Partial purification of a type η protein kinase C from murine brain: separation from other protein kinase C isoenzymes and characterization

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Various murine tissues were tested, by using a protein kinase C-η-specific antiserum, for the expression of type η protein kinase C. Brain was found to be the richest source of a type η isoenzyme. Native protein kinase C-η was partially purified from the cytosol of murine brain by chromatography on DEAE-Sepharose, hydroxyapatite and protamine–agarose. This procedure resulted in a separation of protein kinase C-η from the other phorbol 12-myristate 13-acetate (PMA)-responsive isoenzymes ($\alpha, \beta, \gamma, \delta, \epsilon$) and allowed, for the first time, characterization of the native enzyme. The protein kinase C of type η from mouse brain is a phospholipid-dependent Ca^{2+}-unresponsive protein kinase. Both PMA and bryostatin activate the kinase for phosphorylation of a substrate as well as for autophosphorylation. Various pseudosubstrate-related peptides are suitable as substrates for the η-type kinase, peptide δ being the best and peptides η and ϵ the poorest substrates. The enzyme is inhibited by staurosporine and staurosporine-related compounds, such as K252a and Gö 6976. However, protein kinase C-η, like protein kinase C-δ, is around two orders of magnitude less sensitive towards Gö 6976 than are the Ca^{2+}-responsive isoenzymes (α, β, γ). The η-type protein kinase C exhibits an extreme tendency to lose its PMA-responsiveness. Consequently, purification of the enzyme to homogeneity has not yet been successful.

INTRODUCTION

The protein kinase C (PKC) family, a group of structurally related phospholipid-dependent protein kinases, plays a key role in signal transduction and is involved in the regulation of numerous cellular processes (for reviews see [1–4]). Three classes of PKC isoenzymes are known. The cPKCs (α, β, γ) are Ca^{2+}-responsive and are activated by diacylglycerol (DAG) or the phorbol ester phorbol 12-myristate 13-acetate (PMA; TPA); the nPKCs ($\delta, \epsilon, \eta, \theta$) are Ca^{2+}-unresponsive and are activated by DAG (PMA); the aPKCs (ζ, ι) are Ca^{2+}- and DAG (PMA)-unresponsive. Distinct functions of the different isoenzymes, which vary in their tissue and subcellular distribution, are largely unknown so far. cDNAs of all these isoenzymes were cloned and the corresponding proteins were expressed in transfected cells (see [2–4]). With the exceptions of the more recently discovered PKC-θ and -ι, all recombinant isoenzymes were at least partially purified and characterized. In addition, naturally occurring forms of several PKC subtypes ($\alpha, \beta, \gamma, \delta, \epsilon, \zeta$) were purified from various tissues [5–13]. Rather little information on PKCη is available as yet. mRNA for PKCη was found to be expressed mainly in epithelial tissues [14]. At the protein level, only immunohistochemical studies of PKCη in these tissues, but no quantitative data, were reported [14,15]. In murine epidermis, PKCη was shown to be down-regulated by treatment of the animals with PMA [16]. Recombinant PKCη could be expressed in baculovirus-infected insect cells [17] and in COS cells [18–20]. Recently Dekker et al. [20,21] reported partial purification and characterization of recombinant PKCη from COS cells. In order to investigate the biochemical properties of native PKCη, we have partially purified a type η PKC from mouse brain and separated it from the other isoenzymes.

MATERIALS AND METHODS

Materials

PMA, bryostatin 1 and Gö 6976 were kindly provided by Dr. E. Hecker, German Cancer Research Center, Heidelberg, Germany, Dr. G. R. Pettit, State University of Arizona, Tempe, AZ, U.S.A., and Gödecke A.G., Freiburg, Germany, respectively. Recombinant baculoviruses containing sequences coding for the different PKC isoenzymes were generously given by Dr. S. Stabel, Max-Delbrück-Laboratorium, Köln, Germany. [γ-^{32}P]-ATP (sp. radioactivity 3000 Ci/mmol) was from Du Pont–New England Nuclear (Waltham, MA, U.S.A.). Protamine–agarose, phosphatidylserine (PS) and myelin basic protein (MBP) were from Sigma, Munich, Germany. Staurosporine was from Boehringer, Mannheim, Germany, and K252a from Fluka Chemie A.G., Neu-Ulm, Germany. Second antibodies (goat anti-rabbit) were from Dianova, Hamburg, Germany, and ECL reagents from Amersham–Buchler, Braunschweig, Germany. Pseudosubstrate peptides for PKC isoenzymes with serine replacing alanine (pseudosubstrate-related peptides) were synthesized by R. Pipkorn, German Cancer Research Center, Heidelberg, and had the following sequences: RFARKGSLRQKNV (α), MNRRGSIKQAKI (δ), ERMRPRKRQGSVRRR (ϵ), RKRQRSMRRRVH (η), IYRRGSRRWRKL (ζ). The purity of the peptides was 90–98 %.

