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A New Member of the Protein Kinase C Family, nPKCθ, Predominantly Expressed in Skeletal Muscle

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A new protein kinase C (PKC)-related cDNA with unique tissue distribution has been isolated and characterized. This cDNA encodes a protein, nPKCθ, which consists of 707 amino acid residues and showed the highest sequence similarity to nPKCδ (67.0% in total). nPKCθ has a zinc-finger-like cysteine-rich sequence (C1 region) and a protein kinase domain sequence (C3 region), both of which are common in all PKC family members. However, nPKCθ lacks a putative Ca^{2+} binding region (C2 region) that is seen only in the conventional PKC subfamily (cPKCα, -βI, -βII, and -γ) but not in the novel PKC subfamily (nPKCδ, -ε, -ζ, and -η). Northern (RNA) blot analyses revealed that the mRNA for nPKCθ is expressed predominantly in skeletal muscle. Furthermore, nPKCθ mRNA is the most abundantly expressed PKC isoform in skeletal muscle among the nine PKC family members. nPKCθ expressed in COS1 cells serves as a phorbol ester receptor. By the use of an antipeptide antibody specific to the D2-D3 region of the nPKCθ sequence, nPKCθ was recognized as a 79-kDa protein upon sodium dodecyl sulfate-polyacrylamide gel electrophoresis in mouse skeletal muscle extract and also in an extract from COS1 cells transfected with an nPKCθ cDNA expression plasmid. Autophosphorylation of immunoprecipitated nPKCθ was observed; it was enhanced by phosphatidylserine and 12-*O*-tetradecanoylphorbol-13-acetate but attenuated by the addition of Ca^{2+}. These results clearly demonstrate that nPKCθ should be considered a member of the PKC family of proteins that play crucial roles in the signal transduction pathway.

Protein kinase C (PKC) plays a key regulatory role in a variety of cellular functions, such as cell growth and differentiation, gene expression, secretion of hormones and neurotransmitters, and membrane functions (29, 30). PKC was originally identified as a serine/threonine protein kinase whose activity was dependent on calcium and phospholipids (55). Diacylglycerols (DAG), products of phosphatidylinositol turnover, lower the Ca^{2+} requirement for PKC activity from millimolar to micromolar concentrations in the presence of phosphatidylserine (PS) (18, 56), indicating that PKC is a target of DAG. Tumor-promoting phorbol esters also bind to and activate PKC (5, 15). The pleiotropic action of phorbol esters could be explained as mimicking the activity of PKC by substituting for a physiological PKC activator, DAG. Besides the classical pathway of DAG generation through receptor-mediated hydrolysis of inositol phospholipids, recent experiments have revealed the presence of a novel pathway of DAG generation through hydrolysis of phosphatidylcholine (PC) (9). It was suggested that PKC is also involved in signaling pathways that generate DAG from PC.

Recent molecular cloning experiments have established that PKC molecules consist of a protein family which can be classified into at least two major classes, conventional PKC (cPKC; isoforms α, βI, βII, and γ) and novel PKC (nPKC; isoforms δ, ε, and ζ) (6, 13, 19, 23, 25, 31, 32, 34–38, 41–43, 51). Patterns of expression for each PKC isoform differ among tissues. Biochemical characterization of these PKC family members has revealed clear differences in their cofactor dependencies. In particular, nPKC does not show the classical biochemical properties of PKC; the kinase activities of nPKCδ and -ε are independent of Ca^{2+} (1, 20, 21, 25, 28, 31–33, 34, 38, 48, 51) and had been overlooked by most experiments measuring PKC activities. On the other hand, nPKCδ and -ε, as well as all of the cPKC members, possess phorbol ester-binding activities and kinase activities. Thus, it is evident that cPKC and nPKC are equally available as cellular receptors to DAG and phorbol esters.

In the course of our experiments to identify the PKC isoforms expressed in mouse skin, a target organ of tumor promoters, we have identified nPKCη (45). A human homolog of nPKCη, called PKC-L, was also isolated (3). In marked contrast to the previously identified PKC family members, all of which were isolated from brain cDNA libraries, nPKCη showed unique tissue specificity of expression. That is, nPKCη is expressed predominantly in skin and lung. In this report, we demonstrate the identification of a new member of the nPKC subfamily, nPKCθ, which is expressed predominantly in skeletal muscle and is the most prevalent PKC isoform in this tissue.

MATERIALS AND METHODS

Isolation and characterization of cDNA clones. cDNA clone A-29, encoding the COOH-terminal half of nPKCθ, was isolated from a mouse skin cDNA library constructed in λgt10, from which nPKCη was isolated (45). To isolate a cDNA clone containing the entire nPKCθ coding region, a mouse brain cDNA library was screened by using a 5′-

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terminal restriction fragment of A-29 as a hybridization probe (see Results). Plaque hybridization was performed according to a standard procedure (49). After hybridization, filters were finally washed with 0.1× SSC (1× SSC is 150 mM NaCl plus 15 mM sodium citrate, pH 7.0) containing 0.1% sodium dodecyl sulfate (SDS) at 65°C. Positive plaques with sufficient insert lengths were selected for characterization in detail. Nucleotide sequences of the cDNA clones were determined by the dideoxynucleotide chain termination method on both strands by subcloning overlapping restriction fragments as described previously (45).

