

Tissue and Cellular Distribution of the Extended Family of Protein Kinase C Isoenzymes

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Abstract. Polyclonal isoenzyme-specific antisera were developed against four calcium-independent protein kinase C (PKC) isoenzymes (δ , ϵ , ϵ' , and ζ) as well as the calcium-dependent isoforms (α , β_1 , β_{II} , and γ). These antisera showed high specificities, high titers, and high binding affinities (3–370 nM) for the peptide antigens to which they were raised. Each antiserum detected a species of the predicted molecular weight by Western blot that could be blocked with the immunizing peptide. PKC was sequentially purified from rat brain, and the calcium-dependent forms were finally resolved by hydroxyapatite chromatography. Peak I reacted exclusively with antisera to PKC γ , peak II with PKC β_1 and $-\beta_{II}$, and peak III with PKC α . These same fractions, however, were devoid of immunoreactivity for the calcium-independent isoenzymes. The PKC isoenzymes demonstrated a distinc-

tive tissue distribution when evaluated by Western blot and immunocytochemistry. PKC δ was present in brain, heart, spleen, lung, liver, ovary, pancreas, and adrenal tissues. PKC ϵ was present in brain, kidney, and pancreas, whereas PKC ϵ' was present predominantly in brain. PKC ζ was present in most tissues, particularly the lung, brain, and liver. Both PKC δ and PKC ζ showed some heterogeneity of size among the different tissues. PKC α was present in all organs and tissues examined. PKC β_1 and $-\beta_{II}$ were present in greatest amount in brain and spleen. Although the brain contained the most PKC γ immunoreactivity, some immunostaining was also seen in adrenal tissue. These studies provide the first evidence of selective organ and tissue distributions of the calcium-independent PKC isoenzymes.

PROTEIN kinase C (PKC)¹ plays a major role in transmembrane signal transduction (35). PKC is activated by diacylglycerol, which is generated from membrane phospholipids upon stimulation of cells with various agonists (4). PKC also serves as the receptor for phorbol esters and related tumor promoters (34), which activate the kinase by substituting for endogenous diacylglycerol (8). Because of the pleiotropic actions of diacylglycerol and phorbol esters, PKC has been implicated in the regulation of a variety of cellular processes, including proliferation, differentiation, and release of hormones and neurotransmitters (35, 36).

Molecular cloning studies have revealed that PKC consists of a large family of at least eight different isoenzymes (36, 37) that can be divided into two major groups. Initially, four isoforms of PKC were described. This group consists of PKC α , $-\beta_1$, $-\beta_{II}$, and $-\gamma$, with PKC β_1 and $-\beta_{II}$ arising via alternate splicing of the same gene transcript and resulting in distinct carboxy-terminal regions. All four of these isoen-

zymes show very high sequence homology and similar structural organization with an amino-terminal regulatory region and a carboxy-terminal catalytic domain (22, 36). The regulatory domain is characterized by two highly conserved constant regions C₁ and C₂, with C₁ containing two "zinc finger" cysteine-rich regions. The activity of these four isoforms is characterized by strong dependencies on calcium, phospholipid, and diacylglycerol (7, 36). It is thought that the C₂ region imparts calcium dependence (37), whereas the C₁ region contains the phospholipid and diacylglycerol/phorbol ester binding sites (41). Subsequently, a second group of PKC isoenzymes was identified comprising the PKC δ , $-\epsilon$, $-\epsilon'$, and $-\zeta$ isoforms, with PKC ϵ' arising as a splicing variant of PKC ϵ (40). This novel group of PKC isoenzymes exhibits calcium-independent activity that presumably reflects the absence of the calcium-binding C₂ domain (40).

Although the isoforms of PKC exhibit small differences in their dependencies on lipid cofactors and overlapping substrate specificities (7, 19, 30, 37), a major distinguishing feature of PKC isoforms as a group is their distinct tissue and cellular localization. However, the reported studies are

1. *Abbreviations used in this paper:* PKC, protein kinase C.

limited in scope and have not been extended to calcium-independent isoenzymes. PKC α has been shown to display a ubiquitous tissue distribution in one study (27) and a limited peripheral distribution in another (56). The β isoenzymes show a more restricted distribution (27, 56), although little information is available on the relative distribution of the β_1 and β_{II} isoenzymes. Northern blot analyses (25, 39) and limited immunologic studies (18, 27, 54, 56) indicate that PKC γ may be localized exclusively in the brain. By comparison, analysis of the tissue and cellular distribution of the more recent members of the PKC family is restricted to a limited Northern blot study with a rather conspicuous absence of immunologic determination of the PKC δ , $-\epsilon$, $-\epsilon'$, and $-\zeta$ isoenzymes in different tissues (40).

Because of the lack of information on the tissue distribution of the isoenzymes and because the specificities of most of the available antisera to the conventional isoenzymes of PKC are unknown, this study was undertaken. We describe the development of antisera to eight PKC isoforms: PKC α , $-\beta_1$, $-\beta_{II}$, $-\gamma$, $-\delta$, $-\epsilon$, $-\epsilon'$, and $-\zeta$. We demonstrate specificity of the antisera by ELISA, fast protein liquid chromatography, Western blot, and immunocytochemistry. Finally, we determine the tissue and cellular distribution of the novel PKC isoforms and compare them to the calcium-dependent isoenzymes.

Materials and Methods

Materials

Glutaraldehyde, 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, bovine thyroglobulin, Freund's complete or incomplete adjuvant, *O*-phenylenediamine hydrochloride, and histone III proteins were purchased from Sigma Chemical Co. (St. Louis, MO). Phosphatidylserine was obtained from Avanti Polar Lipids, Inc. (Birmingham, AL). The sn-1,2-dioleoylglycerol was prepared as described (32). PKC peptides were synthesized by the Protein Chemistry Laboratory of the University of North Carolina at Chapel Hill and purified by HPLC on a C₁₈ reverse-phase column using a 0.1% trifluoroacetic acid buffer with acetonitrile gradient elution. Immuno-Plate II 96-well plates for ELISA were bought from Nunc, Inc. (Naperville, IL). Peroxidase-labeled goat anti-rabbit IgG was obtained from Kirkegaard and Perry Labs, Inc. (Gaithersburg, MD). Reagents for SDS-PAGE and nitrocellulose paper were from Bio-Rad Laboratories (Richmond, CA). Additional reagents consisted of sheep anti-rabbit IgG, rabbit peroxidase antiperoxidase, and normal sheep serum (Arnel Inc., Parlin, NJ), DAB and 4-chloro-1-naphthol (Aldrich Chemical Co., Milwaukee, WI), Panatomi X film (Eastman Kodak Co., Rochester, NY), [³²P]-ATP (New England Nuclear, Boston, MA), and GF/c filters (Whatman Inc., Hillsboro, OR).

