA, U.S.A.), and teleocidin was a gift from H. Fujiki (National Cancer Center Research Institute, Tokyo, Japan).

Cell culture

C3H 10T $\frac{1}{2}$ -PKC-4 cells were cultured in Dulbecco's modified Eagle's medium containing 10% (v/v) calf serum. Each frozen stock was subcultured for a maximum of 2 months. Except for the final passage before harvesting, the cells were maintained under selection with G418 (50 $\mu\text{g}/\text{ml}$) [17]. Cells were grown in 150 mm plates for 4 days or until confluent. These cells have an 11-fold increase in PKC activity as compared with control C3H 10T $\frac{1}{2}$ cells [20].

Isolation of PKC- β_1

PKC- β_1 overproduced in C3H 10T $\frac{1}{2}$ cells, or rat brain PKC activity, were isolated and partially purified by chromatography on DEAE-Sephacel, as previously described [17]. Typically, the specific activity of the enzyme at this stage was 11 units/mg for the overproduced PKC- β_1 and 4 units/mg for the mixture of isoenzymes obtained from rat brain. The PKC preparation, which had been eluted in Buffer A [20 mM-Tris, pH 7.5, 2 mM-EGTA, 2 mM-EDTA, 1 mM-dithiothreitol, 1 mM-phenylmethanesulphonyl fluoride, leupeptin (10 $\mu\text{g}/\text{ml}$), soya-bean trypsin inhibitor (10 $\mu\text{g}/\text{ml}$)] that contained 100 mM-NaCl (Buffer A-100), was then adjusted to 1 M-NaCl and placed on ice for at least 1 h. A 1 ml phenyl-Sepharose column was prepared by equilibrating the gel in 10 ml of Buffer A containing 1 M-NaCl (Buffer A-1000). After application of the PKC sample and collection of the flow-through volume, the column was washed with 3 ml

of Buffer A-1000 and then with 3 ml of Buffer A-600; the A-600 wash did not elute PKC activity from the column. Finally, the adsorbed PKC activity was eluted with 4 ml of Buffer A (containing no NaCl).

The enzyme fraction prepared by phenyl-Sepharose chromatography (86 units/mg) was concentrated 10-fold by using a Centricon-30 filtration device (Amicon) and centrifuging at 2500 rev./min for 30 min, at 4 °C. The concentrate (0.9 ml) was then dialysed for 1 h at 8 °C against 100 ml of Buffer H-20 [20 mM-potassium phosphate, pH 7.5, 0.5 mM-EDTA, 0.5 mM-EGTA, 10% (v/v) glycerol, 0.1 mM-dithiothreitol, 1 mM-phenylmethanesulphonyl fluoride, leupeptin (10 $\mu\text{g}/\text{ml}$) and soya-bean trypsin inhibitor (10 $\mu\text{g}/\text{ml}$)].

To prepare hydroxyapatite for chromatography, approx. 1 ml of the free gel was equilibrated in 20 mM-potassium phosphate/1 mM-dithiothreitol/10% glycerol. This was followed by heating the slurry to 80 °C for 15 min in order to de-aerate the gel [21]. The gel was allowed to settle in a 15 ml disposable tube, and the buffer was drained. PKC that had been dialysed against Buffer H-20 was added to 1 ml of hydroxyapatite for batchwise adsorption, which was conducted on ice for 1 h and included frequent rotation. The entire contents of the tube were then transferred to a disposable plastic Bio-Rad column containing a 0.1 ml cushion of hydroxyapatite equilibrated in H-20. The flow-through volume was collected, and the gel was washed with 5 ml of H-20, after which a gradient was initiated consisting of 20 ml of H-20 and 20 ml of H-200 (as Buffer H-20, but contains 200 mM-potassium phosphate). Fractions (1 ml) were collected (ISCO model 328 instrument) and elution was facilitated with a peristaltic pump (Buchler) operated at setting 7 (flow rate 4 ml/h), and by continuous monitoring at 280 nm (ISCO UV-5 instrument). The fractions were assayed for PKC activity and protein content. Fractions containing the peak of PKC activity were pooled; the specific activity was 105 units/mg. The potassium phosphate gradient was defined by assay of free P_i as measured by phosphomolybdate formation [22]. Protein was assayed with the Bio-Rad protein reagent with lysozyme as standard.