Northern (RNA) blot analysis. Total cellular RNAs from mouse embryo and various mouse tissues were extracted by the guanidium thiocyanate-CsCl method (49). Poly(A) RNAs from mouse brain and skeletal muscle were purified by using Oligotex-dT30 (Japan Synthetic Rubber, Tokyo, Japan, and Nippon Roche, Tokyo, Japan) as recommended by the manufacturers. Mouse cDNA clones corresponding to the previously identified cPKC and nPKC isoforms were isolated from cDNA libraries of mouse skin and mouse brain and used as hybridization probes. Specific activities (counts per minute per microgram) of multiprimed ^{32}P-labeled probes used for Fig. 5 and 6 are as follows: α, 7.4×10^8; β, 6.1×10^8; γ, 6.3×10^8; δ, 7.9×10^8; ε, 5.8×10^8; ζ, 6.0×10^8; η, 7.4×10^8; θ, 6.8×10^8; and β-actin, 6.1×10^8. RNA electrophoresis, blotting to nylon membranes (Hybond-N; Amersham International, Amersham, United Kingdom), and hybridization were carried out as described previously (45). After hybridization, the membranes were washed at 45°C in 0.1× SSC containing 0.1% SDS and exposed to X-ray films for 3 days at −70°C with an intensifying screen.

Transfection to COS1 cells. A *Sac*II-*Sac*I cDNA fragment from a cDNA clone, I-7, containing the entire protein coding sequence was introduced into the *Eco*RI site of the SRD expression vector. Construction of the SRD vector was reported earlier (35).

For determination of phorbol ester binding, the expression vectors of cPKCα, nPKCδ, and nPKCθ were transfected into COS1 cells by the DEAE-dextran method as described previously (45). Transfection for immunoblot analysis and autophosphorylation assays was completed by electroporation by using a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) as follows. COS1 cells were suspended in a solution of 308 mM NaCl, 1.2 M KCl, 81 mM $NaHPO_4$, 14.6 mM NaH_2PO_4, and 50 mM $MgCl_2$ at a density of 1.2×10^7 cells per ml. The cell suspension (500 μl) was mixed with ca. 16 μg of expression plasmid DNA in a Gene Pulser cuvette (Bio-Rad) and placed on ice for 10 min. The Gene Pulser apparatus was set to 220 V and 960 μF. The cells in the cuvette were pulsed once (ca. 25 ms) and placed again on ice for 10 min, and then 500 μl of serum-free Dulbecco's modified Eagle's medium (DMEM) was added. After 10 min of incubation at room temperature, the cells were seeded onto 100-mm-diameter dishes and incubated in DMEM supplemented with 10% fetal bovine serum at 37°C.

Phorbol ester binding assay. Intact COS1 cells, transiently expressing various PKC cDNA constructs, were assayed for phorbol ester binding after 60 h of transfection. After two washes with binding medium (DMEM, 1 mg of bovine serum albumin per ml, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES], pH 7.0), COS1 cells (5×10^5 cells per 60-mm-diameter dish) were incubated in 1 ml of phosphate-buffered saline (PBS) containing 10 nM ^{3}H-labeled phorbol 12,13-dibutyrate (PDBu) (Du Pont-NEN) for 30 min at 37°C in the presence (for nonspecific binding) or absence (for total binding) of 100 μM unlabeled PDBu (P-L Biochemicals). The cells were harvested by trypsinization to measure radioactivity after three washes with ice-cold PBS.

Preparation of antisera against nPKCθ. Two peptides, corresponding to the sequences of the D2-D3 region (residues 332 to 353) and the COOH-terminal region (residues 690 to 707) of nPKCθ, were chemically synthesized by using a peptide synthesizer (model 430A; Applied Biosystems). Oligopeptides were coupled to keyhole limpet hemocyanin and used for immunization of rabbits. The antisera were purified by using the peptide antigens with affinity chromatography with Affi-Gel 10 or 15 (Bio-Rad) columns.

Fractionation of COS1 cells and mouse skeletal muscle. COS1 cells were disrupted by sonication in homogenizing buffer [20 mM Tris-HCl, (pH 7.5), 0.25 M sucrose, 2 mM EDTA, 0.5 mM ethyleneglycol-bis(2-aminoethylether)-*N*,*N*,*N*,*N*-tetraacetic acid (EGTA), 50 mM β-mercaptoethanol, 100 μg of leupeptin per ml, 2 mM phenylmethylsulfonyl fluoride (PMSF)]. A cytosolic fraction was obtained by centrifugation at 350,000 × *g* at 4°C for 30 min. The pellet was suspended in the same buffer containing 0.5% Triton X-100 and centrifuged under the conditions described above for separation of Triton-soluble and Triton-insoluble particulate fractions. For SDS-polyacrylamide gel electrophoresis (PAGE), each fraction was diluted with 4× SDS sample buffer (62.5 mM Tris-HCl, 2% SDS, 5% glycerol, 2% 2-mercaptoethanol, 0.02% bromophenol blue).

Mouse thigh muscles were homogenized in 8 volumes of PBS containing 100 μg of leupeptin per ml and 2 mM PMSF and were centrifuged at 8,000 × *g* at 4°C for 30 min to obtain a cytosolic fraction.

Immunoblot analysis. Proteins were subjected to SDS-PAGE (8% polyacrylamide gel) and transferred electrophoretically to Clearblot-P membranes (Atto, Tokyo, Japan). The membranes were incubated overnight at room temperature in PBS containing 1% skim milk and 0.02% NaN_3 and then treated with normal rabbit serum or antipeptide antiserum against nPKCθ at room temperature for 2 h. After being washed with a solution of 20 mM Tris-HCl (pH 7.5), 500 mM NaCl, and 0.05% (vol/vol) Tween 20, the membranes were incubated with anti-rabbit immunoglobulin G conjugated with alkaline phosphatase (Tago, Inc., Burlingame, Calif.). Protein bands were visualized by an alkaline phosphatase substrate kit I (Vector Laboratories, Inc., Burlingame, Calif.).