Antiserum Production

Unique amino acid sequences from each of eight different PKC subspecies were selected and synthesized (Table I). NH₂ terminally derived PKC ϵ and $-\epsilon'$ peptides were conjugated with carbodiimide to thyroglobulin as described (13). After reaction for 18 h at 23°C, the mixture was dialyzed and stored at -20°C in volumes appropriate for the initial injection and subsequent booster injections.

All of the COOH terminally derived PKC peptides were mixed with thyroglobulin as described (10). Glutaraldehyde was added dropwise over a 30-min period to a final concentration of 0.1%. This mixture was allowed to stir for an additional 45 min at 23°C before glycine was added to bring the solution to 0.2 M. This mixture was dialyzed and stored as outlined above.

Antisera to each of the nine PKC-conjugated peptides were raised in male and female New Zealand rabbits (2.3–3.2 kg). Initially, each rabbit was given 0.8 mg of conjugate in multiple intradermal and subcutaneous injections (10). Later, each rabbit was boosted with 0.2 mg of conjugate in 0.5

Table I. Amino Acid Sequences of PKC Peptides Used to Generate Antisera

PKC isoenzyme	Amino acid sequence	Reference	K _d values* nM
Rat PKC α	⁶⁵⁷ SYVNPQFVHPILQSAV ⁶⁷²	22	99.0
Rat PKC β_1	⁶⁶¹ SYTNPEFVINV ⁶⁷¹	22	46.0
Rat PKC β_{II}	⁶⁶⁰ SFVNSEFLKPEVK ⁶⁷³	22	10.5
Rat PKC γ	⁶⁸⁴ DARSPTSPVPVPM ⁶⁹⁷	22	20.0
Rat PKC δ	⁶⁶² SFVNPKYEQFLE ⁶⁷³	40	3.6
Rat PKC ϵ	⁷²⁶ KGFSYFGEDLMP ⁷³⁷	40	28.0
Rat PKC ϵ'	¹ MVVFNGLLKIKI ¹²	40	370.0
Rat PKC ϵ'	¹ MPHKFGIHNK ¹¹	40	180.0
Rat PKC ζ	⁵⁷⁹ EYINPLLLSAEESV ⁵⁹²	40	13.0

The amino acid sequences for PKC α , $-\beta_1$, $-\beta_{II}$, $-\gamma$, $-\delta$, $-\epsilon$, and $-\zeta$ are located at the extreme COOH terminus of these enzymes.

* The peptides were purified by HPLC to a single peak, but no amino acid analysis was run. In these calculations, it is assumed that the peptides were pure.

† The sequences for PKC ϵ and PKC ϵ' are found at the extreme NH₂-terminal region of these enzymes.

K_d: dissociation constant.

ml of Freund's incomplete adjuvant. Rabbits were boosted at 6-wk intervals and bled 10–14 d after each boost.

ELISA Assays

ELISA assays were used to examine the antiserum titer at each bleeding and to determine the affinity and immunological specificity of the antisera. Approximately 6–30 pmol of peptide antigen (Table I) was solubilized with a 3.5 mM sodium bicarbonate/1.5-mM sodium carbonate solution (pH 9.6), and added to wells on Immuno-Plates. All incubations were performed at 23°C. After 2 h, a PBS/0.5% BSA-blocking solution was added, followed by the primary antiserum (1:2,000–1:256,000). The wells were washed in a PBS/0.1% gelatin solution, and then peroxidase-labeled sheep anti-rabbit IgG antiserum was added, and color was developed with a 2.5-mM citrate/5-mM phosphate solution containing *O*-phenylenediamine hydrochloride and hydrogen peroxide. The reaction was stopped with 8 N sulfuric acid and the plates were read at 492 nm with a multiscan MC spectrophotometer (Titertek, Helsinki, Finland).

An antigen-antiserum-binding curve was established by adding increasing concentrations of the antigenic peptide (until absorbance plateaued in the ELISA) to a single dilution of antiserum. After subtraction of background absorbance from each reading, this binding curve was normalized by setting the maximal absorbance for the antigenic peptide equal to 100% binding. Immunological specificity was determined by adding different concentrations (10–5,000 pmol) of the other eight peptides to the assay and comparing their absorbances to the antisera. Cross-reactivity of the antisera for each of these eight comparison PKC peptides was assessed by normalizing the data as described and comparing them with the normalized scores for the antigenic peptide. Since the midpoint on the binding curve approximates the dissociation constant of the antiserum for the peptide antigen, these binding affinities were also estimated.

Preparation of Tissue Extracts for Western Blotting

Rats (200–250 g) were decapitated and the desired organs were immediately removed, weighed, and transferred into ice-cold homogenizing buffer (20 mM Tris-HCl, pH 7.5, 0.25 M sucrose, 10 mM EGTA, 1 mM EDTA, 10 mM β -mercaptoethanol, 1 mM PMSF, 0.02% leupeptin, and 0.1% Triton X-100). The homogenate was incubated for 1 h at 4°C and centrifuged at 100,000 g for 30 min at 4°C. An aliquot of the supernatant was taken for protein determination (5), while the remainder was immediately mixed (1:1) with 2 \times SDS sample buffer (28) and heated in a boiling water bath for 5 min. The boiled samples were either used immediately or frozen at -20°C for SDS-PAGE.

