Purification and characterization of a fatty acid-activated protein kinase (PKN) from rat testis

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PKN, a novel protein kinase with a catalytic domain homologous to that of the protein kinase C (PKC) family and unique N-terminal leucine-zipper-like sequences, was identified by molecular cloning from a human hippocampus cDNA library [Mukai and Ono (1994) *Biochem. Biophys. Res. Commun.* **199**, 897–904]. Recently we partially purified recombinant PKN from COS7 cells transfected with the cDNA construct encoding human PKN, and demonstrated that the recombinant PKN was activated by unsaturated fatty acids and limited proteolysis [Mukai, Kitagawa, Shibata et al. (1994) *Biochem. Biophys. Res. Commun.* **204**, 348–356]. The present work has focused on the further purification and characterization of PKN from native rat tissue. Immunochemical measurement revealed that PKN was found in every tissue, and was especially abundant in testis, spleen and brain; subcellular fractionation of rat brain showed that half of the PKN was localized in the soluble cytosolic fraction. PKN was purified approx. 8000-fold to apparent homogeneity from the cytosolic fraction of rat testis by DEAE-

cellulose chromatography, ammonium sulphate fractionation and chromatography on butyl-Sepharose, heparin-Sepharose, Mono Q and protamine-CH-Sepharose. The enzyme migrates as a band of apparent molecular mass 120 kDa. Using serine-containing peptides based on the pseudosubstrate sequence of PKC- δ as phosphate acceptors, the kinase activity was stimulated several-fold by 40 μ M unsaturated fatty acids or by detergents such as 0.04 % sodium deoxycholate and 0.004 % SDS. In the absence of modifiers, protamine sulphate, myelin basic protein and synthetic peptides based on the pseudosubstrate site of PKCs or ribosomal S6 protein were good substrates for phosphorylation by the kinase. In the presence of 40 μ M arachidonic acid the kinase activity of PKN for these phosphate acceptors was increased 2–18-fold. The autophosphorylation activity of purified PKN was partially inhibited by pretreatment with alkaline phosphatase. These properties appear to distinguish PKN from many protein kinases isolated previously.

INTRODUCTION

Almost all protein kinases have sequence similarities in their catalytic domains, and screening of cDNA libraries with probes corresponding to the catalytic domains of protein kinases has been a basic strategy to identify a novel protein kinase [1]. We screened a human hippocampus cDNA library by utilizing the catalytic domain of protein kinase C (PKC)- β II as a probe, and found a novel protein kinase, designated PKN, with a catalytic domain homologous to that of the PKC family and unique N-terminal leucine-zipper-like sequences [2]. As the primary structure of PKN does not contain consensus Ca^{2+} or phorbol ester binding motifs which are observed in the PKC family [3], PKN may possess a distinct mechanism of regulation from that of PKC.

Using partially purified recombinant PKN from cDNA-construct-transfected COS7 cells, we have shown that Ca^{2+} /phosphatidylserine (PS)/diolein could not activate the kinase activity of PKN, but unsaturated fatty acids enhanced the kinase activity between several-fold and approx. 50-fold [4], suggesting that PKN is a fatty-acid-dependent kinase. Furthermore, studies of substrate specificity showed that the recombinant PKN phosphorylated synthetic peptides based on the PKC pseudosubstrate sequence in the presence of arachidonic acid. For those assays the soluble cytosolic fraction of cDNA-construct-transfected COS7 cells was used as the enzyme source, but most of the recombinant PKN was recovered in the insoluble fraction, suggesting that the high-level expression might result in

incorrectly folded molecules. In addition, this product of transfected cells might be different from native PKN with regard to post-translational modification. Moreover, using partially purified recombinant PKN as the enzyme source, there was the possibility of contamination with other protein kinases or phosphatases or with endogenous inhibitors. As a consequence, purification and characterization of a native PKN was necessary in order to investigate the physiological enzymic properties of the enzyme.

In this paper we show (i) the tissue and subcellular distributions of PKN by immunoblot analysis; (ii) the purification to homogeneity of native PKN from rat testis; (iii) the effects of various modifiers on the kinase activity of PKN using various substrates; and (iv) the effect of preincubation with phosphatase on the autophosphorylation activity of PKN.

EXPERIMENTAL

Materials

Polyvinylidene difluoride membrane (Immobilon) and an enhanced chemiluminescence Western blotting detection kit were purchased from Millipore and Amersham respectively. DEAE-cellulose (DE52) and P81 filter paper were purchased from Whatman. Protein A-Sepharose, butyl-Sepharose, Mono Q HR5/5, activated CH-Sepharose 4B and HiTrap-heparin-Sepharose were purchased from Pharmacia LKB Biotechnology Inc. Alkaline phosphatase (*Escherichia coli* C75) was purchased from Takara Shuzo Co., Kyoto, Japan. Calmodulin was purified

Abbreviations used: PKC, protein kinase C; PS, phosphatidylserine; DTT, dithiothreitol; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PA, phosphatidic acid; PI, phosphatidylinositol; DOC, sodium deoxycholate; PKA, cyclic AMP-dependent protein kinase; MAP kinase, mitogen-activated protein kinase.