Immunoprecipitation and autophosphorylation assay. The supernatant from COS1 cells (100 μl), prepared as described above, was incubated with 2 μl of antiserum against nPKCθ or preimmune rabbit serum at 0°C for 2 h, and then 20 μl of protein A-Sepharose (50%, vol/vol; Pharmacia, Uppsala, Sweden) was added to absorb the immunoprecipitates. After 1 h of incubation at 4°C, the resultant complexes were washed three times with a washing solution (10 mM Tris-HCl [pH 7.5], 500 mM NaCl, 0.5% Nonidet P-40, 1 mM PMSF, 0.01% leupeptin) and once with a final solution (20 mM Tris-HCl [pH 7.5], 0.5 mM EDTA, 0.5 mM EGTA, 50 mM 2-mercaptoethanol, 10% glycerol, 0.01% leupeptin, 2 mM PMSF). An aliquot of the immunoprecipitate solution was mixed with 50 μl of an assay mixture (20 mM Tris-HCl [pH 7.5], 5 mM magnesium acetate, 0.01% leupeptin, 2 mM sodium orthovanadate, 1 μM ATP, 1 μCi of [γ-^{32}P]ATP) in the presence of various combinations of PKC activators (25 μg of PS per ml, 50 ng of 12-*O*-tetradecanoyl phorbol-13-acetate [TPA] per ml, and 0.5 mM Ca^{2+}) and incubated at 0°C for 20 min. After being washed twice with the washing solution, the pellets were dissolved in 15 μl of 1× SDS

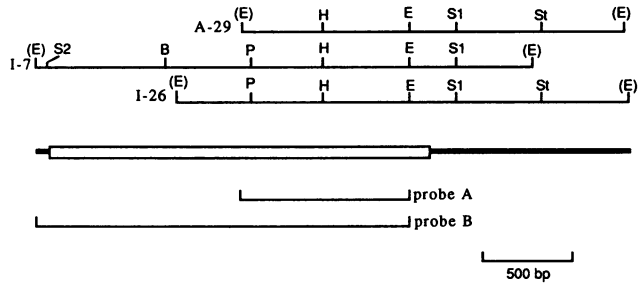

FIG. 1. Schematic structure of overlapping cDNA clones encoding nPKCθ. The open square and solid line indicate the protein-coding and noncoding sequences, respectively. Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; S1, *Sac*I; S2, *Sac*II; St, *Sty*I; (E), *Eco*RI site derived from *Eco*RI linkers used for ligating cDNA fragments.

sample buffer and subjected to SDS-PAGE and autoradiography.

GenBank accession number. The accession number for the cDNA sequence reported in this paper is D11091.

RESULTS

Isolation of cDNA clones for nPKCθ. cDNA clone A-29, encoding a possible PKC-related sequence, was isolated from a mouse skin cDNA library (45). However, this clone did not seem to contain the full length of the protein-coding region, as it lacked an ATG translational initiation codon. Rescreening of 5×10^5 independent phages from the mouse skin cDNA library with a 5′-terminal restriction fragment of A-29 (probe A; Fig. 1) as a hybridization probe yielded four positive clones with sufficient insert lengths, but sequence analyses revealed that three of them encoded nPKCδ and the rest encoded an irrelevant sequence. As all PKC subspecies except nPKCη are enriched in the brain, we then screened approximately 17×10^5 recombinant phages from a mouse brain cDNA library constructed in λgt10 by using the same probe and finally obtained five positive cDNA clones. The insert lengths of these clones were examined by polymerase chain reaction, and two of them with longer inserts, designated I-7 and I-26, were characterized in detail (Fig. 1). The sequences of overlapping portions of these cDNA clones were completely identical (data not shown), indicating that these clones originated from a single mRNA. These three clones covered the cDNA fragment composed of a total of 3,313 nucleotides. The nucleotides contain an open reading frame encoding 707 amino acid residues (Fig. 2) with a calculated molecular weight of 81,571. Although there is no termination codon preceding the ATG at nucleotides 1 to 3, this ATG is most likely the translation initiation codon, considering the following facts: (i) the sequence CAACC preceding the predicted initiation codon is very similar to the consensus sequence for a translational initiation site, CCAC CATG, proposed by Kozak (22) and (ii) an expression plasmid containing a *Sac*II-SacI fragment from I-7 expressed a 79-kDa protein (referred to later). The 3′-terminal untranslated sequence of 1,101 nucleotides contains three potential polyadenylation signals, AATAAA, but no poly(A) tail was found (Fig. 2).

Structural features of nPKCθ. A deduced amino acid sequence of the cDNA fragment was closely related to but clearly distinct from the sequences of previously characterized PKC molecules, indicating that the cDNA encoded a new member of the PKC family. We termed this newly identified PKC nPKCθ.

Figure 3 shows a schematic structure of nPKCθ in comparison with those of the other PKC family members. The presence of the C1 and C3 regions and the lack of a C2 region, which is conserved only among the cPKC subfamily, indicate that nPKCθ should be considered a member of the nPKC subfamily.

In the regulatory domain (C1) of nPKCθ, a zinc-finger-like cysteine-rich sequence is tandemly repeated twice, as seen in the other PKC members except nPKCζ (Fig. 4B). The pseudosubstrate sequence in the NH_2-terminal end of the C1 region, whose motif is an alanine residue flanked by basic amino acids, is also conserved in nPKCθ (Fig. 4B). An ATP-binding consensus sequence, Gly-X-Gly-X_2-Gly-X_{16}-Lys, where X represents any amino acid, is seen in the kinase domain (C3) of nPKCθ, as in the previously characterized PKC molecules (Fig. 4C).