Purification of PKC From Rat Brain

PKC was purified from rat brain cytosol according to the modified fast protein liquid chromatography procedure of Kitano et al. (24). Briefly, rat

brains were homogenized in the homogenizing buffer as described above except Triton X-100 was omitted. The homogenate was centrifuged at 100,000 g for 1 h at 4°C. All the subsequent procedures were performed at 4°C. The supernatant was purified by sequential chromatography on DEAE-sephacryl, threonine-sepharose, and phenyl-sepharose columns. Fractions containing peak activity after the last column were pooled and subjected to hydroxyapatite column chromatography according to Huang et al. (19). The column was equilibrated with buffer B (20 mM potassium phosphate buffer, pH 7.4, 10 mM β -mercaptoethanol, 10% glycerol). The enzyme was eluted by a linear gradient of 20 to 300 mM potassium phosphate in 80 ml buffer B. Every other fraction was assayed for calcium/phosphatidylserine/sn-1,2-diacylglycerol-dependent activity as described (12).

Western Blot Analysis

Tissue extracts previously boiled in sample buffer or fast protein liquid chromatography-purified peak fractions were subjected to 12% SDS-PAGE according to the method of Laemmli (28). The separated proteins were electrophoretically transferred to nitrocellulose at 4°C overnight by the method of Towbin et al. (53). Nonspecific binding sites on nitrocellulose paper were blocked by incubation with 5% nonfat dry milk in 1× PBS for 1 h at room temperature. The nitrocellulose sheets were then incubated with the respective PKC antisera (1:750–1:4,000 dilution) for 2 h at room temperature. After three washes with 5% nonfat milk in 1× PBS at 15-min intervals and a wash with PBS, secondary antiserum (goat anti-rabbit labeled with HRP at 1:1,500 dilution) was added and incubated for 2 h at room temperature. The paper was subjected to three 5-min washes with 5% nonfat dry milk, followed by one 5-min wash with PBS, and then color was developed using hydrogen peroxide and 4-chloro-1-naphthol according to the manufacturer's instruction.

To demonstrate specificity of immunoblotting, the cerebellar extracts were run in the presence or absence of the appropriate antigenic peptide. For this specificity test, 2 μ g/ml of each of the peptide antigens (Table I) was added to the respective primary PKC antisera just before addition to the blot. Subsequent washes, addition of secondary antisera, and color development were performed as described above.

Immunocytochemistry

Male and female Sprague-Dawley rats (250–300 g) were anesthetized with tribromoethanol (1.0 ml/100 g body wt) and perfused through the ascending aorta with 30 ml of 1% phosphate-buffered paraformaldehyde (pH 7.4), followed by 150 ml of 4% paraformaldehyde in the same phosphate buffer. 30 min after perfusion, the brain, cerebellum, ovary, testes, liver, heart, lungs, spleen, pituitary gland, pancreas, and adrenal glands were removed and immersed in the same fixative. The next day, brains or cerebellum were cut into smaller pieces and sliced at 30 μ m with a vibratome (Technical Products International, Inc., St. Louis, MO). The peripheral tissues were dehydrated in a series of graded alcohol steps and embedded in paraffin. These paraffin-embedded tissues were serially cut at 5 to 7 μ m. After deparaffinization, sections were treated with trypsin (11), exposed to 2% normal sheep serum, and then incubated with anti-PKC sera (1:1,000–1:4,000 dilution) for 48 h at 4°C in a humid chamber. Sections were stained by the double peroxidase antiperoxidase technique (44), washed with PBS, and exposed to OsO₄ vapors to enhance the intensity of staining. Finally, the sections were counterstained with toluidine blue.

Brain and cerebellar vibratome sections were collected in glass vials and treated as described (33). The sections were exposed to 2% normal sheep serum and stained while floating in antisera. The rabbit anti-PKC sera (1:1,000–1:4,000 dilution) were added to the sections and incubated for 24 h at 4°C. All subsequent incubations were performed at room temperature for 30 min. After a rinse in PBS, the sections were incubated with sheep anti-rabbit IgG (1:500 dilution), washed again with PBS, and incubated with peroxidase antiperoxidase serum (1:1,000 dilution), and then color was developed with DAB (40 mg/ml) and 0.002% hydrogen peroxide.

Results

ELISA Characterization of Antisera

Antisera to PKC α , β _I, β _{II}, γ , δ , ϵ , and ζ were developed. Three different antisera were developed for PKC ϵ (Table I); two were made to the NH₂-terminal region of this

species to distinguish PKC ϵ from PKC ϵ' (40), whereas a third was targeted to the extreme COOH terminus of PKC ϵ (PKC ϵ and PKC ϵ' have the same COOH-terminal sequence). Titers for all antisera were high on the first bleedings, and they continued to increase over additional bleedings. In initial characterization studies, we used the ELISA to establish binding curves and then examined cross-reactivity of each antiserum to each of the different PKC-derived peptides (Table I). Distinct antisera were developed for each of the isoforms. All antisera showed high titers (1:2,000–1:256,000 dilution) and demonstrated very high affinities for their respective antigenic peptides. The estimated dissociation constants for all of the COOH terminally directed antisera ranged from 3 to 99 nM (Table I). Antisera to the NH₂ terminally derived PKC ϵ and PKC ϵ' had somewhat higher dissociation constants for their respective peptides at 370 and 180 nM (Table I), probably due to the hydrophobic nature of these peptides. All binding curves displayed a sigmoidal shape, suggesting that each polyclonal antiserum was relatively homogeneous with respect to the recognition site for the antigenic peptide (data not shown). Cross-reactivity for the different peptide antigens was always <7% at doses up to 50 μ M (data not shown). Hence, all antisera appeared to be highly specific for the peptide to which they were raised. These results demonstrate the high titers, affinities, and specificities of these antisera for their respective antigenic peptides.

Characterization of Antisera against PKC Isoenzymes

Western blot studies have shown that PKC α , β , and γ are present in rat cerebellum, and gene expression experiments have demonstrated that the mRNAs for PKC δ , ϵ , and ζ are present in rat brain (16, 40). Therefore, in our studies we used a cerebellar extract to further characterize our isoenzyme-specific antisera. Western blot analysis of the cerebellar extract with the COOH-terminally directed PKC α , β _I, β _{II}, γ , δ , ϵ , and ζ antisera showed immunoreactivities at 72–90 kD (Fig. 1). PKC α , β _I, β _{II}, and γ were all detected at \sim 80 kD (Fig. 1). A minor immunoreactive band was recognized at \sim 31 kD by the PKC β _I antiserum. This low molecular mass band and all other bands at \sim 80-kD were displaced by competition with 2 μ g/ml of the respective antigenic peptide (Fig. 1). These results are in agreement with previous Western blot studies suggesting that PKC α , β _I, β _{II}, and γ are \sim 80 kD in size (17, 27, 56). In addition, this size is consistent with the cDNA cloning and sequencing data (9, 25).