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from rat brain using a phenyl-Sepharose column, as described [5]. The following oligopeptides were synthesized with an automated peptide synthesizer (Applied Biosystems, model 430A): PKC- α peptide (corresponding to amino acids 15–31 of PKC- α [6], substituting Ser for Ala²⁵; DVANRFARKGSLRQKNV); PKC- δ peptide (corresponding to amino acids 137–153 of PKC- δ [7], substituting Ser for Ala¹⁴⁷; AMFPTMNRGRSIRQAKI); PKC- ϵ peptide (corresponding to amino acids 149–164 of PKC- ϵ [7], substituting Ser for Ala¹⁵⁹; ERMRPKRQGSVRRRV); and PKC- ξ peptide (corresponding to amino acids 109–125 of PKC- ξ [8], substituting Ser for Ala¹¹⁹; GEDKSIYRRGSRRWRKL). S6 peptide (corresponding to amino acids 231–239 of human 40 S ribosomal protein S6; RRRLLSLRA) was purchased from Santa Cruz Biotechnology, Inc. Other chemicals were obtained from commercial sources. Protamine sulphate (20 mg) was coupled to 3 g of activated CH-Sepharose 4B according to the method suggested by Pharmacia LKB Biotechnology.

Immunoblotting

Antisera against the N-terminal region or C-terminal region of PKN were obtained by immunizing rabbits with bacterially synthesized fragments of rat PKN as described [4]. Samples were subjected to SDS/PAGE and subsequently transferred to a polyvinylidene difluoride membrane. The membrane was then blocked with PBS (20 mM sodium phosphate, pH 7.5, 137 mM NaCl) containing 0.05% Triton X-100 (PBS-T) and 5% normal goat serum for 1 h at room temperature. The membrane was then incubated in PBS-T and the relevant primary antiserum (1:1000 dilution) for 1 h at room temperature. The membrane was washed three times (5 min each time) in PBS-T before incubating the blot in PBS-T containing a goat anti-rabbit IgG conjugated to horseradish peroxidase at 1:1000 dilution for 30 min. After this incubation, the membrane was subjected to three 10 min washes in PBS-T. Blots were developed by the enhanced chemiluminescence method.

Quantification of PKN

Contents of PKN in various tissues and subcellular fractions were determined by immunochemical analysis using antiserum against the N-terminal region of PKN as described [9]. Various amounts (1–10 ng) of purified recombinant PKN from the Triton/salt-insoluble fraction of Sf9 cells [2] were used as standards.

Tissue distribution of PKN

Rat tissues were removed quickly after decapitation and added to 10 vol. of 50 mM Tris/HCl, pH 7.5, containing 5 mM EDTA, 5 mM EGTA, 0.5 mM dithiothreitol (DTT), 10 μ g/ml leupeptin and 1 mM PMSF, and homogenized with 10 strokes of a Teflon/glass homogenizer. The crude homogenates were used for quantification of PKN.

Subcellular fractionation of rat brain

Subcellular fractionation of rat brain was done by the method described by Ueda et al. [10]. Rats were decapitated and brains were quickly removed. All subsequent operations were carried out at 0–4 °C. Starting from 1.6 g of the wet tissue, nuclear (P1 fraction), crude mitochondrial (P2 fraction), microsomal (P3 fraction) and soluble (S fraction) fractions were obtained as presented in Table 2. The P2 fraction was fractionated by the method described by Ueda et al. [10]. Starting from the P2 fraction suspended in 6 ml of sucrose buffer solution, four

subfractions (A, B, C and D) were obtained as presented in Table 2.