PKC assay

PKC activity was taken as the difference in the amount of [^{32}P]P $_i$ transferred from [γ - ^{32}P]ATP to a synthetic peptide sequence of the EGF receptor, R-K-R-T-L-R-R-L (30 μM), or histone III-S (0.75 mg/ml), in the absence or presence of PS (83 $\mu\text{g}/\text{ml}$), as described previously [17]. Assays typically contained 6–12 units/ml. One unit of PKC activity is 1 nmol of [^{32}P]P $_i$ transferred to the peptide substrate/min. For assays in which PKC inhibition was measured, stock solutions of inhibitor (1 mM) were made up in dimethyl sulfoxide. Working solutions were prepared by serial dilution into water. Final concentrations of 10–100 μM were included in the assay medium before initiation of the reaction by addition of [γ - ^{32}P]ATP. For assays in which response to phorbol ester was measured, the incubation medium included the indicated concentration of CaCl_2 (see legends) and PS (83 $\mu\text{g}/\text{ml}$), with or without either TPA or teleocidin (0.1 $\mu\text{g}/\text{ml}$). A stock solution (83 mg/ml) of arachidonic acid was prepared in benzene (purged with argon) and stored at –20 °C. Working solutions were freshly prepared by evaporating a portion (12 μl) of the stock with argon and solubilizing the residue in ethanol. All further

dilutions were carried out in ethanol. A 10 μ l sample of each working solution was added to assay tubes where the final volume was 120 μ l. Control experiments showed that PKC activity was unaffected by the presence of 8% ethanol in the assay medium.

Western-blotting analysis

Samples were subjected to electrophoresis [23] in SDS/8% polyacrylamide gels at 40 V for 12 h. Electroblooming on to nitrocellulose paper was carried out by a standard procedure [24]. Immunoanalysis was conducted with two different polyclonal antisera, diluted 1:2000, the respective epitopes of which are located specifically on either the regulatory or the catalytic subunit of PKC. One preparation, rabbit polyclonal 113, was formulated against a synthetic peptide representing the pseudosubstrate segment [25] in the N-terminal region of bovine PKC- α (amino acids 24–38 in ref. [26]). The other preparation, rabbit polyclonal β_1 A, courtesy of Dr. S. Jaken, W. Alton Jones Cell Science Center, was directed against a peptide representing a C-terminal sequence unique to rat brain PKC- β_1 (amino acids 659–671 in ref. [17]). Detection of immune complexes was by use of an iodinated donkey anti-rabbit IgG preparation (Amersham), followed by autoradiography at -70°C for 2 days.

RESULTS

Purification and immunochemical characterization of PKC- β_1

The C3H 10T $\frac{1}{2}$ -PKC-4 clone selected for the present study exhibited, relative to control cells, an 11-fold increase in total PKC activity [20]. In order to assess the functional and structural integrity of the overproduced protein product, we examined the PKC activity obtained from these cells with respect to the properties commonly ascribed to native rat brain PKC. In the past, hydroxyapatite chromatography has provided a method by which the β isoforms, collected as type II, were resolved from the other PKC isoforms present in rat brain. Using PKC activity that had been partially purified by chromatography on DEAE-Sephacel and phenyl-

Sephacel, we fractionated the enzyme on hydroxyapatite by using conditions of gradient elution sufficient to resolve three distinct peaks of PKC activity when we used a rat brain preparation (results not shown). The results, shown in Fig. 1, indicated that 93% of the total PKC activity obtained from the C3H 10T $\frac{1}{2}$ -PKC-4 cells was eluted as a single peak, representing the overexpressed PKC- β_1 . This major peak, which was eluted between 50 and 75 mM-potassium phosphate, could be distinguished from a second, minor, peak, which accounted for 7% of the total PKC activity and was eluted at approx. 90 mM-potassium phosphate. (Although the relative positions of the peaks were invariant, we observed some variability in the potassium phosphate concentration at which the major peak was eluted. For three independent preparations, the median salt concentration in the eluate containing the major peak was 80 ± 16 mM. A similar effect has been reported elsewhere for the rat brain isoenzymes [12]). The minor peak, which was the only PKC activity detected in the vector control cell line, C3H 10T $\frac{1}{2}$ -pMV7 (results not shown), and was eluted at approx. 100 mM-potassium phosphate, presumably represented the endogenous type III (PKC- α) previously detected in another fibroblast cell line [27].