As shown in Table 1, the amino acid sequence of nPKCθ is the sequence most homologous to that of nPKCδ among the PKC family members, showing sequence identities of 78.1% in the C1 region, 72.9% in the C3 region, and 60.8% in the D4 region. The sequence similarity to nPKCδ also extends into the D1 region (Fig. 4A).

nPKCθ is predominantly expressed in skeletal muscle. Figure 5 shows the tissue distribution of the mRNA for nPKCθ examined by Northern blot analysis in comparison with that of nPKCδ, whose sequence is the most similar to that of nPKCθ. Expression of the mRNA for nPKCθ is highly characteristic, being predominantly expressed in skeletal muscle (Fig. 5A). In a longer exposure, faint bands of nPKCθ mRNA were detected in brain, lung, and spleen (Fig. 5B). Two transcripts with different sizes (4.0 and 3.6 kb) seem to originate from the alternative use of polyadenylation signals in the 3′-untranslated region (Fig. 2). In contrast to nPKCθ, the mRNA for nPKCδ is expressed in all tissues examined, although the expression level varies from tissue to tissue (Fig. 5C).

These results led us to analyze expression of the other PKC mRNA species in skeletal muscle. As shown in Fig. 6, among the eight PKC subspecies, nPKCθ mRNA is the most abundantly expressed PKC isoform in skeletal muscle. The mRNA for cPKCα is also expressed in skeletal muscle, but its expression level is much lower than that of nPKCθ. In brain tissue, however, the mRNA for nPKCθ as well as that for nPKCη showed only faint bands, while the rest of the PKC mRNA species were enriched. These results indicate that nPKCθ is a major PKC mRNA subspecies in skeletal muscle.

nPKCθ serves as a phorbol ester receptor. The presence of the cysteine-rich repeat sequence, which is known to be essential for phorbol ester binding (40), led us to examine the ability of nPKCθ to bind a phorbol ester, [^{3}H]PDBu. As shown in Fig. 7, the [^{3}H]PDBu binding of COS1 cells transfected with the nPKCθ cDNA construct was 3.9-fold higher than that of mock-transfected cells and nearly the same as that of cells transfected with the cPKCα cDNA construct. These results indicate that nPKCθ is a phorbol ester receptor.

nPKCθ protein is present in skeletal muscle. An affinity-purified antibody against a peptide from the D2-D3 region of nPKCθ was prepared and used for immunological detection of nPKCθ at the protein level. A protein band with an apparent molecular mass of 79 kDa was detected by this antibody in the Triton-insoluble particulate, the Triton-soluble particulate, and the cytosolic fractions of nPKCθ-