Purification of PKN

All procedures were carried out at 0–4 °C. Rat testis (60 g fresh weight) was added to 300 ml of homogenization buffer (50 mM Tris/HCl, pH 7.5, 5 mM EDTA, 5 mM EGTA, 0.5 mM DTT, 10 μ g/ml leupeptin, 0.02% Na₂S₂O₈ and 1 mM PMSF) and homogenized for 10 min with a Polytron homogenizer (Kinematica). The resulting crude homogenate was then centrifuged at 10000 *g* for 1 h. The supernatant was recentrifuged at 24000 *g* for 1 h, and the supernatant was loaded on to a 180 ml DE52 column equilibrated in buffer B (25 mM Tris/HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.5 mM DTT, 100 ng/ml leupeptin and 0.02% Na₂S₂O₈). The column was then washed with 20 column vol. of buffer B containing 50 mM NaCl and eluted with buffer B containing 200 mM NaCl. The DE52 fraction was immediately subjected to ammonium sulphate fractionation. Solid ammonium sulphate was added to the fraction to obtain 40% saturation. The solution was left to stand, with stirring, for 30 min and was then centrifuged at 15000 *g* for 1 h. The precipitate was resuspended in buffer B containing 20% ammonium sulphate and loaded on to a 20 ml butyl-Sepharose column equilibrated with the same buffer. The column was washed with 20 column vol. of the same buffer and 3 column vol. of buffer B containing 15% ammonium sulphate, and PKN was eluted with 3 column vol. of buffer B containing 10% ammonium sulphate. The fraction from butyl-Sepharose was dialysed against buffer B, and then divided into two equal portions. One sample was loaded on to a 5 ml HiTrap-heparin-Sepharose column equilibrated with buffer B, which was connected to a Pharmacia FPLC system. The column was then washed with 5 column vol. of the same buffer, and PKN was eluted from the column by application of a 50 ml linear concentration gradient of NaCl (0–0.6 M) in buffer B at a flow rate of 60 ml/h. Fractions of 1 ml each were collected, and a major peak of PKN appeared in fractions 34–41; these fractions were pooled. Another sample was subjected to the same chromatography, and the resultant fractions were pooled as described above. The two pooled fractions were added together, dialysed against buffer B, and then loaded on to a MonoQ 5/5HR column equilibrated with the same buffer using an FPLC system. The column was then washed with 10 column vol. of the same buffer, and PKN was eluted from the column by application of a 40 ml linear concentration gradient of NaCl (0–0.4 M) in buffer B at a flow rate of 120 ml/h. Fractions of 1 ml were collected, and a major peak of PKN appeared in fractions 25–30. These fractions were pooled and loaded on to a 4 ml protamine-CH-Sepharose column equilibrated with buffer B. The column was washed with 3 vol. of the same buffer and 6 vol. of buffer B containing 0.5 M NaCl, and eluted with a 16 ml linear gradient of NaCl (0.5–2 M) and 10 ml of buffer B containing 2 M NaCl. Fractions of 1 ml were collected, and a major peak of PKN appeared in fractions 16–20.

Enzyme assays

The enzyme was incubated for 5 min at 30 °C in a reaction mixture (final volume 25 μ l) containing 20 mM Tris/HCl, pH 7.5, 8 mM MgCl₂, 20 μ M ATP, 18.5 kBq of [γ -³²P]ATP, phosphate acceptors and modifiers as indicated in each experiment. At this time point, kinase activity was linear with incubation time. The reaction was started by addition of the enzyme and terminated by spotting on to Whatman P81 paper. The paper was washed five times in 75 mM phosphate. Incorporation of [³²P]phosphate into phosphate acceptors was assessed by scintillation counting.

Table 1 Distribution of PKN in various rat tissues

Each tissue was homogenized; homogenates were separated by SDS/PAGE and amounts of PKN were measured by immunoblotting using antiserum against the N-terminal region of PKN, as described in the Experimental section. The data are representative of three experiments performed in duplicate producing similar results.

Tissue	PKN/wet tissue ($\mu\text{g/g}$ of tissue)	PKN/total protein ($\mu\text{g/g}$ of protein)
Brain	11.3	112
Heart	8.4	46
Kidney	7.6	40
Liver	14.7	53
Lung	14.3	97
Spleen	25.5	134
Testis	23.7	193
Thymus	11.7	103

Immunoprecipitation and phosphatase treatment of PKN

A 200 μl aliquot of fraction 17 from protamine-CH-Sepharose chromatography was incubated with 3 μl of antiserum against the N-terminal region of PKN for 2 h at 4 °C in 200 μl of buffer C (10 mM Tris/HCl, pH 7.5, 0.5 M NaCl, 0.5 % Nonidet P40,

1 mM PMSF, 0.05 mM sodium orthovanadate, 5 mM NaF and 0.5 $\mu\text{g/ml}$ leupeptin), and then 200 μl of 20 % (w/v) Protein A-Sepharose was added. After a 1 h incubation at 4 °C, the immunoprecipitates adsorbed on to Protein A-Sepharose were washed three times with buffer C and twice with buffer D (50 mM Tris/HCl, pH 9.0, 1 mM MgCl_2). Finally, the pellets were resuspended in 60 μl of buffer D. Then 10 μl of resuspended immunoprecipitate was incubated with 40 μl of alkaline phosphatase (0.025 unit/ μl) in buffer D for 20 min at 30 °C. After incubation under the various conditions indicated in the legend of Figure 5, the reactions were terminated by washing the immunoprecipitates twice with buffer C and once with buffer B. Autophosphorylation was performed by adding 50 μl of reaction mixture containing 20 mM Tris/HCl, pH 7.5, 8 mM MgCl_2 , 20 μM ATP, 18.5 kBq of [γ - ^{32}P]ATP and 8 mM sodium phosphate (pH 7.5) to the immunoprecipitates. After incubation at 30 °C for 5 min, reactions were stopped by the addition of 5 $\times$ Laemmli's sample buffer [11] and boiling, and then a 20 μl aliquot was subjected to SDS/PAGE (10 % gel) followed by autoradiography.

