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Protein kinase Cζ and η in murine epidermis

TPA induces down-regulation of PKCη but not PKCζ

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Murine epidermis contains PKCζ and η as evidenced by the application of specific antisera. PKCζ predominates in the cytosol and PKCη in the particulate fraction. PKCζ is shown to be present also in other murine tissues, with large amounts found in lung. Whereas epidermal PKCη is completely down-regulated by treatment of mouse skin with TPA or bryostatin 1 for 18 h, PKCζ is neither translocated by treatment with TPA for 20 min, nor down-regulated by treatment with TPA or bryostatin 1 for 18 h. PKCζ is activated by phosphatidyl serine alone and does neither respond to Ca^{2+} nor to TPA. It is inhibited by staurosporine with an IC_{50} of 16 nM, which is within the same range of other PKC isoenzymes. The sensitivity of PKCζ towards the staurosporine derivative K252a is similar to that of PKCα,β,γ but much higher than that of PKCδ and ε.

Murine epidermis; Protein kinase ζ; Protein kinase η

1. INTRODUCTION

Protein kinace C (PKC) plays a key role in intracellular signal transduction in that it constitutes a link between the receptor-mediated phosphatidylinositol and phosphatidylcholin breakdown and protein phosphorylation (for a review see [1]). Today, PKC is known to cover a family of phospholipid-dependent isoenzymes catalyzing the phosphorylation of proteins at serine and threomine [1,2]. There are two classes of PKC isoenzymes; one contains the putative Ca^{2+}-binding region C-2 in the regulatory domain and is Ca^{2+}-responsive (PKSα,β,γ) and the other lacks this region and is Ca^{2+}-unresponsive (PKCδ,ε,ζ,η). cDNAs of all these isoenzymes were cloned and sequenced and the corresponding proteins were expressed in transfected cells (for a review see [2]). As the phorbol ester TPA is known to activate PKC, mimicking the physiological activator diacylglycerol, PKC is thought to mediate TPA-induced biological effects including tumor promotion in mouse skin [1]. In this context, it is mandatory to know which PKC isoenzymes are present in mouse epidermis and which of these respond to TPA. PKCα,β,γ [3] and predominantly δ [4,5] are expressed as protein in murine epidermis. The presence of PKCα,β and δ, but not of PKCγ in murine epidermis was confirmed in terms of mRNA expression [6]. Our own unpublished data indicate that murine epidermis lacks PKCγ. Human skin also lacks this PKC isoenzyme [7]. Epidermal PKCα,β [8] and δ [4] were found to be down-regulated upon treatment of the skin with TPA. Recently, the expression of PKCη mRNA in mouse [6,9] and rat [10] skin was reported. Data on the expression of the respective protein in tissues are not available as yet. The same is true for PKCζ.

Here we demonstrate by the use of specific antisera that PKCη and ζ are present in mouse epidermis. Whereas PKCη predominated in the particulate fraction and, similar to other PKC isoenzymes, was down-regulated by TPA, PKCζ was mainly cytosolic and resistant to TPA with respect to translocation and down-regulation.

Abbreviations: PKC, protein kinase C; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PS, phosphatidyl serine

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2. MATERIALS AND METHODS

2.1. *Materials*

12-*O*-Tetradecanoylphorbol-13-acetate (TPA) and bryostatin 1 were kindly provided by Dr. E. Hecker, German Cancer Research Center, Heidelberg, Germany and Dr. G.R. Pettit, State University of Arizona, Tempe, USA, respectively. [γ^{32}p]ATP (spec. act. 3000 Ci/mmol) was from DuPont-New England Nuclear (Waltham, Ma, USA). Protamine agarose, phosphatidyl serine and histone III-S were from Sigma, Munich, Germany. Staurosporine was from Boehringer, Mannheim, Germany and K252a from Fluka Chemie AG, Neu-Ulm, Germany.

2.2. *Animals*

Female NMRI mice (7 weeks old) were used and fed a standard diet ad libitum. The back skin was shaved 3 days before treatment with 100 μl acetone (control) or 10 nmol TPA or bryostatin 1 in acetone.

2.3. *PKC antisera*

PKCζ antiserum was obtained from Gibco BRL, Eggenstein, Germany. PKCα,β,γ antiserum was prepared as described previously [4].