Buffers

A: 50 mM Tris/HCl (pH 8.0), 1 mM phenylmethanesulphonyl fluoride (PMSF), 50 mM β-mercaptoethanol, 10 % glycerol. B: 20 mM Tris/HCl (pH 7.5), 5 mM EGTA, 2 mM EDTA, 1 mM

Abbreviations used: PKC, protein kinase C; PMA, phorbol 12-myristate 13-acetate; DAG, diacylglycerol; PS, phosphatidylserine; MBP, myelin basic protein; PMSF, phenylmethanesulphonyl fluoride.

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PMSF, 50 mM β-mercaptoethanol, 0.2% Triton X-100, 10% glycerol. C: as buffer A, but with 1 mM EDTA. D: 20 mM Tris/HCl (pH 7.5), 50 mM β-mercaptoethanol.

cPKC and PKCδ

cPKC (a mixture of PKC-α, -β and -γ) and PKCδ were purified from mouse brain and pig spleen, respectively, as described previously [8,9].

Anti-PKC antisera

Antisera against peptides with sequences specific for PKC-α, -β and -γ [9], -δ [9], -ϵ [22] and -η [16] were raised in rabbits as described previously.

Preparations of cytosol and particulate extract from various murine tissues

Tissues were homogenized in buffer A (70 mg of tissue/ml) by using an Ultra-Turrax homogenizer. The homogenate was separated into cytosol and particulate fraction by centrifugation at 100000 *g* for 30 min. Extraction of the particulate fraction was carried out with buffer B at 4 °C for 30 min, and centrifugation at 100000 *g* for 30 min yielded the particulate extract.

Chromatography on DEAE-Sepharose

Cytosol (40 ml) was applied to a DEAE-Sepharose column (1.6 cm × 7.5 cm) equilibrated with buffer C. The column was washed with 1 vol. of buffer C and eluted with 1 vol. each of buffer C containing 100 mM, 400 mM and 1 M NaCl. The fractions (2.5 ml each) were tested for PKCη by immunoblotting (see Figure 3a).

Chromatography on hydroxyapatite

Fractions 8–13 of the 400 mM NaCl eluate (from DEAE-Sepharose) were pooled and applied to a hydroxyapatite column (1 cm × 5 cm) equilibrated with buffer C. After washing the column with 1 vol. of buffer C, the run-through and wash fractions (2.5 ml each) were tested for PKC-η and -ϵ by immunoblotting (see Figure 3b).

Chromatography on protamine–agarose

Fractions 3–8 from the hydroxyapatite chromatography were pooled and applied to a protamine–agarose column (0.5 cm × 5 cm) equilibrated with buffer C. After washing with 10 vol. of buffer C containing 150 mM NaCl, PKCη was eluted with a linear 0.15–1 M NaCl gradient. The fractions (2 ml each) were tested for PKC-η and -ϵ by immunoblotting, and their kinase activity was measured (see Figure 3c).

PKC assay

The assay was performed as described previously [23]. Cytosol or partially purified preparations of PKCη served as a source for the kinase. MBP or pseudosubstrate-related peptides were used as substrates. Where indicated, PS (100 μg/ml), $CaCl_2$ (5.1 mM) and PMA (1 μM) were added.

Autophosphorylation

Phosphorylation was as described for the PKC assay, but in the absence of a substrate. Proteins were precipitated with 10% trichloroacetic acid, separated by SDS/PAGE and detected by autoradiography.

Immunoblots

Immunoblots using alkaline-phosphatase-conjugated goat anti-rabbit IgG as second antibody were performed as described previously [16]. For application of the ECL detection system, blots were incubated with a horseradish-peroxidase-conjugated second antibody and the immunoreactive bands were detected according to the manufacturer's instructions.

Immunoprecipitation

Cytosol (200 μl) was incubated with 10–30 μl of anti-PKCη antiserum at 0–4 °C for 2 h and then with 100 μl of Protein A–agarose for another 1 h. After centrifugation at 12000 *g* for 2 min, the pellet was washed with 3 × 1 ml of 50 mM Tris/HCl (pH 7.5)/400 mM NaCl. The washed pellet was used for determination of the kinase activity by the PKC assay and the amount of PKCη by immunoblotting.

Recombinant PKC isoenzymes

Sf9 cells were infected with the recombinant baculovirus essentially as described by Stabel et al. [24]. Cell extracts in buffer D containing 1 mM PMSF and 0.2% Triton X-100 were used as a source for each PKC isoenzyme.