The COOH terminally directed PKC δ , ϵ , and ζ antisera detected immunoreactive bands at \sim 80, 90, and 72 kD, respectively (Fig. 1). The PKC δ antiserum recognized a single immunoreactive species, and this 80-kD band was successfully competed with peptide. Frequently, the PKC ϵ antiserum immunostained materials as a doublet at \sim 90–94 kD. A third faint band can also be seen at \sim 50 kD in this blot. The higher molecular mass bands were displaced by the addition of antigenic peptide, whereas immunoreactivity remained at the lower molecular mass. The PKC ζ antiserum recognized materials at \sim 50, 72, and 80 kD, with the predominant band migrating at 72 kD. The 50- and 72-kD bands disappeared with the addition of the antigenic peptide. Two points should be made. First, the sizes of the materials detected by our PKC δ and ϵ antisera are consistent with the

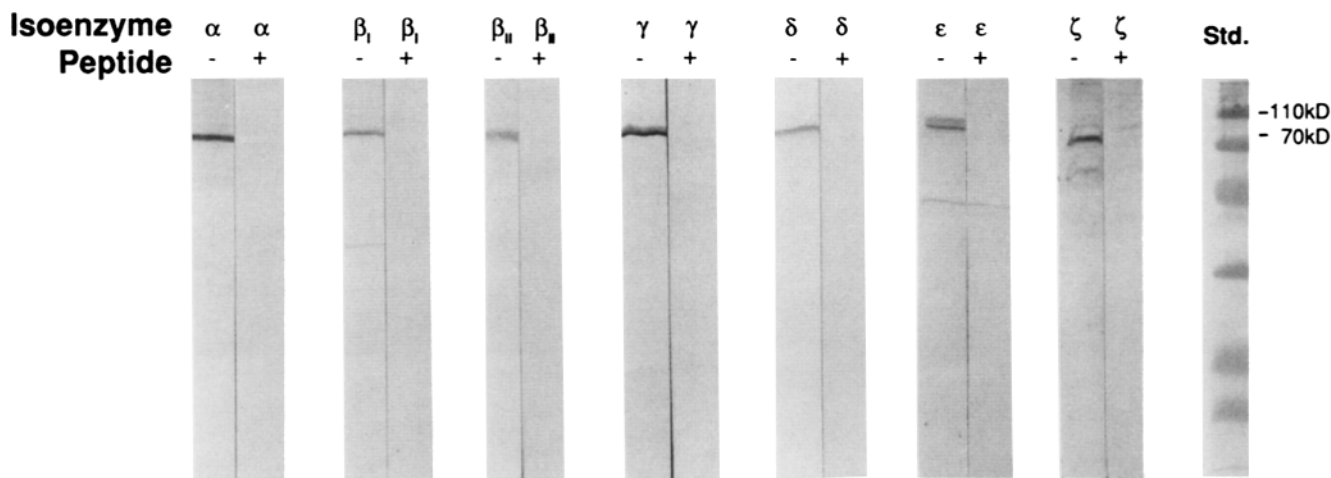


Figure 1. Specificity of the different C-terminally derived PKC antisera. Fresh cerebellar extract (28 μg protein) was subjected to SDS-PAGE and transferred to nitrocellulose paper as described in Materials and Methods. Immunoblotting was performed using each antiserum (1:750–1:4,000 dilution) with (+) or without (–) the respective peptide (2 $\mu\text{g}/\text{ml}$) added just before addition to the blot. Std., standard.

sizes (e.g., 80–90 kD) observed both with Western blots (23, 42, 49) and predicted from the cDNA cloning data (39, 40). The PKC ζ isoform identified in cerebellum was found to be somewhat larger than that reported in transfected COS cells (42). This discrepancy may be attributed to differences in posttranslational processing, because we observed the PKC ζ immunoreactive species to be of differing sizes in various tissues (see below). Second, protein bands of 70–90 kD were all successfully competed with the corresponding antigenic

peptide. Hence, these antisera appeared to recognize the appropriate PKC species to which they were raised.

In addition to the PKC antisera, which were targeted to the COOH terminal regions of these seven PKC species, we also developed antisera to the NH₂-terminal regions of PKC ϵ and PKC ϵ' . The PKC ϵ antiserum bound material at ~ 90 kD, whereas the PKC ϵ' antiserum recognized a major band at ~ 55 kD and a minor band at ~ 90 kD (data not shown). This high molecular mass band detected at 90 kD