Other techniques

Protein content was determined by the method of Peterson [12], with BSA as standard. Silver staining of SDS/polyacrylamide

Table 2 Subcellular localization of PKN in rat brain

Subcellular fractions were obtained and designated as described in the text. An aliquot of each fraction was separated by SDS/PAGE, and the amount of PKN was measured by immunoblotting using antibody against the N-terminal region of PKN, as described in the Experimental section. The data are representative of three experiments performed in duplicate producing similar results.

Fraction	Protein (mg/g of tissue)	Specific content (ng/mg of protein)	Total content ($\mu\text{g/g}$ of tissue)	Distribution (%)
Homogenate	100.6	112.0	11.3	—
P1	23.0	55.0	1.3	13.3
P2	31.0	103.2	3.2	33.7
P3	8.1	61.2	0.5	5.2
S	27.0	168.2	4.5	47.8
(P1 + P2 + P3 + S)	89.1	—	9.5	100.0
P2				
P2A	6.8	106.0	0.7	26.0
P2B	4.4	137.6	0.6	21.5
P2C	10.7	101.0	1.1	38.6
P2D	6.6	58.1	0.4	13.8
(P2A + P2B + P2C + P2D)	28.5	—	2.8	100.0

Table 3 Purification of PKN

PKN was purified from 60 g of rat testis. The kinase activity of PKN was assayed as described in the Experimental section.

Fraction	Volume (ml)	Protein concn. (mg/ml)	Total protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min per mg)	Yield (%)	Purification (fold)*
Low-speed supernatant	292	16.50	4818	379	0.08	100	—
DE52	180	8.75	1575	313	0.20	82	3
$(\text{NH}_4)_2\text{SO}_4$ precipitate	119	4.75	565	552	0.98	146	12
Butyl-Sepharose	60	2.70	162	126	0.78	33	10
Heparin-Sepharose	16	1.13	18.0	39.3	2.18	10	28
Mono Q	8	0.75	6.0	34.8	5.79	9	74
Protamine-CH-Sepharose	3	0.004†	0.012	7.5	625	2	7944

* Estimated from specific activity.

† Protein content was determined after concentration using a centrifugal concentrator.

gels was done using a silver stain kit from Wako Pure Chemical, Tokyo, Japan.

RESULTS

Tissue distribution and subcellular distribution of PKN

PKN was found in every tissue studied and was most enriched in the testis, making up 0.02% of the total protein in this tissue (Table 1). To investigate the subcellular distribution of PKN in native rat tissues, we performed subcellular fractionation of PKN from rat brain (Table 2). When the homogenates of rat brain were fractionated into P1 (nuclei and cell debris), P2 (myelin, mitochondria and synaptosomes), P3 (microsomes) and S (soluble cytosol) fractions, 48% of the PKN was found in the S fraction. Among the three particulate fractions, approx. 60% of the PKN was in the P2 fraction. Further fractionation of P2 into myelin (fraction A), endoplasmic reticulum and Golgi complex (fraction B), synaptosomes (fraction C) and mitochondria (fraction D) revealed that each subfraction contained a roughly equal amount of PKN. The subcellular fractionation pattern of PKN was distinct from those of PKC or cyclic AMP-dependent protein kinase (PKA), which are mostly associated with synaptosomes [13,14]. Although in cDNA-construct-transfected COS7 cells or insect cells most of the PKN was recovered in the Triton/salt-insoluble fraction as previously described [4], native PKN was localized in the soluble cytosol rather than in insoluble particulate fractions. Thus we used the S fraction of rat testis homogenate for purification of native PKN.

Purification of PKN

In order to monitor the efficiency of purification of PKN we used a specific antiserum raised against the N-terminal region of the enzyme, and the catalytic activity of PKN was assessed by the arachidonic acid-dependent phosphorylation of PKC- δ peptide. The purification of PKN from rat testis is summarized in Table 3 (see the Experimental section for details). Five column chromatography steps yielded approx. 12 μ g of PKN purified about 8000-fold to apparent homogeneity from 60 g of starting material. As shown in Table 3, the total PKC- δ peptide kinase activity of the fraction from ammonium sulphate precipitation was higher than that of the fraction from the DE52 column, and the specific activity was decreased after butyl-Sepharose chromatography. This was due to contaminating kinases that were present at early stages of purification and were activated by ammonium sulphate, as shown by the following. (i) The addition of ammonium sulphate to 20% saturation stimulated the PKC- δ peptide kinase activity of the fraction from the DE52 column by 1.6–2.0-fold, but could not increase the activity of fractions after the butyl-Sepharose column (results not shown). (ii) We confirmed that each step was effective for purification of immunoreactive PKN by Western blotting.