The immunoreactivity of the PKC activity present in the eluate from DEAE-Sephacel was examined by Western-blotting analysis using two different antisera. One antibody preparation (polyclonal 113) recognized an epitope in the pseudo-substrate segment of the regulatory subunit of both PKC- α and PKC- β [24]. The second antibody preparation, polyclonal β_1 A, recognized a sequence in the C-terminal region of PKC- β_1 (see the Materials and methods section), which is unique to the β_1 isoform. Shown in Fig. 2 are the results of testing the immunoreactivity of both antisera with PKC activity from C3H 10T $\frac{1}{2}$ -PKC-4 cells, and the C3H 10T $\frac{1}{2}$ -pMV7 cells (vector control).

Bands that demonstrated immunoreactivity with antibody 113 were detected in both PKC preparations. In the vector control cells (lane 1) there was a moderately intense band present at 80 kDa, whereas an equal amount of protein from PKC- β_1 -overproducing cells (lane 2)

Fig. 1. Hydroxyapatite fractionation of partially purified PKC activity from C3H 10T $\frac{1}{2}$ -PKC-4 cells

PKC activity from C3H 10T $\frac{1}{2}$ -PKC-4 cells was isolated by DEAE-Sephacel chromatography [17], followed by chromatography on phenyl-Sepharose and hydroxyapatite, as described in the Materials and methods section. A representative elution profile is shown here: $\diamond$, PKC activity; $\circ$, A_{280} ; $\bullet$, [potassium phosphate] (mM).

Fig. 2. Immunoreactivity of total PKC activity from C3H 10T¹-PKC-4 cells

Western-blotting analysis was performed with eluates from DEAE-Sephacel and probed with two different antisera. Immunoreactivity was analysed with antibody 113 (lanes 1 and 2), which recognizes the pseudo-substrate segment in the *N*-terminal region of both PKC- α and PKC- β_1 , and with antiserum β_1A (lanes 3–5), which recognizes a *C*-terminal segment that is unique to the β_1 -isoform. PKC activities from the vector control cells C3H 10T¹-pMV7 (50 μ g; lanes 1 and 3), C3H 10T¹-PKC-4 cells (50 μ g; lanes 2 and 4) and rat brain (300 μ g; lane 5) were compared. See the Materials and methods section for further details.

there was a very prominent band at 77 kDa. In both control and overproducing cells there was a band at 90 kDa, whose significance is not known. Portions of these protein samples (each 50 μ g), and of a mixture of rat brain PKC isoenzymes (300 μ g), were run in parallel and immunoblotted with the β_1 -specific polyclonal antibody β_1A (lanes 3–5). Consistent with the hydroxyapatite-elution profile (results not shown), no signal was detected with the sample from control cells (lane 3), indicating the absence of PKC- β_1 in these cells. The same amount of protein from PKC- β_1 -overproducing cells, however, produced a single very dense band at 77 kDa (lane 4) which had a distinctly different position from the signal at 82 kDa given by a mixture of rat brain PKC isoenzymes (lane 5).

Functional properties of PKC- β_1

Having established that about 93% of the PKC activity in eluates from DEAE-Sephacel is attributable to PKC- β_1 , we used this fraction to determine some of the functional properties of the overproduced enzyme. We first examined its responsiveness to Ca^{2+} and the phorbol ester tumour promoter TPA. Typically, a preparation of PKC from rat brain is stimulated by increasing Ca^{2+} concentrations in the presence of PS, with an optimum Ca^{2+} value of 0.5–1.0 mM; the optimal Ca^{2+} shifts dramatically to lower Ca^{2+} concentrations in the presence of TPA or related compounds [28]. Shown in Fig. 3 is the Ca^{2+} -dependence of the PKC- β_1 protein product. In the absence of TPA, the PS-dependent enzyme activity increased gradually with increasing Ca^{2+} concentration, and maximal activity was attained at 500 μ M- Ca^{2+} . When TPA (0.1 μ g/ml) was present, the optimal Ca^{2+} con-

Fig. 3. Ca^{2+} -dependence in the absence and presence of TPA of PKC- β_1 isolated from C3H 10T¹-PKC-4 cells

PKC- β_1 was prepared by elution from DEAE-Sephacel (see the Materials and methods section). PKC activity was assayed at the indicated concentrations of Ca^{2+} , plus phosphatidylserine (83 μ g/ml) without (○) or with (●) TPA (0.1 μ g/ml). The data plotted highlight the effect of TPA on PKC activity at low concentrations of Ca^{2+} .

centration was shifted to 20 μ M. In a similar experiment with teleocidin, a tumour-promoting indole alkaloid known to activate rat brain PKC in a manner similar to that of TPA [29], we observed that the optimal Ca^{2+} concentration was shifted to 10 μ M (results not shown). We note that when no Ca^{2+} was added to the reaction mixture (ordinate value), TPA plus PS produced near-maximal PKC stimulation. This Ca^{2+} -independent activity has been observed previously with both TPA and teleocidin with a preparation of rat brain PKC [29], and specifically for type II PKC activity [14].