RESULTS

An antiserum raised in rabbits against a PKCη oligopeptide [16] was tested with the recombinant PKC isoenzymes α, β_1, β_2, γ, δ, ϵ, ζ and η by immunoblotting and proved to recognize the PKCη isoenzyme selectively (Figure 1a).

As the aim of this investigation was to purify native PKCη from a tissue, various murine tissues were studied with respect to their PKCη content using the PKCη-specific antiserum. As shown in Figure 2, brain was found to be the richest source of PKCη. Regarding the amount of PKCη per mg of total protein, the order of the tissues tested was as follows: brain ≫ spleen ≫ liver, lung > heart. The enzyme was not detectable in the forestomach by this method. In most tissues PKCη was located almost exclusively in the cytosol. Only brain contained some of

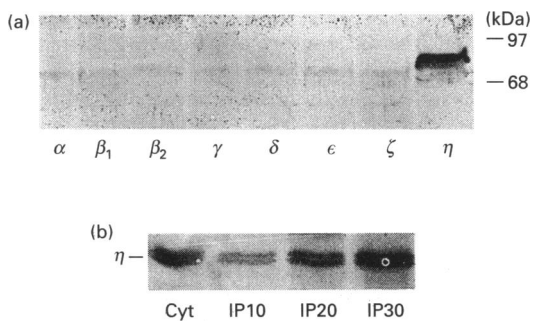

Figure 1 Specific recognition and immunoprecipitation of PKCη by the anti-PKCη antiserum

(**a**) Immunoblots of the recombinant PKC isoenzymes α, β_1, β_2, γ, δ, ϵ, ζ and η from baculovirus-infected insect cells with the anti-PKCη antiserum. Extracts from the isoenzyme-producing cells were used for the blot. (**b**) Immunoblots of PKCη from brain cytosol (Cyt) and PKCη immunoprecipitated from brain cytosol with 10 μl (IP10), 20 μl (IP20) and 30 μl (IP30) of anti-PKCη antiserum.

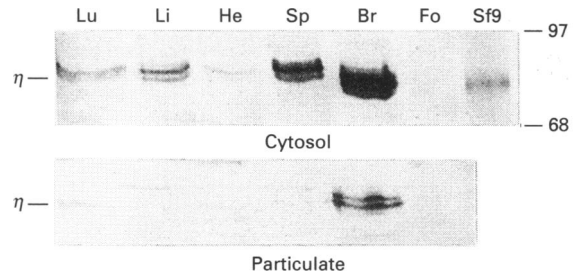

Figure 2 PKCη in murine tissues

Immunoblots of cytosol and particulate fractions from various murine tissues with the anti-PKCη antiserum. Abbreviations: Lu, lung; Li, liver; He, heart; Sp, spleen; Br, brain; Fo, forestomach; Sf9, extract of baculovirus-infected insect cells producing PKCη.

the enzyme in the particulate fraction. The molecular mass of PKCη from brain (83 kDa) was identical with that of recombinant PKCη from baculovirus-infected Sf9 insect cells, but somewhat lower than that of other murine tissues (86 kDa), including epidermis, which we had previously shown to contain PKCη [16]. Compared with brain, however, epidermis, like liver and lung, contained a rather small amount of this PKC isoenzyme. On the basis of these results, we decided to purify PKCη from mouse brain.

Partial purification of PKCη could be performed by immunoprecipitation as well as by precipitation with 30%-satd. $(NH_4)_2SO_4$. As shown in Figure 1(b), the PKCη-specific antiserum could be used for immunoprecipitation of PKCη from the cytosol of murine brain. The efficacy of the precipitation depended on the amount of antiserum. Precipitation was almost complete when 30 μl of antiserum was used per 200 μl of cytosol (600 μg of protein). No precipitation of PKCη was observed when either a preimmune serum or an anti-PKCα antiserum was used (results not shown). The immunoprecipitation and the precipitation with 30% $(NH_4)_2SO_4$, however, caused PKCη to become largely independent of PMA (or DAG) for activation and to behave similarly to PKCζ. Therefore, both methods were not applicable for the purification of native PKCη from mouse brain.