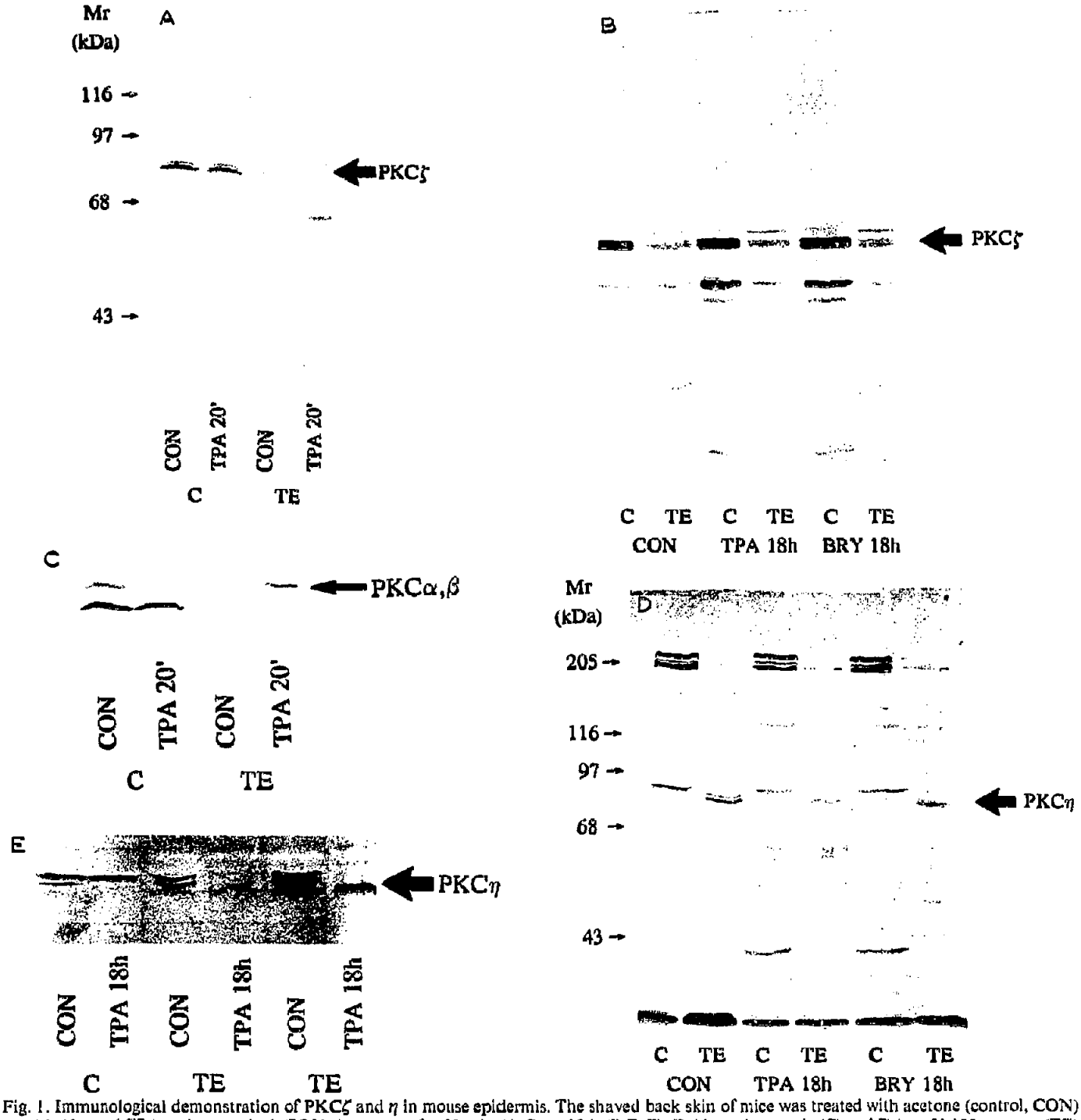


Fig. 1. Immunological demonstration of PKCζ and η in mouse epidermis. The shaved back skin of mice was treated with acetone (control, CON) or with 10 nmol TPA or bryostatin 1 (BRY) in acetone for 20 min (A,C) or 18 h (B,D,E). Epidermal cytosols (C) and Triton X-100 extracts (TE) of particulate fractions were prepared and applied to protamine agarose as described in section 2. Protamine agarose eluates (partially purified PKC preparations) or cytosol and extract directly (see 1E) were used for immunoblotting with PKCα,β,γ (C), PKCζ (A,B) or PKCη (D,E) specific antisera (see section 2). A. Immunoblotting of cytosols (C) and Triton X-100 extracts (TE) of particulate fractions after protamine agarose chromatography, using the PKCζ antiserum. CON, control, TPA 20', after treatment with TPA for 20 min. B. As in A, but after treatment with TPA or bryostatin 1 for 18 h (TPA 18 h, BRY 18 h). C. As in A, but using the PKCα,β,γ antiserum. D. As in B, but using the PKCη antiserum. E. Immunoblotting of cytosols (C) and Triton X-100 extracts (TE) of particulate fractions (lanes 1–4) or protamine agarose eluates of extracts (lanes 5,6), using the PKCη antiserum.