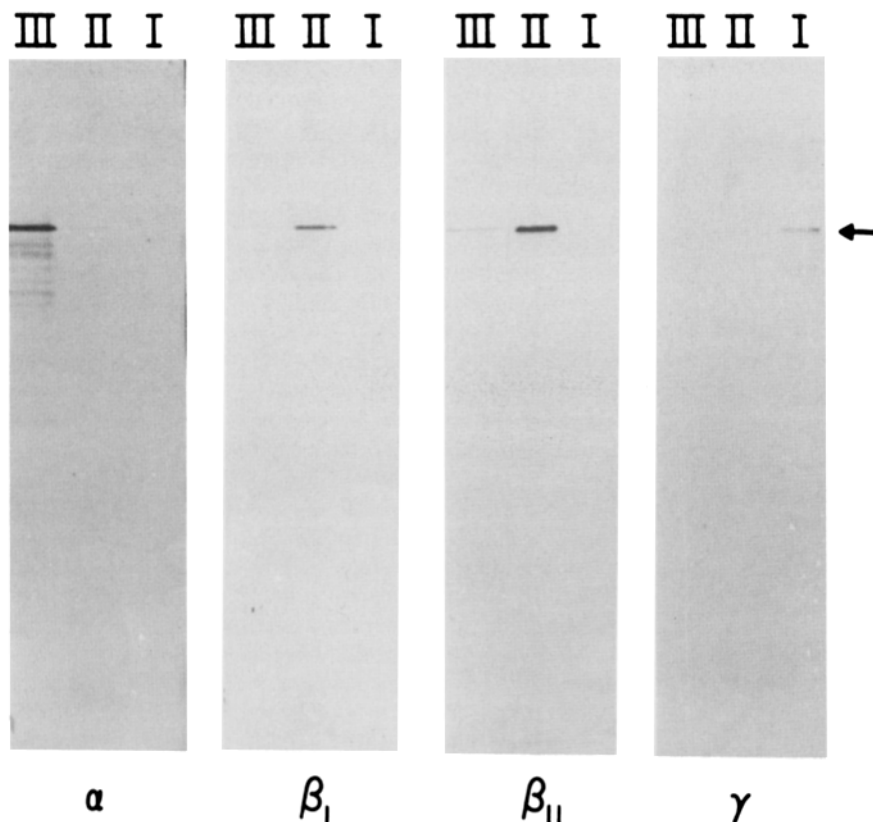


Figure 2. Specificity of PKC α , $-\beta_1$, $-\beta_{II}$, and $-\gamma$ antisera against hydroxyapatite-resolved PKC isoenzymes. Immunoblot analysis of type I, II, and III isoenzymes: pooled fractions containing equal kinase activity were subjected to SDS-PAGE and then transferred to nitrocellulose paper and immunoblotted with each of the antisera. The arrow indicates the position of PKC.

by the PKC ϵ' antiserum may represent PKC ϵ , because this PKC isoform contains an internal amino acid sequence corresponding to ϵ' (ϵ' is a truncated splice variant of ϵ and does not offer unique sequence [40]). In addition, these immunoreactive bands were successfully competed by the respective peptide antigen (data not shown). However, these antisera were difficult to use for Western blotting, and, as a result, the COOH terminally directed PKC ϵ antiserum was used in all subsequent immunoblots for detection of PKC ϵ .

Specificity of Antisera Using Purified PKC Isoenzymes
PKC α , $-\beta$, and $-\gamma$ isoenzymes can be resolved chro-

matographically on hydroxyapatite with increasing phosphate concentrations (19). In our studies, we used sequential chromatography followed by a hydroxyapatite separation at the final step. This procedure permitted us to separate, as a group, the calcium-independent forms of PKC from the dependent forms, because the calcium-independent isoenzymes elute at a higher salt on DEAE. Hence, during hydroxyapatite separation, the remaining calcium-dependent forms were resolved into three different peaks (data not shown).

Western blot analysis of these peaks is shown in Fig. 2. It is evident from these results that type I PKC reacted with

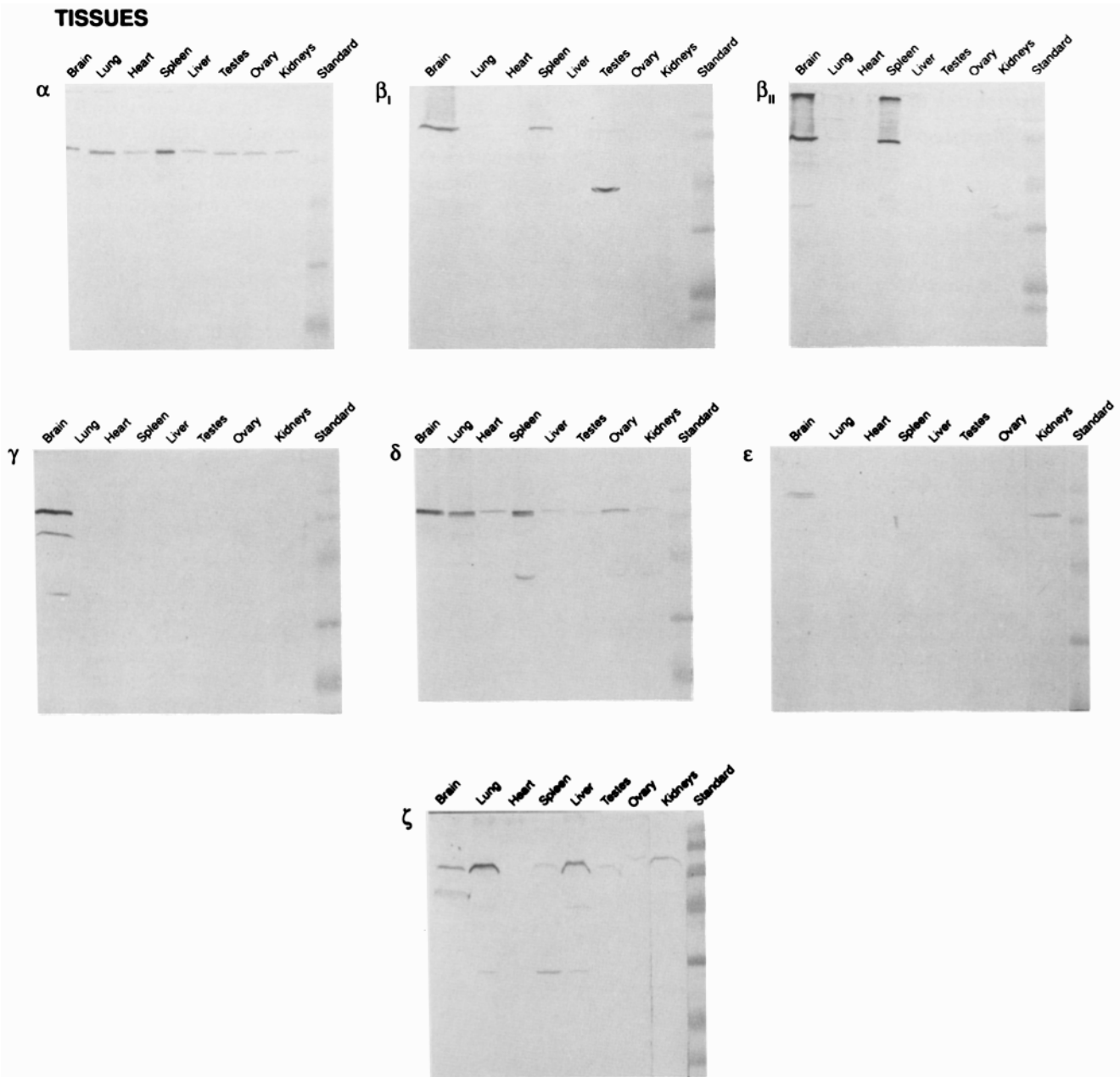


Figure 3. Tissue distribution of different PKC isoenzymes in the rat. Total tissue extract (28 μ g protein) was separated by SDS-PAGE and immunoblotted as described in Materials and Methods. The PKC α and $-\zeta$ antisera were used at 1:4,000 and 1:750 dilutions, respectively. All other antisera were used at a 1:1,000 dilution.