For partial purification of brain PKCη and its complete separation from other PMA- (or DAG)-responsive PKC isoenzymes we developed a four-step procedure. In the first step mouse brain was homogenized in an EDTA-free buffer, in order to keep a large part of the Ca^{2+}-dependent PKC isoenzymes (α, β, γ) bound to the particulate fraction. Next, the cytosol containing the major portion of PKCη was applied to DEAE-Sepharose. PKCη could be eluted from this column with 0.4 M NaCl (Figure 3a). By this, more than 75% of the protein was removed. Then followed chromatography on hydroxyapatite. This material bound PKC-α, -β, -γ and -δ almost completely and PKCε partially, whereas PKCη and about 20% of PKCε were found in the flow-through fraction (Figure 3b). Finally, fractions 3–8 of this flow-through fraction containing the largest amount of PKCη and just a small portion of PKCε were applied to protamine–agarose. PKCη was eluted by a linear 0.15–1 M NaCl gradient at 0.4–0.65 M NaCl (Figure 3c). This final step of the purification procedure resulted in a complete separation of PKCε from PKCη and the removal of around 75% of the protein. Figure 4 demonstrates the gradual separation of the other PMA-responsive PKC isoenzymes (α, β, γ, δ, ε) from PKCη in the course of the purification procedure. PKCη in the protamine–agarose eluate (fractions 4–9) was no longer contaminated with

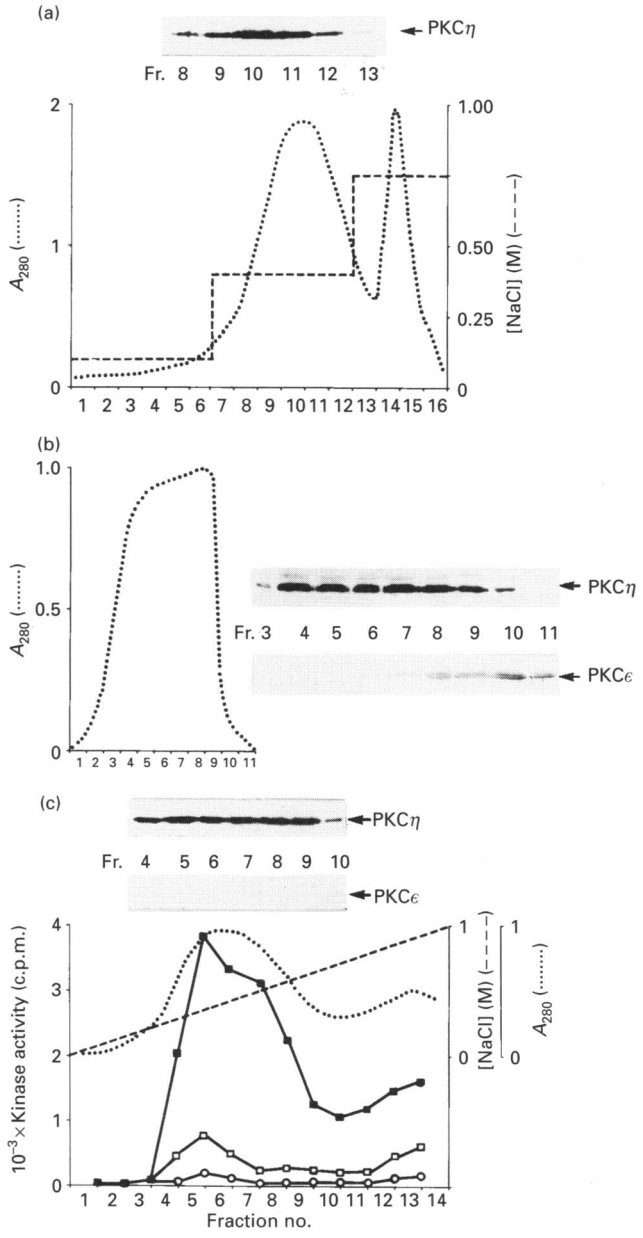

Figure 3 Partial purification of PKCη from mouse brain cytosol by chromatography on (a) DEAE-Sepharose, (b) hydroxyapatite and (c) protamine–agarose

Details of the procedures were as described in the Materials and methods section. Inserts show immunoblots of fractions (Fr.) containing PKC-η and -ε with the respective antisera. Fractions eluted from protamine–agarose (c) by NaCl were assayed for their kinase activity in the absence of cofactors (○) and in the presence of PS (□) or PS and PMA (■) as described in the Materials and methods section.

these isoenzymes, and thus the PMA-dependent activity of PKCη could be determined in each fraction (Figure 3c).

The partially purified enzyme had a specific activity of 21 m-units (1 unit of the enzyme incorporates 1 nmol of ^{32}P into MBP/min)/mg of protein. The specific activity of PKCη in the brain extract could not be determined because of the presence of other PKC isoenzymes. Therefore, the specific activity that should have been obtained after the partial purification of the enzymes could not be calculated. Nevertheless, the specific

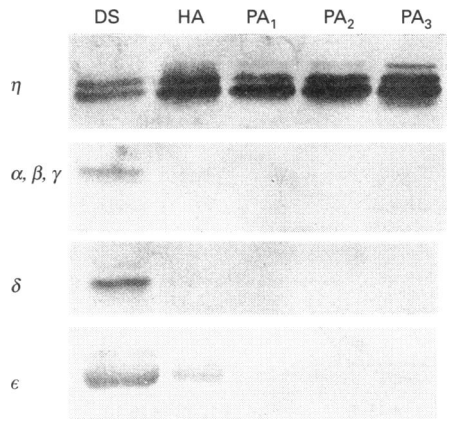

Figure 4 Separation of PKCη from other PKC isoenzymes

Immunoblots of pooled fractions containing PKCη of DEAE-Sepharose (DS), hydroxyapatite (HA) and protamine–agarose (PA_1, PA_2, PA_3) chromatography: PA_1, fractions 4+5; PA_2, fractions 6+7; PA_3, fractions 8+9 (see Figure 3c). Antisera specific for the indicated PKC isoenzymes were used for the blots.