The activation of native PKC by PS is common to all of the known PKC isoenzymes [14]. We also found that

Fig. 4. Activation of PKC- β_1 by arachidonic acid and Ca^{2+}

PKC- β_1 , isolated by elution from DEAE-Sephacel, was assayed as described in the Materials and methods section, except that the indicated concentration of arachidonic acid was substituted for phosphatidylserine and all assays were done in the presence of either 0.5 mM- Ca^{2+} (●) or 0.5 mM-EGTA (○). For each condition, activity is reported as the difference between radioactivity (c.p.m.) observed in the presence of the indicated concentration of arachidonic acid and that in the absence of arachidonic acid.

Table 1. Effects of various inhibitors on PKC- β_1

PKC was isolated by elution from DEAE-Sephacel, as described in the Materials and methods section, and assayed over a range of concentrations of the indicated inhibitor. IC₅₀ is the concentration that produced 50% inhibition of enzyme activity.

PKC source	Compound	IC ₅₀
C3H 10T $\frac{1}{2}$ -PKC-4	H-7	10 μ M
Rat brain	H-7	6 μ M*
C3H 10T $\frac{1}{2}$ -PKC-4	Tamoxifen	15 μ M
Rat brain	Tamoxifen	15 μ M
C3H 10T $\frac{1}{2}$ -PKC-4	Staurosporine	2 nM†
Rat brain	Staurosporine	6 nM†‡

* As reported in [32], in which histone H2B was used as substrate.

† Assays were conducted with histone III-S as substrate.

‡ As reported in [33].

the individual rat brain isoenzymes, types I, II and III, resolved by hydroxyapatite chromatography were maximally stimulated by the same range of PS concentrations (results not shown). When the overproduced PKC- β_1 isoenzyme was studied, we observed that, at 0.5 mM-Ca²⁺, it was activated by PS over the same concentration range (20–83 μ g/ml) as for the rat brain isoenzymes. Maximal stimulation of PKC- β_1 by PS was attained with concentrations of 83 μ g/ml (100 μ M) or higher (results not shown).

The differential stimulation of rat brain PKC types I, II and III by arachidonic acid [14,16] offered another criterion by which to assess the behaviour of the overproduced PKC enzyme. When PKC- β_1 activity was assayed at 0.5 mM-Ca²⁺ in the absence of PS, we observed pronounced activation with increasing arachidonic acid concentrations (Fig. 4). The maximal response occurred with 70 μ M-arachidonic acid and represented 55% of the total activity observed with a comparable concentration of PS. In agreement with other studies, we found that the activation by arachidonic acid was Ca²⁺-dependent. It is noteworthy that the biphasic nature of the activation curve shown here for PKC- β_1 was reported for PKC types I, II and III in a previous study [16]. Furthermore, the peak stimulation detected at 70 μ M was attained at a much lower concentration of arachidonic acid than that previously reported (400 μ M).

Another functional property of the overproduced isoenzyme that we explored was its sensitivity to known PKC inhibitors. Since there are no known PKC inhibitors that discriminate among the type I, II and III isoforms, we used a preparation of a mixture of rat brain isoenzymes for comparison with the overproduced PKC- β_1 enzyme. Table 1 shows the results of testing three common PKC inhibitors on the overproduced PKC- β_1 and rat brain enzymes. The compounds tested were H-7 and staurosporine, each of which has been shown to act exclusively at the catalytic subunit [30], and tamoxifen, which influences both regulatory and catalytic domains [30,31]. All of these compounds inhibited the overproduced PKC- β_1 with about the same potency displayed with the mixture of isoenzymes from rat brain.