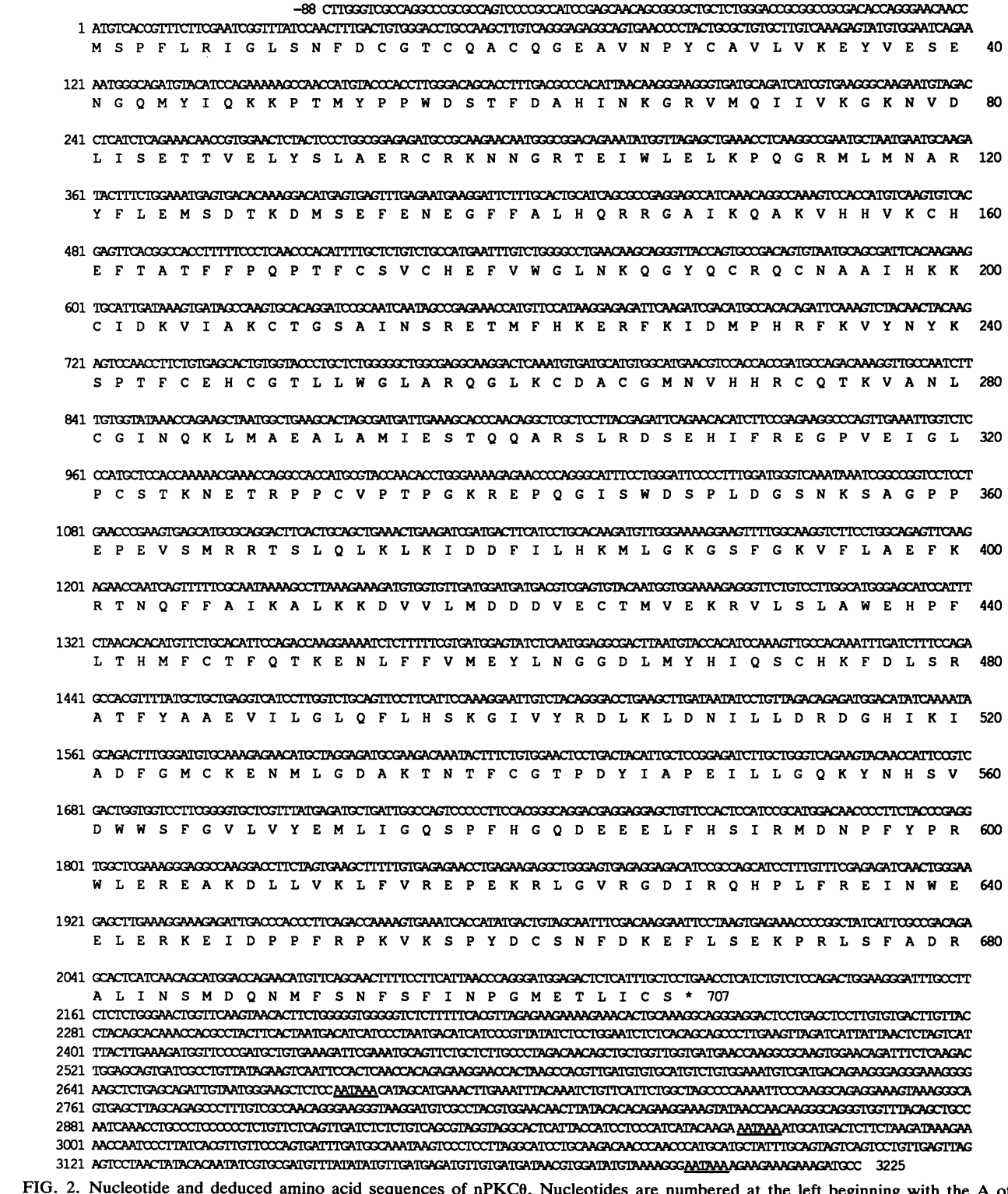

FIG. 2. Nucleotide and deduced amino acid sequences of nPKCθ. Nucleotides are numbered at the left beginning with the A of the predicted translation initiation codon; amino acid residues are numbered at the right. The asterisk indicates a termination codon. Polyadenylation signals (AATAAA) are underlined.

expressing COS1 cells (Fig. 8A, lanes 4 to 6, respectively). Preimmune rabbit serum could not react to this band (Fig. 8A, lanes 7 to 9). A smaller band in lane 4 seems to be a proteolytic product of this protein. The 79-kDa protein band specifically recognized by this antibody was also identified in the cytosolic fraction of mouse skeletal muscle (Fig. 8B, lane 2), indicating that nPKCθ is expressed in this tissue.

nPKCθ is a protein kinase. nPKCθ expressed in COS1 cells

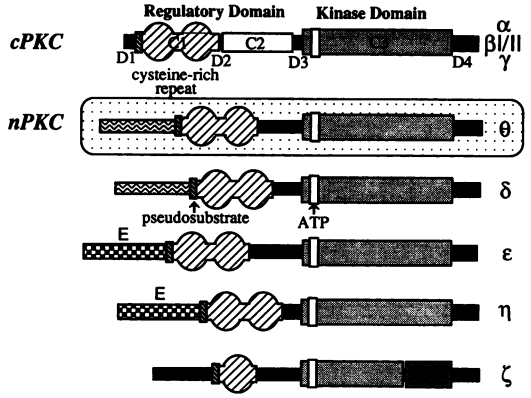

FIG. 3. Schematic structures of the PKC family. C1 to C3 and D1 to D4 indicate the conserved and divergent regions, respectively. Homologous regions among the PKC subspecies are emphasized. Pseudosubstrate and ATP binding sequences are indicated by arrows. E represents the E region in nPKCε and nPKCη as reported previously (45).

was immunoprecipitated with the nPKCθ-specific antipeptide antibody. Autophosphorylation of the immunoprecipitates was examined in the presence of various combinations of the known PKC activators Ca^{2+} (0.5 mM), PS (25 μg/ml), and TPA (50 ng/ml).

As shown in Fig. 9, the 79-kDa protein bands were specifically immunoprecipitated and phosphorylated in lysates prepared from COS1 cells transfected with the nPKCθ cDNA construct (lanes 4 to 8) but not in lysates from controls (lanes 1 to 3), indicating that nPKCθ is a protein kinase. The level of phosphorylation was enhanced by the presence of TPA and PS (lanes 5 and 6) but attenuated by the addition of Ca^{2+} (lane 8), suggesting that the kinase activity of nPKCθ is Ca^{2+} independent, like the activities of other nPKC molecules. The relative ratios to the basal phosphorylation level (lane 4) were 2.7 in lane 5, 2.2 in lane 6, 2.9 in lane 7, and 1.3 in lane 8. In addition to the autophosphorylation, the heavy chain of immunoglobulin G was also phosphorylated. Amounts of proteins applied to the gel were monitored by immunoglobulin G (Fig. 9B). The equal densities of Coomassie brilliant blue staining indicated that the difference in autophosphorylation levels was due to the addition of the PKC activators and not to the amounts of the loaded proteins.

DISCUSSION

In this study, we clearly demonstrated the presence of a new member of the PKC family, nPKCθ, which shows the closest amino acid sequence similarity to nPKCδ, and also demonstrated a unique pattern of expression of nPKCθ. The mRNA for nPKCθ is expressed predominantly in skeletal muscle, in clear contrast to that from nPKCδ, which is expressed in all cells and tissues examined (Fig. 5). Furthermore, nPKCθ mRNA is the dominant PKC isoform mRNA among the nine PKC family members in skeletal muscle (Fig. 6). Antibodies raised against a peptide deduced from the cDNA sequence specifically recognized a 79-kDa protein in an extract from mouse skeletal muscle as well as in an extract from COS cells transfected with the nPKCθ cDNA expression plasmid (Fig. 8). nPKCθ serves as a phorbol ester receptor when expressed in COS1 cells (Fig. 7) and shows protein kinase activity which is activated by phospholipid and phorbol ester (Fig. 9).