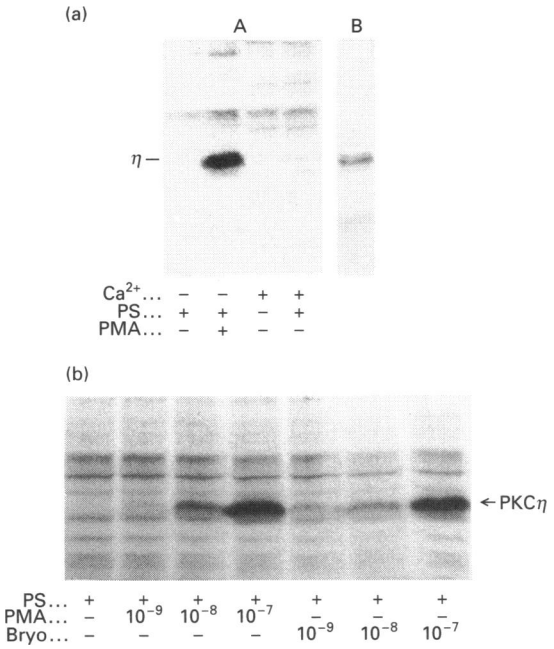

Figure 5 Autophosphorylation of partially purified PKCη

(**a**) PKCη (0.6 m-unit) was autophosphorylated in the presence of PS, Ca^{2+}, Ca^{2+}/PS and PS/PMA as described in the Materials and methods section. (A) Autoradiography; (B) immunoblot of lane 2 in (A) with the PKCη-specific antiserum. (**b**) Autophosphorylation of PKCη (0.6 m-unit) was performed in the presence of various concentrations (M) of PMA or bryostatin (Bryo).

activity of 21 m-units/mg of protein determined after purification appeared to be very low. This was probably due to a loss of activity during purification, since the enzyme proved to be extremely unstable. Partially purified PKCη lost its PMA-responsiveness and subsequently its kinase activity completely within 2 days, and therefore further purification of the enzyme was not possible.

As shown in Figure 5(a), PKCη was able to phosphorylate itself. The autophosphorylation was strictly dependent on PS/PMA and could not be observed in the presence of Ca^{2+}, PS or Ca^{2+}/PS. The location of the PKCη activity, as demonstrated by autophosphorylation, was identical with the location of the enzyme recognized by the PKCη-specific antiserum. Moreover, PKCη activity could be depleted by 92 % by immunoprecipitation with this antiserum. PMA and bryostatin both activated the enzyme for autophosphorylation in a concentration-dependent manner. The two activators were similarly effective (Figure 5b).

Phosphorylation of MBP with PKCη was stimulated by PMA approx. 9-fold, whereas PS alone had no effect (Table 1). Bryostatin appeared to be slightly less efficient than PMA in activating PKCη for phosphorylation of MBP (Figure 6). Large differences in the phosphorylation capacity of the enzyme with various pseudosubstrate-related peptides were observed (Figure 7a). Surprisingly, the η and ϵ peptides proved to be very poor substrates for PKCη, incorporating just 5 and 8 %, respectively, of the phosphate that was incorporated into the most effectively phosphorylated peptide, the δ peptide.

In contrast, recombinant PKCη from baculovirus-infected insect cells was able to phosphorylate the η peptide at a concentration of 100 μM somewhat more efficiently than the other peptides (Figure 7b). Similarly, the η peptide was a suitable substrate for cPKC (Figure 7c) and PKCδ (Figure 7d) purified from murine brain and pig spleen respectively. Thus the poor phosphorylation of the η peptide by the native type η PKC could not be a function of peptide purity.

Staurosporine and two staurosporine-related inhibitors, K252a and Gö 6976, were tested for their ability to suppress PKCη-

Table 1 Phosphorylation of MBP by PKCη

Phosphorylation was performed with 0.3 m-unit of PKCη in the absence of cofactors and in the presence of PS or PS/PMA as described in the Materials and methods section.