PKCη antiserum was raised in rabbits against the oligopeptide INQDEFRNFSYVSPELQL that had an amino acid sequence corresponding to the sequence 666–683 of PKCη [9]. Immunization of rabbits was performed essentially as described previously [4]. However, the oligopeptide was used without coupling it to thyroglobulin.

2.4. *Preparation of cytosol and particulate extract from various murine tissues*

Tissues were homogenized in buffer A (20 mM Tris-HCl, pH 7.5, 10 mM β-mercaptoethanol, 1 mM PMSF) using an Ultra-Turax homogenizer. The homogenate was separated into cytosol and particulate fraction by centrifugation at 100,000 × g for 30 min. Extraction of the particulate fraction was carried out with buffer B (20 mM Tris-HCl, pH 7.5, 5 mM EGTA, 2 mM EDTA, 1 mM PMSF, 0.2% Triton X-100, 10% glycerol) at 4°C for 30 min, and centrifugation at 100,000 × g for 30 min resulted in the particulate extract.

2.5. *Chromatography on protamine agarose*

2 to 3 ml (3 to 5 mg protein) of cytosol or particulate extract were applied to protamine-agarose column (1 × 0.8 cm) equilibrated with buffer A. The column was washed with 4 ml buffer A containing 0.5 M NaCl and eluted with 2 ml buffer A containing 1.5 M NaCl. The eluate was either used directly for the PKC assay, or proteins in the eluate were precipitated with 10% trichloroacetic acid and then used for immunoblotting.

2.6. *Immunoblots*

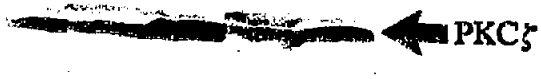
Immunoblotting was performed essentially as described previously [11]. However, alkaline phosphatase-conjugated goat anti-rabbit IgG was used for immunostaining [5].

2.7. *PKC assay*

The assay was performed as described previously [8]. However, instead of purified PKC, 20 μl of the protamine agarose eluates of epidermal cytosol or particulate extract were used as enzyme preparations. Ca^{2+}, PS and TPA were added as indicated in the text.

3. RESULTS AND DISCUSSION

A polyclonal antiserum raised against a PKCζ peptide was used to test mouse epidermis for the presence of PKCζ. A tissue homogenate was separated into cytosol and particulate fraction and the latter was extracted with a buffer containing 0.2% Triton X-100. Chromatography of the cytosol and the particulate extract on protamine agarose resulted in partially purified PKC preparations, which were used for immunoblotting. PKCζ was recognized by the antiserum and appeared in the immunoblot as a doublet with a molecular weight of around 80 to 82 kDa. It predominated in the cytosol. In addition, a third protein band corresponding to a molecular weight of 84 kDa was observed (Fig. 1A and 1B). The immunostaining of all three protein bands, but not of the bands around 67 kDa, could be prevented by addition of the PKCζ peptide, which had been used to raise the antibody (data not shown). A molecular weight of around 80 kDa was reported by Ways et al. [12] for PKCζ expressed in several cell types, including COS cells, transfected with a PKCζ cDNA. Quite similar to our results, Wooten et al [13] observed in PC12 cells three protein bands in the 80 kDa range that were recognized by a PKCζ antibody. However, in the first report by Ono et al. [14] on PKCζ from COS cells transfected with a PKCζ cDNA the enzyme showed a molecular weight of 64 kDa. This low molecular weight might have been due to a degraded form of PKCζ. Expression of the PKCζ protein was demonstrated in platelets [15] and in several cell types [12,13] but not in tissues so far. According to the data shown in Fig. 2, PKCζ appears to be rather an ubiquitous PKC isoenzyme. It was found to be present, besides in epidermis, in all murine tissues tested, i.e. in spleen, lung, brain and



E S L B P

Fig. 2. Immunoblotting of various tissue extracts using the PKCη antiserum. Equal amounts of protein were applied to the gel. E, epidermis; S, spleen; L, lung; B, brain; P, pancreas.