TABLE 1. Amino acid sequence identities between nPKCθ and other PKC family members

Form of PKC	% Identity			
	D1	C1	C3	D4
α	—[a]	45.2	61.4	—
βI/II	—	45.2	61.0	38.0/—
γ	—	45.5	58.9	—
δ	49.3	78.1	72.9	60.8
ε	—	47.7	59.1	31.3
η	—	47.4	55.2	37.5
ζ	—	30.1	45.3	34.0

[a] —, sequence identity to nPKCθ is less than 30%.

nPKCθ contains two characteristic sequences, the cysteine-rich repeat sequence (C1) and the protein kinase sequence (C3), that are conserved among all members of the PKC family except nPKCζ, in which the cysteine-rich sequence is not repeated. The cysteine-rich repeat sequence is required for zinc binding (14) and is involved in phorbol ester binding (40). The sequence His-X_{12}-Cys-X_2-Cys-X_{10-14}-Cys-X_2-Cys-X_4-His-X_2-Cys-X_7-Cys, which is involved in zinc binding, is conserved in both repeats of the nPKCθ sequence. The asparagine residue between the fourth and fifth cysteine residues of the second repeat, which has been suggested to be involved in the interaction with TPA or DAG (10), is also seen in the nPKCθ sequence. nPKCθ belongs to the nPKC subfamily. The nPKC members so far identified (δ, ε, η, ζ, and θ) lack the C2 region that is conserved in all conventional PKC members (α, βI, βII, and γ) and has been suggested to be involved in Ca^{2+} dependencies of kinase activities, phorbol ester binding activities, and association with the membranes (1). The lack of the C2 region in the nPKCθ sequence is consistent with results showing that its kinase activity, measured by means of autophosphorylation, seems to be independent of Ca^{2+} (Fig. 9). An additional structural feature of the nPKC subfamily is the presence of a long N-terminal sequence (D1) which is preceding the cysteine-rich sequence. The function(s) of this extended D1 region in nPKCs remains to be identified, but the importance of the catalytic domain, including the D1 region, for substrate specificity was reported previously (46, 50). The N-terminal portion of the cysteine-rich repeat sequence is also conserved in all PKC members. This region contains an alanine residue flanked by basic amino acid residues and has been indicated to be involved in the regulation of cPKCα activity by acting as a pseudosubstrate sequence (12). Although nPKCθ contains this characteristic sequence in the corresponding region, the sequence is clearly distinct from those in the other PKC members except nPKCδ; both nPKCδ and -θ share a closely related sequence in this region. This finding suggests that differences in substrate specificity and activity regulation between nPKCθ and -δ and other PKC members may arise from structural differences associated with this region.

One of the most interesting points in the discovery of nPKCθ is its characteristic expression in skeletal muscle. This property is in marked contrast to the ubiquitous expression of nPKCδ. A survey of the expression of mRNAs for the PKC family members in mouse skeletal muscle revealed that nPKCθ is the major PKC isoform expressed in this tissue. The second most abundant PKC isoform expressed in skeletal muscle is cPKCα, but very weak or no signals were detected for the other mRNA species (Fig. 6). This finding