Cofactors	Kinase activity (% of control)
–	100
PS	140
PS/PMA	982

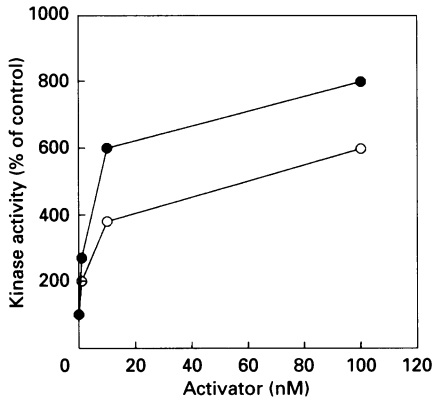

Figure 6 Phosphorylation of MBP by PKCη

Phosphorylation was performed in the presence of various concentrations of PMA (●) or bryostatin (○).

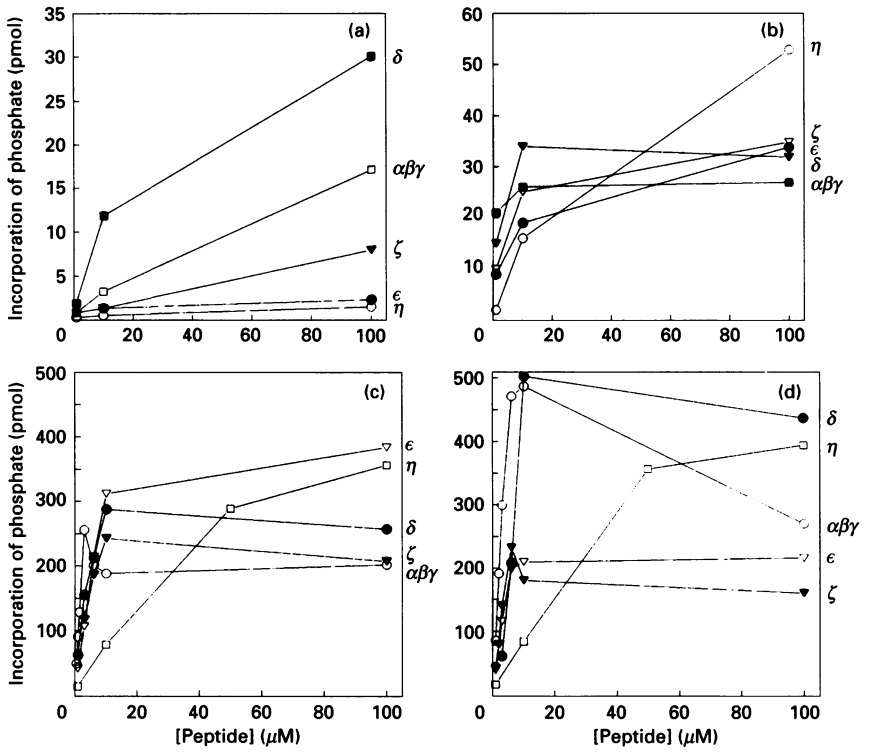

Figure 7 Phosphorylation of various pseudosubstrate-related peptides by partially purified native PKCη (a; 0.3 m-unit), recombinant PKCη (b; cell extract; 0.3 m-unit), cPKC (c; 8 m-unit) and PKCδ (d; 8 m-unit)

Peptides (α, β, γ), δ, ϵ, η and ζ (for the sequences, see under 'Materials'), were phosphorylated at concentrations of 1, 10 and 100 μM in the presence of PS/PMA as described in the Materials and methods section.

catalysed phosphorylation of MBP (Figure 8a) and PKCη autophosphorylation (Figure 8b). Phosphorylation of MBP by PKCη was inhibited most potently by staurosporine, with an IC_{50} of 5 ± 2.9 nM. K252a and Gö 6976 were somewhat less effective, with IC_{50} values of 56 ± 9 nM and 247 ± 173 nM respectively. For the inhibition of PKCη autophosphorylation a nearly 10-fold higher concentration of each inhibitor than for the inhibition of substrate phosphorylation was required. We tested these three inhibitors also with recombinant PKCη expressed in baculovirus-infected insect cells. Additionally, we compared the data on recombinant and native PKCη with data on the inhibition of other PKC isoenzymes (Table 2). PKCη was suppressed by staurosporine comparably with the other isoenzymes. K252a inhibited PKCη and most of the other isoenzymes (especially the native enzymes) slightly less effectively than staurosporine. Only PKCδ was two to three orders of magnitude less sensitive to K252a than to staurosporine. According to Martiny-Baron et al. [25], Gö 6976 was able to differentiate between the Ca^{2+}-responsive (α, β, γ) and the Ca^{2+}-unresponsive (δ, ϵ, ζ) isoenzymes, merely suppressing the Ca^{2+}-responsive ones. However, we found the Ca^{2+}-unresponsive isoenzyme PKCη to be inhibited by Gö 6976, even though with a 50–100-times higher IC_{50} (247 nM for the natural and 685 nM for the recombinant enzyme) than for PKC-α, -β or -γ (IC_{50} around 5 nM).