the PKC γ antiserum, and peak II reacted with both the PKC β_1 and $-\beta_{II}$ antisera, with PKC β_{II} showing higher reactivity than PKC β_1 . Peak III reacted with the PKC α antiserum and, to a much lesser extent, with the PKC β_1 antiserum. A similar finding has been reported by others (19). These results demonstrate that the PKC α , $-\beta_1$, $-\beta_{II}$, and $-\gamma$ antisera are selective and biochemically specific for the isoenzymes against which they were raised.

Because of our separation scheme, the separated peaks on hydroxyapatite did not show any cross-reactivity with the PKC δ , $-\epsilon$, and $-\zeta$ antisera (data not shown), again emphasizing the immunospecificity of each of the antisera. These results also demonstrate that the purified and separated PKC α , $-\beta$, and $-\gamma$ isoenzymes are not contaminated with PKC δ , $-\epsilon$, or $-\zeta$ when sequential chromatography precedes the hydroxyapatite separation. Hence, this separation scheme removes PKC ϵ as a possible coeluting species in the PKC β peak when only hydroxyapatite chromatography is used (see [26, 49]).

Tissue Distribution of PKC Isoenzymes

We used isoenzyme-specific antisera for Western blotting to detect the presence of the PKC α , $-\beta_1$, $-\beta_{II}$, $-\gamma$, $-\delta$, $-\epsilon$, and $-\zeta$ isoforms in extracts from rat tissues prepared from brain, lung, heart, spleen, liver, testes, ovary, and kidney. These results are depicted in Fig. 3 and Table II. Because the antisera have differing affinities for their peptide antigens and, presumably, for the corresponding PKC isoform, comparisons in relative immunostaining among tissues can be made for a given antiserum but not among antisera. During the preparation of samples for immunoblotting, we found that rapid processing of the organs was essential to minimize the appearance of low molecular mass bands on the gels (indicative of PKC degradation because they could be competed with antigenic peptides). In Fig. 3, the immunoreactive bands from the different tissues were the same size as those found for the cerebellum (see Fig. 1), with few exceptions as noted below.

When the blot was stained with the PKC α antiserum, an 80-kD band was visualized in all tissues examined (Fig. 3). The spleen was particularly enriched with this isoform, and dense bands were also obtained from lung and brain extracts (Table II). Lighter bands were found in the ovaries, testes, kidney, heart, and liver. The ubiquity of PKC α has been reported by other investigators (1, 27, 29, 46, 55, 56). This study corroborates these findings.

When blots were stained with the PKC β_1 antiserum, the darkest staining occurred with the brain extract; somewhat lighter staining was present in the spleen, and very faint immunoreactivity was visualized in the testes, lung, heart, and ovary (Fig. 3; Table II). Dark immunostaining with PKC β_1 antiserum, which was observed at 39 kD in testis extract, disappeared with addition of antigenic peptide (data not shown). The identity of this band is unknown; however, it could represent a proteolytic fragment of PKC β_1 . Immunoblotting for PKC β_{II} revealed a dense band in brain extract and spleen and very light staining in other tissues (Table II). No staining was detected in liver with either PKC β_1 or $-\beta_{II}$ antisera. Although PKC β_1 and $-\beta_{II}$ have been shown by Western blotting to be present in rabbit and rat brain (1, 51), they have not been localized specifically to these other tissues. It should be noted, however, that other investigators have detected PKC β (with no distinction between PKC β_1 and $-\beta_{II}$) in spleen, testes, and kidney (27).

The PKC γ immunoblots revealed this isoform to be present exclusively in brain (Fig. 3; Table II). Faint bands at ~ 65 and 35 kD could not be competed with peptide in brain (data not shown). Our findings are consistent with previous studies using Northern or Western blot analyses (6, 9, 25, 46).

PKC δ was present as an 80–84-kD doublet in brain, lung, spleen, ovary, and kidney (Fig. 3). In heart, liver, and testes, it appeared as a single 80-kD band. A minor band at ~ 50 kD was detected in spleen. All bands could be competed with the antigenic peptide (data not shown). Hence, PKC δ may have multiple molecular forms in different tissues. PKC δ was particularly prominent in brain (Fig. 3; Table II). Spleen and lung contained significant levels of PKC δ , with somewhat lower levels in ovary and heart, whereas testes, kidney, and liver were found to express the least amount of the PKC δ isoenzyme. Although Western blot analyses have not been previously applied to these tissues, Northern blots have revealed PKC δ to be expressed in brain, lung, spleen, and kidney (40).

The PKC ϵ antiserum bound material at ~ 90 –94 kD as a doublet in brain and as an 80-kD species in kidney (Fig. 3). A very faint band at 50 kD was visualized in spleen and testes that could not be competed with antigenic peptide (data not shown). Hence, this low molecular mass material must represent nonspecific binding. The immunostaining in the brain was darker than in kidney, and little staining was detected in the other tissues examined (Table II). Although our results

Table II. Relative Band Density of PKC Isoenzymes in Various Tissues of the Rat as Detected by Western Blot Analysis

Isoenzymes	Relative density in organs*							
	Brain	Lung	Heart	Spleen	Liver	Testes	Ovary	Kidney
α	3	4	1	5	1	2	2	2
β_1	5	1	1	3	ND	1 [†]	1	ND
β_{II}	5	1	1	4	ND	1	1	1
γ	5	ND	ND	ND	ND	ND	ND	ND
δ	5	4	3	5	2	1	3	1
ϵ	5	ND	ND	ND	ND	ND	ND	3
ζ	3	5	1	2	4	2	2	3

* Equal amounts of protein were loaded from each organ as described in Materials and Methods. The numbers indicate the relative density of immunostaining with 5 being highest and 1 the least detectable staining.