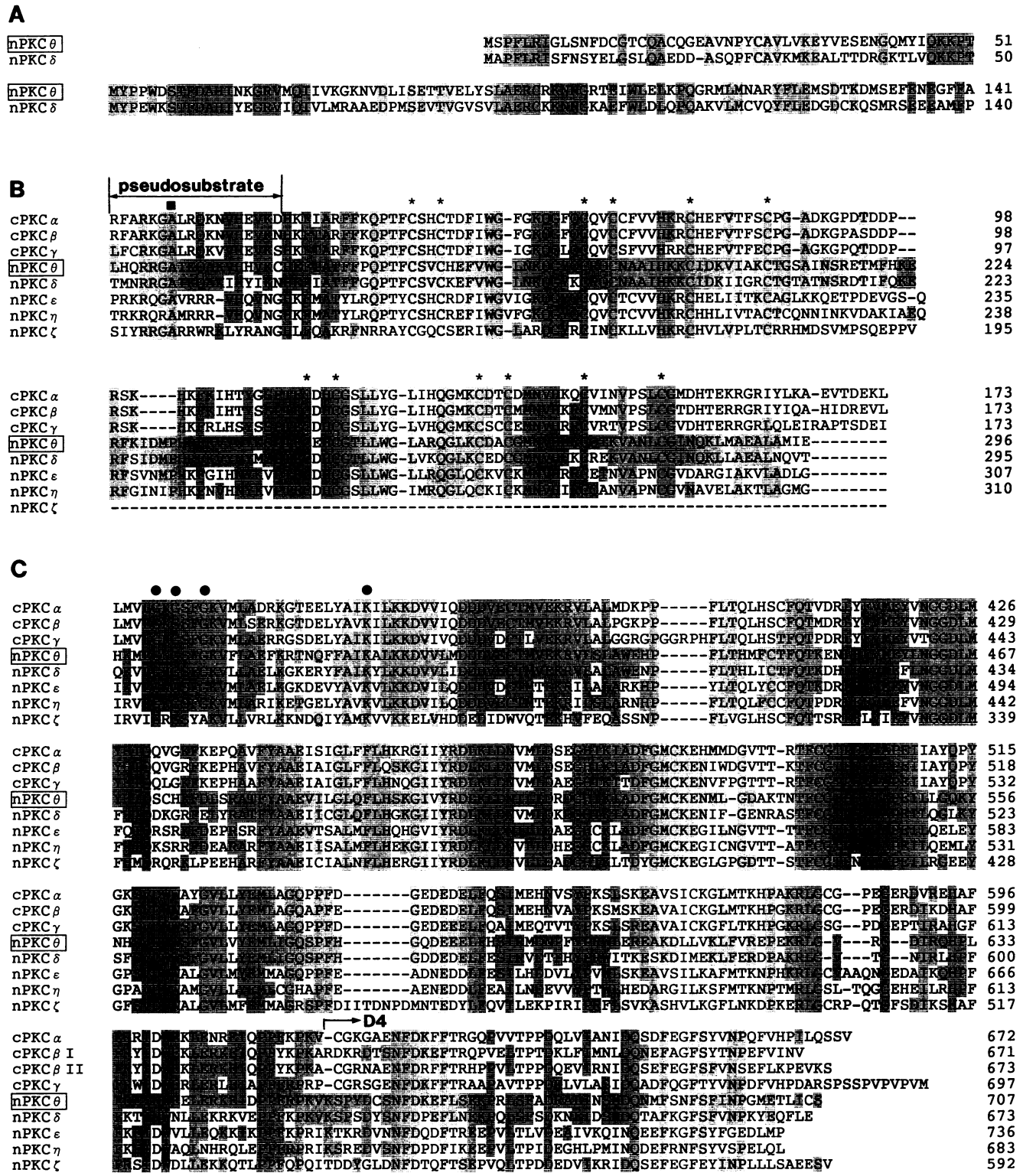

FIG. 4. Sequence comparison of nPKCθ with those of other PKC subspecies. (A) Comparison of the D1 region sequences of nPKCθ and nPKCδ. Identical residues are shaded. (B) Comparison of the cysteine-rich sequences (C1) of the PKC family members. Amino acid residues identical to those of nPKCθ are shaded. Asterisks indicate the conserved cysteine residues in the cysteine-rich sequence. A closed square shows the conserved alanine residue in the pseudosubstrate sequence. (C) Comparison of the kinase domain sequence (C3 and D4) of the PKC family members. Residues are indicated as in panel B. Sequences used for alignment are rabbit cPKCα, rabbit cPKCβI and -βII, rabbit cPKCγ, rat nPKCδ, rabbit nPKCε, rat nPKCζ, and mouse nPKCη.

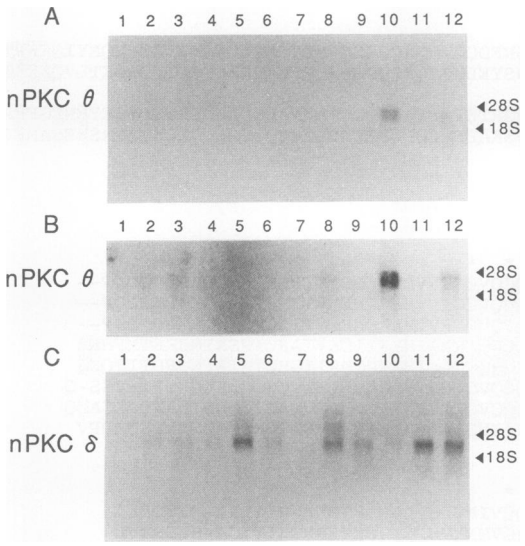

FIG. 5. Northern blot analysis of nPKCθ and nPKCδ in mouse embryo and various mouse tissues. (A) Northern blot analysis using a cDNA fragment specific to mouse nPKCθ (nucleotide −88 to 2004). Sizes of transcript: 4.0 and 3.6 kb. (B) Long exposure (10 days) of the Northern blot shown in panel A. (C) Northern blot analysis using a cDNA fragment encoding the entire protein coding region of mouse nPKCδ. Size of transcript: 3.2 kb. Lanes; 1, COS1 cells; 2, embryo (12 days); 3, brain (4 weeks); 4, heart; 5, intestine; 6, kidney; 7, liver; 8, lung; 9, ovary; 10, skeletal muscle; 11, skin; 12, spleen. Five micrograms of total cellular RNA was loaded in each lane. The positions of rRNAs (18S and 28S), used as standards, are indicated by arrowheads.

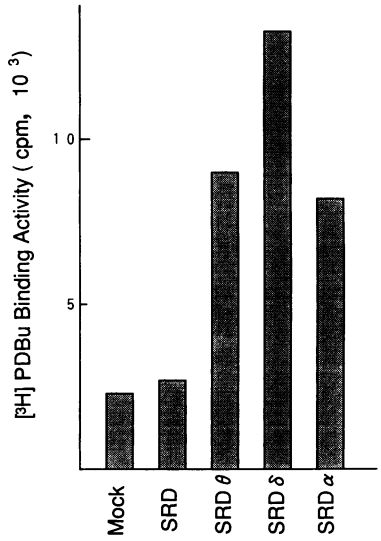

FIG. 7. Phorbol ester binding to intact COS1 cells transfected with various PKC cDNA constructs. The assay was carried out in the presence of 10 nM [3H]PDBu. Each column represents the mean of triplicate measurements.

suggests that nPKCθ is the major phorbol ester receptor in this tissue and that nPKCθ is involved in a certain pathway of signal transduction mediated by DAG and mimicked by phorbol esters. There are several reports on physiological roles of PKC in skeletal muscle. The involvement of PKC in differentiation of skeletal muscle, especially in myoblast fusion, is suggested by studies with chicken and mouse myoblasts (7, 58). Involvement of PKC in insulin-stimulated glucose transport in BC3H-1 myocytes was also reported (53). However, contrary results from skeletal muscle in vivo were obtained (11, 57). Much more work is needed on this subject. Within the myogenic master gene family, MyoD and myogenin are phosphorylated (39). Furthermore, the modulation of myogenin gene expression by TPA in cultured chicken myoblasts has also been reported (24). The MyoD family commonly contains a serine/threonine-rich region near the COOH terminus in which some potential phosphorylation sites for PKC are present (4, 8, 47, 59). It is important