The elution profiles of the last three chromatographic steps shown in Figure 1 were highly reproducible, and the PKC- δ peptide kinase activity was closely correlated with the appearance of immunoreactive bands (Figure 2). The final step, i.e. chromatography on protamine-CH-Sepharose, fractions from which were analysed by SDS/10%-PAGE and silver staining (Figure 2), resulted in a marked purification (approx. 100-fold). PKN bound to the column very tightly and was eluted by 2 M NaCl. Purified PKN eluted from the final chromatography column was resolved in a single band, of apparent molecular mass 120 kDa, which reacted with the antibody.

Figure 1 Sequential chromatography steps in the purification of PKN

Assays were carried out of arachidonic acid-independent (○) and arachidonic acid-dependent (●) PKN activity with PKC- δ peptide as substrate under the conditions described in the Experimental section. Protein concentration (dotted line) was monitored by absorbance at 280 nm. (a) Heparin-Sepharose (HiTrap heparin) column; (b) Mono Q column; (c) protamine-CH-Sepharose 4B column.

Effects of modifiers on PKN activity

As shown in Table 4, the kinase activity of PKN was not enhanced by various modifiers of protein kinases such as cyclic AMP, cyclic GMP, guanosine 5'-[γ -thio]triphosphate, Ca^{2+} /calmodulin and Ca^{2+} /PS/diolein. Although DNA-activated protein kinase is activated at least 10-fold by double-stranded DNA [15], the kinase activity of PKN was enhanced only

Figure 2 Polypeptide composition and immunoblot of column fractions from the protamine-CH-Sepharose column

Fractions of protamine-CH-Sepharose were analysed by SDS/PAGE. (a) Silver-stained SDS/polyacrylamide gel and (b) Western blot using polyclonal antiserum against N-terminal region of PKN as described in the Experimental section. All lanes contain 4 μ l of the fraction. Numbers above the lanes indicate gradient fractions from the column. Numbers on the right indicate molecular masses of marker proteins in kDa. The arrows indicate the polypeptide that co-eluted with PKC- δ peptide kinase activity.

about 2-fold by single-stranded or double-stranded DNA. Unsaturated fatty acids, including arachidonic acid, linoleic acid and oleic acid, enhanced the kinase activity about 7–9-fold, whereas saturated fatty acids such as palmitic acid were less effective. When the PKC- δ peptide was employed as the phosphate acceptor, half-maximal activation ($A_{0.5}$) was achieved at about 7–8 μ M arachidonic acid, and maximal activation occurred at 40 μ M (Figure 3a). Lysophospholipids such as lysophosphatidylcholine (lyso-PC), lyso-PS, lysophosphatidic acid (lyso-PA) and lysophosphatidylinositol (lyso-PI) at 8 μ g/ml were capable of enhancing the kinase activity by 2–5-fold (Table 4). The $A_{0.5}$ values of these modifiers are shown in Table 5.

We investigated the effects of various detergents on the kinase activity of PKN (Table 6). Interestingly, steroid-type detergents such as sodium cholate, digitonin, CHAPS and especially sodium deoxycholate (DOC) at 0.04% (w/v) enhanced the kinase activity approx. 7-fold. Moreover, ionic detergents such as SDS at 0.04% (w/v) enhanced the kinase activity, whereas non-ionic detergents, such as Triton X-100, octyl glucoside, dodecyl maltoside and Lubrol PX, were only slightly stimulatory. Higher concentrations of detergents inhibited kinase activity (Figure 3b). Several compounds were observed that inhibited the kinase activity of PKN (Table 4). As shown in Figure 3(c), Ca^{2+} and Mn^{2+} inhibited PKN activity in a dose-dependent manner, with IC_{50} values of 610 μ M and 42 μ M respectively for basal kinase activity (Table 5). The kinase activity of PKN was inhibited

Table 4 Effects of various modifiers on PKN activity

Samples of 0.5 μ l of fraction 17 from protamine-CH-Sepharose column chromatography were assayed for PKC- δ peptide kinase activity in the presence of various modifiers as described in the Experimental section. Phosphate incorporation in the absence of modifiers was 73 nmol/min per mg of PKN, which was taken as 100% activity. Each value represents the mean $\pm$ S.E.M. of three separate observations. Abbreviations: GTP[S], guanosine 5'-[γ -thio]triphosphate; ssDNA and dsDNA, single- and double-stranded DNA respectively.