[†] Strong specific staining of a 39-kD band was detected in testes.

ND, no staining detected.

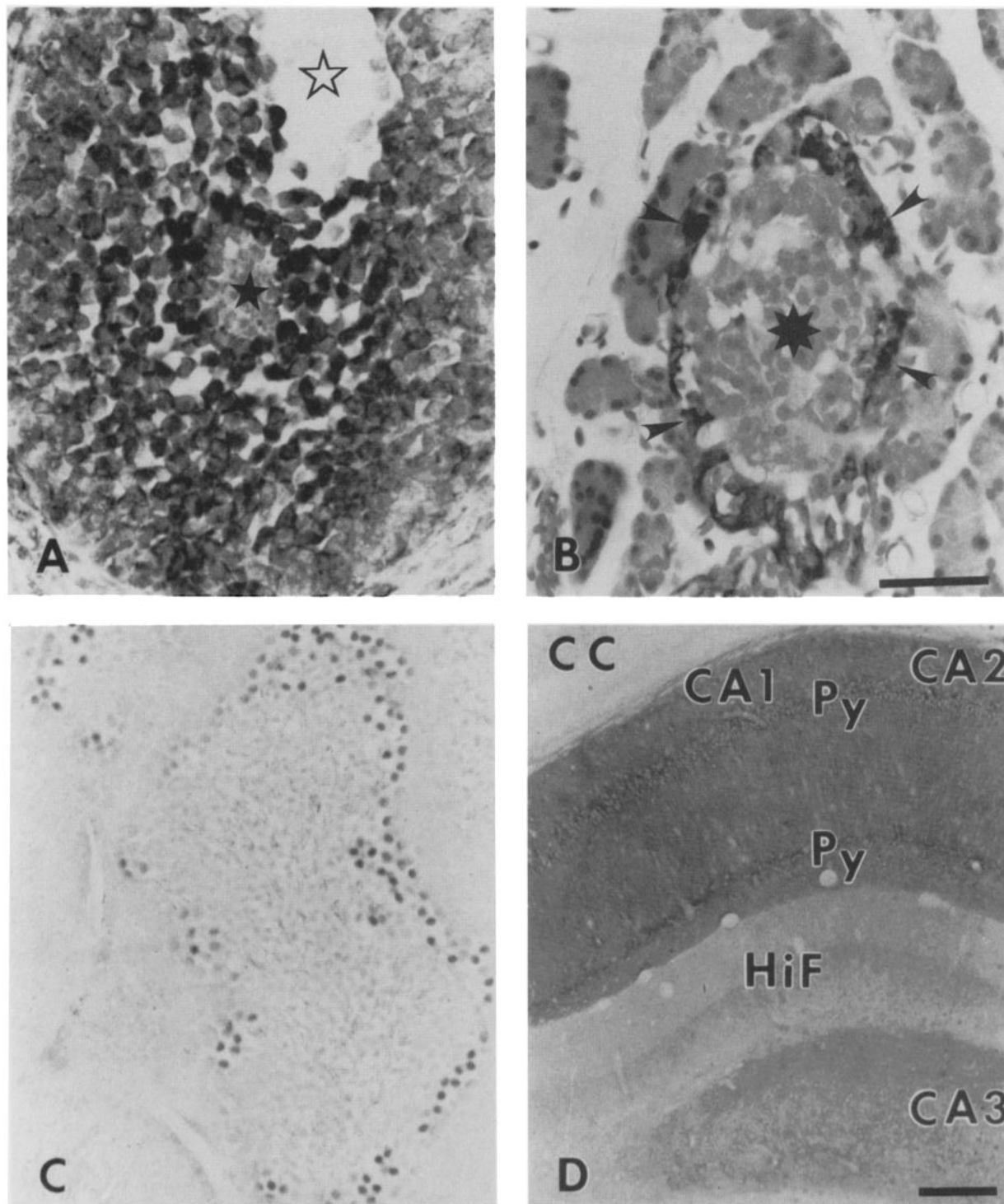


Figure 4. Immunocytochemical staining of the rat ovary, pancreas, testes, and hippocampus with polyclonal antisera to PKC α , PKC β_1 , PKC β_n , and PKC γ , respectively. In the ovary (A), the granulosa cells of the ovarian follicle are darkly stained. The open star represents the antrum folliculi; the solid star indicates the ovum with the cumulus oophorus. In the pancreas (B), only peripherally located cells of the islets of Langerhans (asterisk) are immunostained (arrowheads). The exocrine glands are not stained. In the testes (C), the nuclei of the Sertoli cells are immunostained. In the hippocampus (D), the CA1-CA3 regions are darkly stained. The immunostaining is located in nerve terminals throughout these regions. The pyramidal cells (Py) seem to be surrounded by immunoreactive nerve terminals. The paraffin sections (A-C) were counterstained with toluidine blue. The vibratome section is not stained. CC, corpus callosum; HiF, hippocampal fissure. Bars, 100 μ m.

from brain are in agreement with other Western and Northern blot data (1, 39, 40), the occurrence of PKC ϵ in other organs has not been studied previously. In addition, since multiple transcripts for PKC ϵ can exist both within the same

tissue and among tissues (40, 50), PKC ϵ isoenzymes of differing sizes may exist in these tissues.