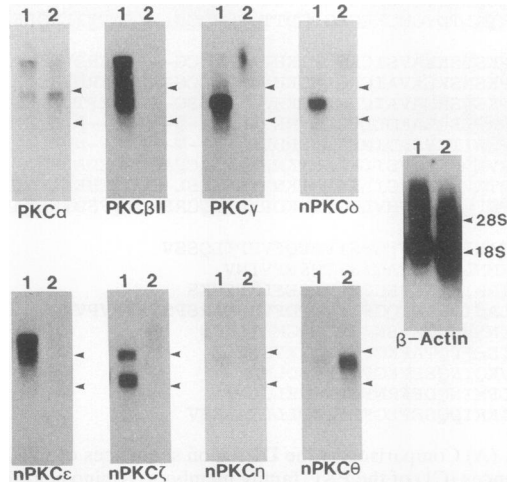

FIG. 6. Northern blot analysis of the PKC isoforms in brain and skeletal muscle. Two-microgram samples of poly(A) RNA from brain (lane 1) and skeletal muscle (lane 2) were loaded. Amounts of loaded RNA samples were monitored by the expression of β-actin. The positions of rRNAs (18S and 28S), used as standards, are indicated by arrowheads.

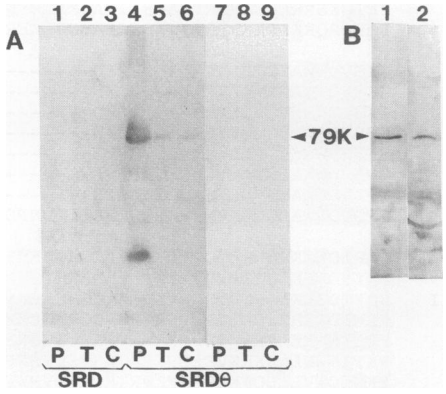

FIG. 8. Immunoblot analysis of nPKCθ in extracts from COS1 cells and skeletal muscle. (A) Extracts from COS1 cells transfected with the SRD vector alone (lanes 1 to 3) or SRDθ (lanes 4 to 9) were treated with antiserum specific to nPKCθ (lanes 1 to 6) or preimmune rabbit serum (lanes 7 to 9). P, Triton-insoluble particulate fractions; T, Triton-soluble particulate fractions; C, cytosolic fractions. (B) Immunological detection of nPKCθ protein in skeletal muscle (lane 2). The cytosolic fraction of COS1 cells expressing nPKCθ (lane 1) was included as a positive control. The same antiserum was used as in panel A.

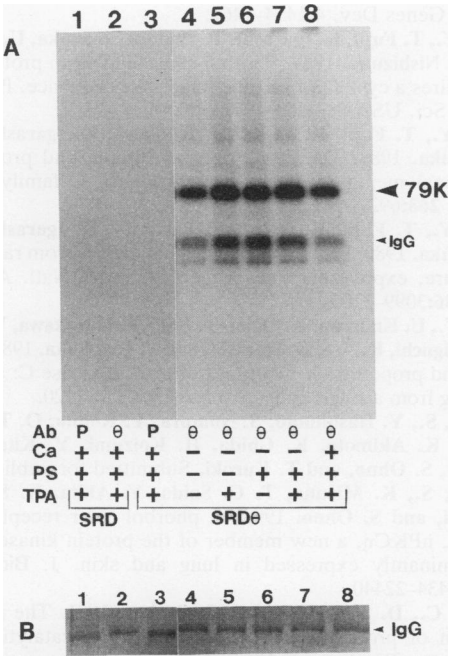

FIG. 9. Autophosphorylation of nPKCθ expressed in COS1 cells. (A) The cytosolic fractions from COS1 cells transfected with SRD (lanes 1 and 2) or SRDθ (lanes 3 to 8) were immunoprecipitated with preimmune rabbit serum (P) (lanes 1 and 3) or antiserum against nPKCθ (θ) (lanes 2 and 4 to 8). The resultant immunoprecipitates were subjected to SDS-PAGE and autoradiography. Equally divided immunoprecipitates were loaded in lanes 4 to 8. The assay was performed in the presence (+) or absence (−) of the known PKC activators Ca^{2+} (0.5 mM), PS (25 μg/ml), and TPA (50 ng/ml). (B) Coomassie brilliant blue staining of the gel used for autoradiography in panel A. Arrowheads indicate the position of the heavy chain of immunoglobulin G (IgG).

to clarify the physiological relationships between nPKCθ and these potential substrates.

Although the presence of Ca^{2+}-independent nPKC molecules has been demonstrated by molecular cloning studies, little attention has been paid to their physiological significance. We have previously reported the presence of nPKCη (PKC-L), which is predominantly expressed in skin and lung (3, 45). In situ hybridization and immunohistochemical analyses of the expression of nPKCη clearly showed that nPKCη is widely expressed in epithelia but not in mesenchyme (44). nPKCδ is also enriched in skin (25) and is the major PKC isoform expressed in hemopoietic cell lines (27). The potential importance of nPKCε in thyrotropin-induced signal transduction in GH4C1 and GH3 rat pituitary tumor cells has also been reported (2, 16, 17). nPKC-like molecules are also found in lower organisms, such as *Drosophila* sp. (52), nematodes (54), and *Saccharomyces cerevisiae* (26), suggesting their evolutionary relationship and universal importance in signal transduction. These results, in conjunction with the findings of this study, indicate that nPKC members but not cPKC members are the major PKCs (phorbol ester receptor/protein kinases) expressed in certain cells and tissues. Recent demonstrations indicating the significance of the novel pathway of DAG generation through phosphatidylcholine breakdown (9), which results in the long-lasting generation of DAG in a variety of signaling systems, may account for the involvement of a specific type(s) of PKC. Functional identification of the PKC type which is involved in a certain signaling pathway is one of the most promising steps toward an understanding of the molecular mechanism underlying signal transduction pathways mediated by the PKC family members.

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