DISCUSSION

A comparison of PKCη expression at the protein level in various tissues has not been reported so far. According to our results, among several murine tissues brain expresses the largest amount of PKCη protein, followed by spleen. Compared with brain, lung and epidermis contain much less of this PKC isoenzyme, and in forestomach it is not detectable at all with our method. This result is in contrast with data on the respective mRNA levels reported by Osada et al. [14]. According to these authors, mRNA for mouse PKCη is strongly expressed in skin, lung and forestomach, but only slightly in brain and spleen. Provided that this mRNA codes for the PKCη recognized by our antiserum, the discrepancy in PKCη mRNA and protein levels might be due to a regulation of PKCη expression at the translational level and/or to an instability of mRNA for PKCη in certain tisues, e.g. brain.

Native PKCη has not yet been purified and studied. Therefore, as a first approach, we aimed at separating PKCη from the other PMA (DAG)-activated PKC isoenzymes by partial purification from mouse brain. Chromatography of brain cytosol on DEAE-Sepharose, hydroxyapatite and protamine–agarose results in a PKCη preparation that is free of PKC-α, -β, -γ, -δ and -ϵ. The PMA-dependent activity measured in this preparation can thus be attributed to PKCη. In the absence of any cofactors or in the presence of PS alone, the freshly prepared enzyme exhibits very low basal activity. On addition of PMA, i.e. in the presence of PS and PMA, a 9-fold increase in enzyme activity is observed. Autophosphorylation of PKCη also occurs just in the presence of PS/PMA. Native PKCη does not respond to Ca^{2+}, and thus behaves like the other Ca^{2+}-unresponsive PKC isoenzymes.

It had been suggested that some differences in the induction of biological effects by PMA and bryostatin might be due to a differential action of bryostatin on various PMA-activatable PKC isoenzymes [28]. Here we show that at least native PKCη is activated by bryostatin almost as effectively as by PMA.

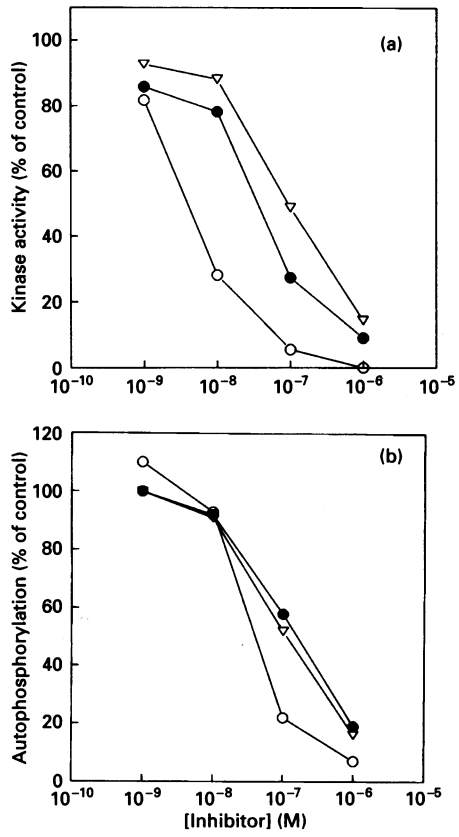

Figure 8 Suppression of PKCη activity by various inhibitors

(**a**) The activity of PKCη (0.3 m-unit) was determined in the presence of PS/PMA with MBP as substrate. Staurosporine (○), K252a (●) and Gö 6976 (▽) were added at various concentrations to the kinase assay mixture. (**b**) Autophosphorylation of PKCη (0.6 m-unit) was performed in the presence of staurosporine (○), K252a (▽) and Gö 6976 (●) as described in Figure 5. The intensity of PKCη phosphorylation was determined by densitometric analysis of the autoradiograms.

Table 2 Inhibition of PKC isoenzymes by staurosporine and staurosporine-related compounds

The activity of recombinant PKC isoenzymes was determined with extracts of baculovirus-infected insect cells or with partially purified preparations from cell extracts[a]. Native isoenzymes α, β, γ [8], δ [9], ϵ [27], ζ [16] and η were from murine brain, pig spleen, rabbit brain, murine epidermis and murine brain, respectively. References: a, [25]; b, [16]; c, [26]; d, M. Gschwendt, G. Fürstenberger, H. Leibersperger, W. Kittstein, D. Lindner, C. Rudolph, H. Barth, J. Kleinschroth, D. Marmé, C. Schächtele and F. Marks, unpublished work; e, [27]. Abbreviation: n.d., not determined.