DISCUSSION

This study reports the biochemical characteristics of PKC- β_1 , an isoform of PKC which had been genetically engineered to be overproduced in murine C3H 10T $\frac{1}{2}$ cells. We show here that 93% of the total PKC activity from this overproducing cell line occurs as a single major peak, not present in control cells, when eluted from hydroxyapatite. Immunoanalysis with a polyclonal antiserum that recognizes both PKC- α and PKC- β_1 also demonstrated the abundances of these two forms of PKC in overproducing cells and control C3H 10T $\frac{1}{2}$ cells. Further analysis with an antiserum that reacted with only the β_1 -isoform clarified that PKC- β_1 was present in the overproducing cells and was absent from the control cells. We have also obtained evidence that the overproduced PKC- β_1 protein has several functional properties previously described for native PKC activity, with respect to phospholipid-dependence, Ca²⁺-dependence in the absence and presence of TPA, and sensitivity to certain PKC inhibitors. From these findings, we conclude that the overproduced protein product exhibits the structural and enzymic properties characteristic of previously studied native PKC isoforms.

The overproduced PKC- β_1 exhibited certain notable differences in behaviour as compared with rat brain PKC or with the endogenous PKC- α activity in C3H 10T $\frac{1}{2}$ cells. One major difference was the higher electrophoretic mobility of the overproduced PKC- β_1 (77 kDa) as compared with PKC from rat brain (82 kDa). It is possible that this apparent difference in size reflects cell type-specific differences in the post-translational processing of PKC. The difference in electrophoretic mobility seen between the overproduced PKC- β_1 and the endogenous PKC- α activity present in C3H 10T $\frac{1}{2}$ cells (80 kDa) further implies that the C3H 10T $\frac{1}{2}$ cell system may subject the α - and β_1 -isoforms to different post-translational processing, e.g. the extent of phosphorylation. Further characterization of this phenomenon is needed.

Immunoblotting of the overproduced protein by using 50 μ g/lane and the β_1 -specific polyclonal antibody gave a much stronger signal than that given by the rat brain sample at 300 μ g/lane (Fig. 2). The weaker signal obtained with the rat brain sample is consistent with a previous study [14], in which immunoblotting with a subspecies-specific antiserum showed that the β_1 -isoform comprised only about 6% of the total PKC activity from rat brain.

The activation of type II PKC by arachidonic acid was previously reported to be Ca²⁺-dependent and required an arachidonic acid concentration of 400 μ M to produce a maximal response [14,16]. In the present study we found that in the presence of 0.5 mM-Ca²⁺ the overexpressed PKC- β_1 was maximally stimulated by 70 μ M-arachidonic acid. Therefore, it appears to be almost 6 times more sensitive to arachidonic acid stimulation than the type II activity. Moreover, with PKC- β_1 the maximal response obtained with arachidonic acid was about 55% of the maximal response obtained with PS plus Ca²⁺. The higher sensitivity of PKC- β_1 to arachidonic acid may simply be due to the higher purity of our PKC- β_1 sample. Previous studies of type II activation by arachidonic acid typically examined a mixture of β_1 and β_2 isoforms, which generally are eluted as a single peak from hydroxyapatite; in one study [14],

the β_1 -isoform appeared to constitute only 12.5 % of the total type II activity. Another factor could be the purity and nature of the arachidonic acid suspension and the possible formation of lipid hydroperoxides by autoxidation [34], since various hydroperoxides can affect PKC activity [35].

The biphasic effect on PKC- β_1 activity of increasing concentrations of arachidonic acid has been observed previously for the PKC isoforms [16]. By contrast with the previous study, the curve shown in Fig. 4 describes a relatively well-defined peak, followed by a rapid fall that reaches zero at a lipid concentration of 280 μ M. An explanation for this effect is not obvious. At concentrations above 70 μ M, arachidonic acid either (1) ceases to be an activator, perhaps owing to the formation of lipid micellar structure that render the lipid unavailable for activation, or (2) acquires inhibitory character by blocking the enzyme's active site, or by disturbing the binding of the cationic substrate, which at 30 μ M could be sequestered by the carboxyl groups of the lipid. A more extensive kinetic analysis would be required to characterize the biphasic effect of arachidonic acid fully.

The biochemical characterization of the overproduced PKC- β_1 isoform with respect to its structural and functional properties lays the foundation for investigations of the intracellular activities of this PKC subspecies. In future studies it will be of interest to explore the effects of site-specific mutagenesis on the functional properties of this isoform, and eventually to extend this general approach to other isoforms of PKC.

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