Modifier(s)	Activity (% of control)
Cyclic AMP (10 μ M)	114 $\pm$ 11
Cyclic GMP (10 μ M)	104 $\pm$ 14
GTP[S] (0.4 mM)	70 $\pm$ 9
ssDNA (50 μ g/ml)	225 $\pm$ 30
dsDNA (50 μ g/ml)	220 $\pm$ 22
Calmodulin (1 mg/ml)	135 $\pm$ 31
Calmodulin (1 mg/ml) + Ca^{2+} (1 mM)	4 $\pm$ 1
PS (8 μ g/ml) + dioleoin (0.8 μ g/ml) + Ca^{2+} (1 mM)	9 $\pm$ 4
PC (8 μ g/ml)	89 $\pm$ 23
PE (8 μ g/ml)	85 $\pm$ 18
Lyso-PC (8 μ g/ml)	235 $\pm$ 14
Lyso-PS (8 μ g/ml)	342 $\pm$ 27
Lyso-PA (8 μ g/ml)	427 $\pm$ 30
Lyso-PI (8 μ g/ml)	490 $\pm$ 17
Lyso-PE (8 μ g/ml)	74 $\pm$ 17
Arachidonic acid (40 μ M)	852 $\pm$ 29
Linoleic acid (40 μ M)	899 $\pm$ 35
Oleic acid (40 μ M)	731 $\pm$ 20
Palmitic acid (40 μ M)	100 $\pm$ 15
NaCl (150 mM)	54 $\pm$ 12
NaCl (300 mM)	16 $\pm$ 5

> 97% by 1 mM Mn^{2+} or by 10 mM Ca^{2+} (Figure 3c). The enzyme was also inhibited by NaCl in a dose-dependent manner, with an IC_{50} value of 160 mM (Figure 3d, Table 5).

Effects of dilution of assay mixture on the activation of PKN by modifiers

The reversibility of the PKN activation reaction was examined in the following way. A complex between PKN and the modifier was formed by mixing the two. The ability of the PKN-modifier complex to dissociate and thus restore PKN basal activity was then assessed by diluting the mixture and determining the extent of PKN activation. The results presented in Figure 4 show that PKN activity was decreased upon dilution of the PKN-modifier complex, suggesting that the modifier reversibly binds to PKN to form an active PKN-modifier complex.

Substrate specificity

As described above, the C-terminal region of PKN is homologous to the catalytic domain of the PKC family [2]. Therefore several proteins and peptides which have been shown to be good substrates for PKC were tested as substrates for purified PKN. As expected, we found the PKC pseudosubstrate sequence-derived peptides to be excellent substrates for native PKN (Table 7). In the absence of arachidonic acid, protamine sulphate and PKC- δ peptide were good substrates for phosphorylation by the kinase. In the presence of 40 μ M arachidonic acid, the kinase activity of PKN for these phosphate acceptors was increased about 2–18-fold over control activity. Of all the synthetic peptides tested, PKC- δ peptide was most effective substrate, with a PKN specific activity of about 600 nmol/min per mg of protein in the

Figure 3 Effects of fatty acids, detergents, bivalent cations and NaCl on PKN activity

Assays were performed as described in the Experimental section with PKC- δ peptide as substrate. (a) Effect of fatty acid concentration (○, linoleic acid; □, arachidonic acid; △, oleic acid). (b) Effect of detergent concentration (○, DOC; □, SDS). (c) Effect of bivalent cation concentration (○, Ca²⁺; □, Mn²⁺). (d) Effect of NaCl concentration

Table 5 $A_{0.5}$ and IC_{50} values for the various agents

Samples of 0.5 μ l of fraction 17 from protamine-CH-Sepharose column chromatography were assayed for PKC- δ peptide kinase activity with various concentrations of the compounds listed, as described in the Experimental section. For agents that activate kinase activity, the concentration of that agent that causes 50% maximal activation of kinase activity ($A_{0.5}$) is listed. For agents that inhibit kinase activity (*), the concentration of that agent that causes 50% maximal inhibition of kinase activity (IC_{50}) is listed. Each value represents the mean $\pm$ S.E.M. of three separate observations.

Agent	$A_{0.5}$ or IC_{50} *
Arachidonic acid	$7.2 \pm 1.8 \mu\text{M}$
Linoleic acid	$10.5 \pm 2.0 \mu\text{M}$
Oleic acid	$7.2 \pm 2.5 \mu\text{M}$
Lyso-PI	$4.3 \pm 2.0 \mu\text{g/ml}$
Lyso-PA	$4.5 \pm 2.4 \mu\text{g/ml}$
DOC	$0.005 \pm 0.0008 \%$
Sodium cholate	$0.01 \pm 0.002 \%$
Digitonin	$0.001 \pm 0.0005 \%$
SDS	$0.0004 \pm 0.00007 \%$
CHAPS	$0.004 \pm 0.0009 \%$
NaCl*	$158 \pm 19 \text{ mM}$
CaCl ₂ *	$610 \pm 75 \mu\text{M}$
MnCl ₂ *	$42 \pm 19 \mu\text{M}$

Table 6 Effects of various detergents on PKN activity

Samples of 0.5 μ l of fraction 17 from protamine-CH-Sepharose column chromatography were assayed for PKC- δ peptide kinase activity in the presence of various detergents as described in the Experimental section. Phosphate incorporation in the absence of detergents was 73 nmol/min per mg of PKN, which was taken as 100% activity. Each value represents the mean $\pm$ S.E.M. of three separate observations.