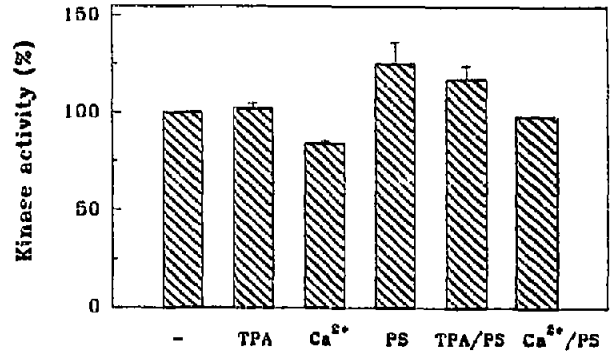


Fig. 3. Dependence of PKCζ activity on various cofactors. Epidermal cytosol of TPA-treated (18 h) mouse skin was applied to protamine agarose and the 1.5 M NaCl eluate was used for determination of the PKCζ activity with histone III-S as substrate. 400 μM Ca^{2+}, 350 μg/ml PS and 1 μM TPA were added to the assay as indicated. Values are the mean of two determinations using different cytosol preparations. The basal enzyme activity in the absence of any cofactor (100%) was 1362 cpm and 1070 cpm in the two experiments.

Table I

Inhibition of PKCζ by staurosporine and K252a (K252b) compared to other PKC isoenzymes.

Isoenzyme	IC_{50}(μM) Staurosporine	IC_{50} (μM) K252a (K252b)	$\frac{IC_{50}\text{ isoenzyme 1}}{IC_{50}\text{ isoenzyme 2}}$ of K252a (K252b)					
			δ/ε	δ/α,β,γ	δ/ζ	ε/α,β,γ	ε/ζ	α,β,γ/ζ
δ	0.009	10 (2)[b]	(1)[b]	50	250			
ε	0.009[a]	(2)[b]				(25)[b]	[50][c]	
α,β,γ	0.007	0.2 (0.08)[b]						5
ζ	0.016	0.04						

[a] This value was taken from Koide et al. [19].
[b] Values in brackets represent inhibition by K252b and were taken from Koide et al. [19].
[c] This value represents a ratio of IC_{50} values of K252b (ε) and K252a (ζ) and therefore can be taken as a rough estimate only.

pancreas. Whereas lung contained the largest amounts of this isoenzyme, it was rather poorly represented in brain. This is intriguing, since brain is a rich source for most of the other PKC isoenzymes.

An antiserum raised in rabbits against a PKCη-specific peptide, with an amino acid sequence corresponding to the sequence 666 to 683 of PKCη [9], recognized PKCη selectively. None of the other isoenzymes ($\alpha,\beta,\gamma,\delta,\varepsilon,\zeta$) showed any reaction with this antiserum (data not shown). With the η-specific antiserum the presence of PKCη could be demonstrated to some extent in the cytosol, but predominantly in the particulate fraction of mouse epidermis (Fig. 1D and E). It appeared in the immunoblot as a doublet with a molecular weight of around 86 kDa. Recently, Odasa et al [9] and Bacher et al. [10] reported on the expression of PKCη (L-PKC) mRNA in mouse and rat skin, respectively. No data on the expression of the respective protein in skin and epidermis are available as yet. The molecular weight of 86 kDa is in accordance with that determined by Livneh and coworkers [10,15] for PKCη expressed in transfected COS cells and in several other cell lines. Osada et al. [9], reported on a molecular weight of 82 kDa for PKCη expressed in transfected COS cells. Recently, a strictly nuclear localization of PKCη was found in various cell types [16]. Respective data on the cellular distribution of PKCη in tissues have not been reported as yet. Even though we find PKCη to predominate in the particulate fraction, preliminary data do not indicate a nuclear but rather a membrane localization.