	IC_{50} (nM)		
Isoenzyme	Staurosporine	K252a	Gö 6976
Recombinant			
α	7.5	17	2.3[a]
β	10	15	6.2[a]
γ	10	18	n.d.
δ	65	3000	> 10000[a]
ϵ	40	700	> 10000[a]
ζ	10	10	> 10000[a]
η	25	165	685
Native			
α, β, γ	7[b]	200[b,c]	5[d]
δ	9[b]	10000[b,c]	1000[d]
ϵ	0.6[e]	10[e]	n.d.
ζ	16[b]	40[b]	n.d.
η	5	56	247

Native PKCη is an extremely unstable PKC isoenzyme, especially regarding its PMA-responsiveness. Precipitation with $(NH_4)_2SO_4$ as well as with the PKCη-specific antiserum causes the enzyme to become independent of activation factors. $(NH_4)_2SO_4$ precipitation has been used by Dekker et al. [20] for partial purification of recombinant PKCη from transfected COS cells. An effect of the precipitation on the enzyme activity has not been described. However, the activity of the recombinant PKCη was only 3 times higher when stimulated by PS/PMA than in the presence of PS alone, possibly indicating a partial loss of the PMA-responsiveness. Similarly, we observed a greatly diminished PMA responsiveness with recombinant PKCη from baculovirus-infected insect cells as compared with the native enzyme (R. Zang and M. Gschwendt, unpublished work).

Moreover, after partial purification native PKCη loses 50 % of its PMA-dependent activity within 24 h. Concomitantly, the basal cofactor-independent activity rises. In this regard, the behaviour of PKCη is clearly different from that of other PKC isoenzymes. It might be due to a rather unstable binding of the pseudosubstrate region to the substrate-binding site, allowing the enzyme to switch more easily to an active conformation even in the absence of cofactors. We have observed similar behaviour with PKCδ purified from pig spleen, though only after storage for several months (H. Leibersperger and M. Gschwendt, unpublished work).

Pseudosubstrate-related peptides with sequences derived from various PKC isoenzymes are phosphorylated by PKCη with extremely different degrees of efficiency. Unexpectedly, the peptides η and ϵ are most poorly phosphorylated by PKCη, whereas peptide δ is the best substrate for PKCη among the pseudosubstrate peptides. In this respect, the native PKCη acts in an entirely different way from the recombinant enzyme from transfected COS cells, which phosphorylates peptide η and ϵ more efficiently than peptide δ [20]. In accordance with the latter results, we have observed that peptide η serves as a good substrate for recombinant PKCη from baculovirus-infected insect cells. This apparent difference in substrate specificity is not yet understood, and would require a more detailed investigation before any conclusions could be drawn. One possible explanation of these results, which cannot be excluded completely, would be that the partially purified enzyme is a novel PKC gene product. However, this is rather unlikely, since this Ca^{2+}-unresponsive PMA-activated kinase is recognized, and its activity is depleted, by a PKCη-specific antiserum that does not cross-react with other Ca^{2+}-unresponsive PMA-activated PKC isoenzymes, such as PKC-δ and -ϵ.

Results on the inhibition of native or recombinant PKCη are not available as yet. Staurosporine is the most effective inhibitor, but does not differentiate between PKCη and the other isoenzymes. This is in agreement with the generally poor selectivity of staurosporine. The staurosporine-related compound K252a is around one order of magnitude less active than staurosporine. Other PKC isoenzymes also exhibit a lower sensitivity to K252a than staurosporine. Only PKCδ is extremely insensitive, with a difference in the IC_{50} values for K252a and staurosporine of up to three orders of magnitude. Another staurosporine-related compound, Gö 6976, has been reported to exhibit a pronounced selectivity for the Ca^{2+}-responsive isoenzymes α, β and γ [25]. When we tested this inhibitor with native and recombinant

PKCη as well as native PKCδ, we found that there is a significantly decreased sensitivity of these isoenzymes towards Gö 6976 as compared with the Ca^{2+}-responsive isoenzymes. However, the difference (around two orders of magnitude) is not as dramatic as reported for recombinant PKC-δ, -ϵ and -ζ [25].

According to our results, native PKC of the η type from mouse brain behaves with regard to its activation by PMA and bryostatin, suppression by staurosporine and staurosporine-related inhibitors, autophosphorylation and phosphorylation of the substrate MBP, as could be expected from a member of the Ca^{2+}-unresponsive PKC group of the PKC family. Unlike the other PKC isoenzymes, the η type exhibits an extreme tendency to lose its PMA-responsiveness, a property that is not at all understood so far.

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Received 20 April 1994/7 July 1994; accepted 15 July 1994