Detergent	Concentration (% w/v)	Activity (% of control)
DOC	0.04	732 ± 20
DOC	0.4	20 ± 5
Sodium cholate	0.04	505 ± 23
Digitonin	0.004	446 ± 28
SDS	0.004	689 ± 34
SDS	0.04	0 ± 3
CHAPS	0.04	413 ± 37
Triton X-100	0.004	192 ± 22
Triton X-100	0.04	51 ± 14
Octyl glucoside	0.04	199 ± 18
Dodecyl maltoside	0.004	206 ± 13
Lubrol PX	0.004	180 ± 15

Inhibition of autophosphorylation activity of PKN by phosphatase pretreatment

presence of 40 μ M arachidonic acid. On the other hand, PKC- ξ peptide was less effective even in the presence of arachidonic acid. These data suggest that arachidonic acid-activated PKN has a high affinity for a specific sequence within substrates.

To determine whether the kinase activity of PKN is regulated by phosphorylation of PKN itself, we examined the autophosphorylation activity of purified PKN after dephosphorylation of the enzyme by alkaline phosphatase. It was observed that intact

Figure 4 Reversibility of the activation of PKN by arachidonic acid, DOC and SDS

Fractions from protamine-CH-Sepharose chromatography were preincubated for 2 min at 30 °C in 20 mM Tris/HCl (pH 7.5) containing 20 μ M arachidonic acid (black bars), 0.0256% DOC (shaded bars) or 0.0016% SDS (white bars); portions of the mixture were then diluted 8- or 16-fold in 20 mM Tris/HCl (pH 7.5) and further incubated for 2 min at 30 °C. Diluted and undiluted mixtures were then assayed for kinase activity with PKC- δ peptide as substrate.

Table 7 Substrate specificity of PKN

Samples of 0.5 μ l of fraction 17 from protamine-CH-Sepharose column chromatography were assayed under various substrate conditions. Kinase assays were as described in the Experimental section. Each value represents the mean $\pm$ S.E.M. of three separate observations. Abbreviation: MBP, myelin basic protein.

Substrate	Kinase activity (pmol/min per ml)	
	— Arachidonic acid	+ Arachidonic acid (40 μ M)
Histone H1 (0.2 mg/ml)	120 $\pm$ 15	241 $\pm$ 40
Whole histone (0.2 mg/ml)	74 $\pm$ 20	140 $\pm$ 24
Protamine sulphate (0.2 mg/ml)	698 $\pm$ 51	850 $\pm$ 48
MBP (0.2 mg/ml)	106 $\pm$ 17	408 $\pm$ 25
Casein (0.2 mg/ml)	7 $\pm$ 4	14 $\pm$ 4
PKC- α peptide (100 μ M)	90 $\pm$ 16	1693 $\pm$ 110
PKC- δ peptide (100 μ M)	340 $\pm$ 32	2898 $\pm$ 119
PKC- ϵ peptide (100 μ M)	137 $\pm$ 17	944 $\pm$ 86
PKC- ζ peptide (100 μ M)	159 $\pm$ 23	396 $\pm$ 35
S6 peptide (100 μ M)	88 $\pm$ 20	939 $\pm$ 57

Figure 5 Effect of phosphatase treatment on PKN autophosphorylation activity

Fractions from protamine-CH-Sepharose chromatography were immunoprecipitated and preincubated for 20 min under various conditions. Lane 1, preincubated with buffer; lane 2, preincubated with 1 unit of alkaline phosphatase; lane 3, as in lane 2 except that sodium phosphate was added at 8 mM final concentration; lane 4, preincubated with sodium phosphate at 8 mM final concentration. After preincubation, autophosphorylation was assayed as described in the Experimental section. An autoradiogram of the dried gel is shown.

purified PKN was autophosphorylated *in vitro*, and pretreatment of PKN with alkaline phosphatase resulted in a decrease in the autophosphorylation activity of PKN (Figure 5). This effect of phosphatase could be blocked almost completely by adding a phosphatase inhibitor (sodium phosphate) to the reaction mixture. This result suggests that the purified PKN was already phosphorylated to some extent and that its phosphorylation was required for sufficient activation of the enzyme.

DISCUSSION

In this study we first investigated the tissue and subcellular distributions of native PKN. PKN was expressed ubiquitously in every tissue, and was especially abundant in the brain, testis and spleen. The subcellular distribution of PKN, by immunoblot analysis, indicated that the level of PKN in the soluble cytosolic fraction (S fraction) was higher than in other fractions. PKN was purified approx. 8,000-fold to homogeneity from native rat tissue and employed for kinase assays. The effects of various modifiers and of limited proteolysis (results not shown) and the substrate specificity showed that the properties of purified native PKN were mostly consistent with those of recombinant PKN obtained from previous studies [4].