Treatment of mouse skin with TPA (10 nmol) for 20 min caused a decrease of the amount of PKCα,β in the cytosol and an increase in the particulate fraction, i.e. a translocation of PKCα,β from the cytosol to the particulate fraction (Fig. 1C). Contrary to PKCα,β, the amount of PKCζ was not altered upon this treatment, neither in the cytosol nor in the particulate fraction (Fig. 1A). Thus, TPA did not induce translocation of PKCζ. Furthermore, treatment of mouse skin with TPA of bryostatin 1 for 18 h did not cause down-regulation of PKCζ (Fig. 1B). In accordance with our findings in murine epidermis, Ways et al. [12] were also unable to cause translocation and down-regulation of PKCζ by treatment of several cell types with TPA, and Huwiler et al. [17] found that PKCζ was not affected by phorbol-dibutyrate in rat renal mesangial cells. Crabos et al. [15], however, reported on a translocation of PKCζ in human platelets upon treatment with TPA. Similar to PKCα,β [8] and PKCδ [4], epidermal PKCη was completely down-regulated by treatment of mouse skin with TPA or bryostatin 1 for 18 h (Fig. 1D). Our finding of a TPA-induced down-regulation of PKCη in murine epidermis was in contrast to a report of Greif et al. [16] on the lack of down-regulation of PKCη in several cell types upon TPA-treatment. Therefore, the experiment was repeated with another group of animals. Epidermal cytosol and particulate fraction as well as the particulate fraction after partial purification and concentration on protamine agarose were used for the immunoblot (Fig. 1E). Again, complete down-regulation of PKCη by TPA could be clearly demonstrated and was especially evident in the concentrated preparation (Fig. 1E, lanes 5 and 6). An immunostained protein in the cytosol, with a molecular weight slightly larger than that of the PKCη doublet, was not down-regulated by the TPA or bryostatin 1 treatment (Fig. 1D and E), suggesting it to be a cross-reacting protein different from PKCη. As our data indicate that PKCη in tissues behaves 'normally' compared to most other PKC isoenzymes with respect to localization and down-regulation, the exclusive nuclear localization and the lack of down-regulation described by Greif et al. [16] is difficult to understand but might be due to a special property of PKCη present in certain cell lines. Epidermal cytosol of TPA-treated (18h) mouse skin contained just PKCζ, since all other PKC isoenzymes were down-regulated (see above). The lack of all PKC isoenzymes besides PKCζ was proved by immunoblotting of a respective cytosol using antisera specific for PKCα,β,γ, PKCδ, PKCη and PKCζ (data not shown). After partial purification of PKCζ from such a cytosol by chromatography on protamine agarose, we determined the activity of this isoenzyme with histone III-S as substrate (Fig. 3) and the inhibition of the enzyme activity by staurosporine and K252a (Fig. 4). As previously shown for PKCζ expressed in transfected COS cells [14], PKCζ exhibited a rather high basal activity (in the absence of any cofactor or activator), could be activated slightly by PS alone and was not further activated by the addition of Ca^{2+} or TPA to PS (Fig. 3). As long as PKCζ has not been purified to homogeneity and the enzyme preparation might contain some other protein kinases, the actual basal activity of PKCζ and capacity of PS to activate the enzyme remains unknown. It seems evident from these results, however, that Ca^{2+} and TPA are unable to activate PKCζ. The inability of TPA to activate this

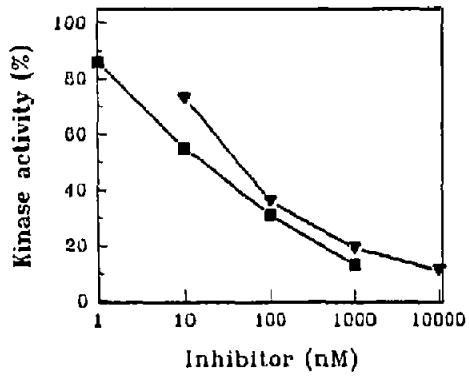


Fig. 4. Inhibition of PKCζ by staurosporine (■-■) and K252a (▲-▲). The same preparation of PKCζ as described in Fig. 3 was used. The enzyme assay was performed in the presence of 350 μg/ml PS and various concentrations of the inhibitors as indicated.

PKC isoenzyme might be due to the lack of the second cystein-rich region in the regulatory domain of PKCζ, as we had suggested previously [18]. Thus, PKCζ apparently does not respond to TPA at all, neither by activation nor by translocation and down-regulation. PKCζ was inhibited by staurosporine with an IC50 of 16 nM and by K252a with an IC50 of 42 nM (Fig. 4). Thus, the inhibition of PKCζ by staurosporine was comparable to that of other PKC isoenzymes (see Table I). The staurosporinge derivative K252a, however, differentiated between the isoenzymes, the inhibition of PKCζ being similar to that of PKCα,β,γ but considerably more effective than that of PKCδ and PKCε (see Table I). Data for PKCε [19] were obtained with K252b, a staurosporine derivative very similar to K252a.

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