As shown in a previous study [4], PKN was activated by unsaturated fatty acids. In the present paper we have also shown that several detergents stimulated the activity of PKN. Moreover, we observed that lysophospholipids that are known as biological detergents also enhanced the activity, whereas phospholipids such as PC or PE did not. It is well known that fatty acids and detergents aggregate to form micelles in aqueous solution due to their amphiphilic structure, but are in monomeric form under critical micelle concentrations [16]. Thus we suggest that the activation of PKN by fatty acids or detergents at low concentrations is due to the interaction of PKN with these amphiphilic molecules, since PKN binds to hydrophobic affinity resins, demonstrating the presence of exposed hydrophobic sites. High concentrations of detergents inhibited PKN, which may be caused by the denaturation of the enzyme or its incorporation into detergent micelles. The observations that fatty acids and detergents can alter PKN activity suggest that endogenous surfactants such as lyso-PC or amphiphiles such as arachidonic acid might be modulators of PKN *in vivo*.

Arachidonic acid is a major component of lipid, and is found abundantly in the *sn*-2 chains of phospholipids. Arachidonic acid is liberated to the unesterified form during selective hydrolysis by the action of phospholipase A₂ [17]. In its unesterified state arachidonic acid is believed to be important in signal transduction as a precursor of a variety of biological products, such as prostaglandins and leukotrienes [18]. Several laboratories have reported that arachidonic acid, and to a lesser extent related fatty acids, are able to activate several signal transduction mechanisms independent of enzymic pathways, such as the activation of K⁺ channels in cardiac and smooth muscle cells [19,20]; amplification of Ca²⁺ entry through *N*-methyl-D-aspartate receptor-oriented Ca²⁺ channels [21]; enhancement of long-term potentiation [22]; inhibition of Ras-GTPase activating protein [23]; stimulation of Ras-GTPase inhibiting protein [24]; activation of a GTP binding protein in the neutrophil plasma membrane [25]; and exocytosis in the pituitary gland [26], placenta [27] and pancreas [28]. As a result, several investigators have suggested that arachidonic acid may be acting as a 'second messenger' [29,30] according to criteria advanced by Sutherland and co-workers [31]. In most cases, second messengers stimulate the activities of specific protein kinases, which phosphorylate and hence modulate the activity of a large number of enzymes, membrane ion channels

and structural proteins [32,33]. In this context, there are a number of publications showing activation of PKC by arachidonic acid [34–36]. A major obstacle to the assignment of a PKC as a physiological target of arachidonic acid is the very high concentration needed for enzyme activation (100–600 μ M). This obstacle seems to have been overcome with the isolation of PKC- γ , and the subsequent re-evaluation of the effect of arachidonic acid on this isoenzyme. PKC- γ is activated by very low, possibly physiological, concentrations of arachidonic acid (approx. 10 μ M), independent of Ca^{2+} , PS and DG [37,38]. However, PKC- γ is present only in central nervous system tissues [39,40], and considerable evidence has accumulated to suggest that arachidonic acid exerts its biological effect as a messenger in other tissues independent of PKC. For example, arachidonic acid stimulates insulin secretion from pancreatic β -cells, but not via activation of PKC [41–44]. Recently the presence of arachidonic acid-activated kinases distinct from PKC, calmodulin-dependent protein kinase and PKA was reported in pancreatic β -cells [45], human platelets [46] and rat liver [47], suggesting the presence of another target for arachidonic acid as a second messenger, although the molecular aspects of these kinases have not yet been fully clarified. In the present studies we showed that native PKN is distributed ubiquitously and is activated by a low, possibly physiological, concentration of arachidonic acid, suggesting the possibility that PKN is a target for arachidonic acid. Implicating arachidonic acid as a physiological activator requires additional studies at a cellular level and the use of specific inhibitors.

In this study we show that the autophosphorylation activity of PKN is inhibited by pretreatment with alkaline phosphatase. These data suggest that phosphorylation of PKN plays an important role in the activation of PKN activity. A similar case can be presented in support of a regulatory role for phosphorylation in the mitogen-activated protein kinase (MAP kinase) pathway, which uses sequential protein kinase reactions to phosphorylate and activate the next kinase in the pathway. For example, the autophosphorylation activity of purified MAP kinase kinase, a key intermediate in the MAP kinase cascade [48,49], is inactivated by phosphatase treatment, and this is correlated with its activator activity [50]. If the PKN activation mechanism is analogous to what is observed in the MAP kinase activation of the MAP kinase pathway, PKN phosphorylation may be the initiating event in PKN activation. A more probable possibility is that the phosphorylation of PKN is either a necessary precursor for cofactor-induced PKN activation and/or a secondary event that stabilizes an active conformation generated by the cofactor–PKN interaction. Further studies to examine the physiological regulation of PKN are under way in our laboratory and should be facilitated by the availability of the purified preparation of the enzyme.

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