PKC ζ migrated as a 72-80-kD band in different tissues (Fig. 3). PKC ζ was maximally expressed in lung and at lower

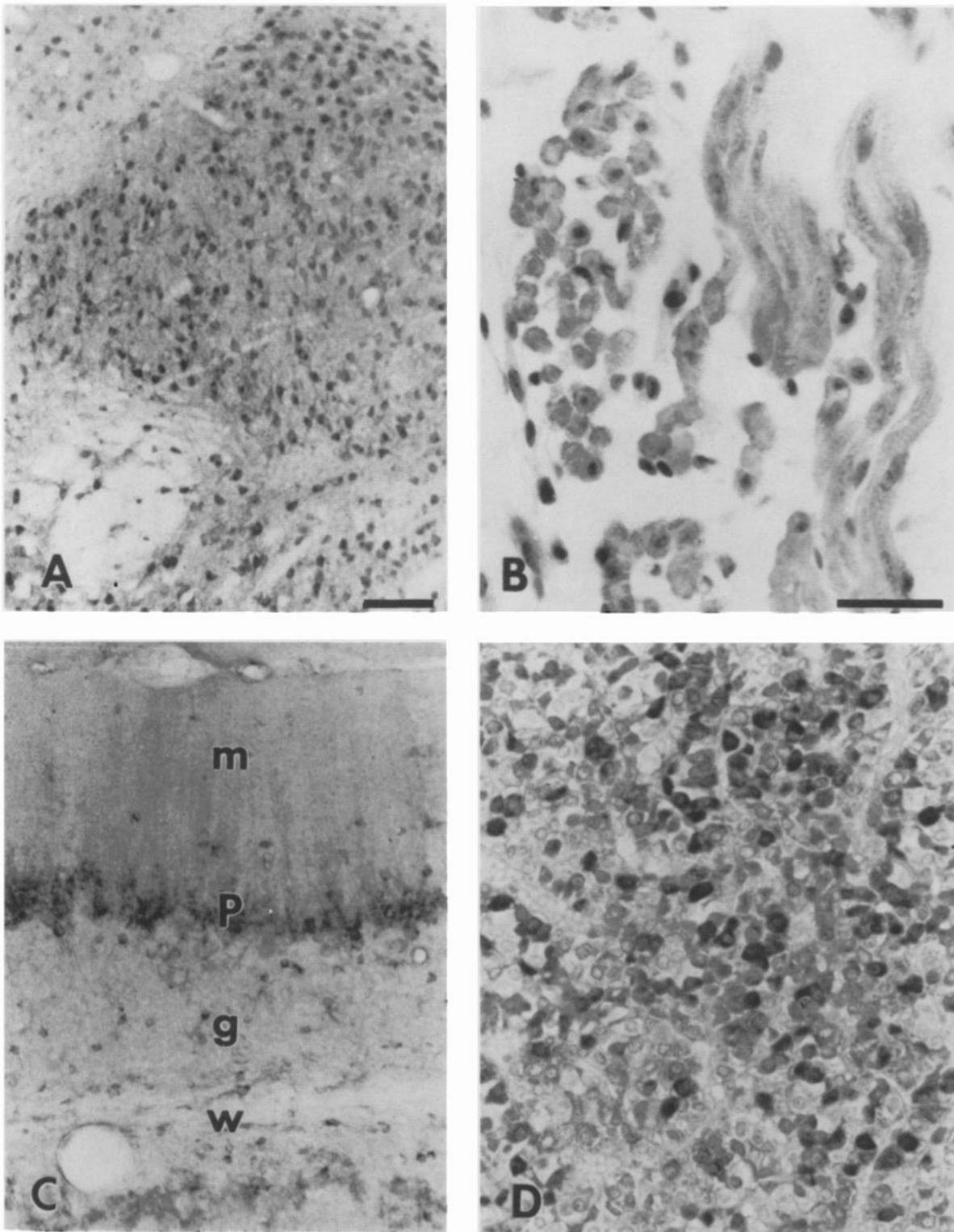


Figure 5. Immunostaining of rat thalamus, heart, cerebellum, and anterior pituitary gland with antisera to PKC δ , PKC ϵ , PKC ϵ' , and PKC ζ , respectively. The PKC δ antiserum stains all neuronal cells in the thalamus, especially in the nucleus posterolateralis (A). The terminal portion of these neurons in the cortex is also immunostained (data not shown). In the heart (B), longitudinally and cross-sectioned muscle fibers are immunostained. The PKC ϵ' antiserum stains cells in the molecular layer (m), the granular layer (g), and fibers and nerve terminals around Purkinje cells (P) in the cerebellum (C). Within the pituitary gland, the medium and large round cells are immunostained (D). The paraffin sections are counterstained with toluidine blue (B and D); the vibratome sections are not counterstained (A and C). w, white matter of the cerebellum. Bars, 100 μ m.

levels in liver, brain, kidney, testes, spleen, and ovary (Table II). Extremely faint immunostaining was detected in heart. The only published reports of PKC ζ have localized it by Western blots to brain and by Northern blots to brain, lung, and kidney (40, 42). Additional tissues were not examined in those studies. Our results demonstrate the selective tissue distribution not only of the PKC α , $-\beta_1$, $-\beta_{II}$, and $-\gamma$ isoenzymes, but also of the newly described PKC δ , $-\epsilon$, and $-\zeta$ isoforms.

Immunocytochemistry

To characterize the tissue and cellular distribution of these PKC isoenzymes, immunocytochemical analyses were performed with eight of the isoenzyme-specific antisera. For the purposes of this analysis, all COOH terminally derived antisera, except that for PKC ϵ , were used. In the latter case, the NH $_2$ terminally derived antisera for PKC ϵ and $-\epsilon'$ were used because they provided the better comparison for immunostaining. These cytochemical studies were performed on sections from cerebellum, thalamus, pituitary gland, pancreas, pineal gland, liver, spleen, ovary, testes, adrenal glands, lung, and heart. Immunoreactivity was determined in the absence or presence of excess immunizing peptide. Figs. 4–6 show representative tissues for each of the isoenzymes. We also examined immunostaining in cerebellar sections (Fig. 7), a tissue in which some of this information is partially known.

Antisera raised against PKC α stained granulosa cells of the ovarian follicles (Fig. 4 A), Purkinje cells in the cerebellum (Fig. 7 A), cells located peripherally to the islets of

Langerhans in the pancreas, and muscle cells of the heart. Staining in the lung and spleen was related to stromal and epithelioid cells, respectively. Staining was also observed in adrenal and pineal glands, and faint immunoreactivity was detected in testes and pituitary gland. This antiserum did not stain structures in the liver at the dilution used. Although many of these findings are novel, other investigators have reported the presence of PKC α immunoreactivity in the pancreas (21, 43) and either the presence or absence of mRNA expression in spleen and liver (6, 21, 43). The immunostaining of Purkinje cells in the cerebellum is also controversial (14, 18, 52).

The PKC β_1 antisera stained peripheral cells within the islets of Langerhans of the pancreas most intensely (Fig. 4 B). It also stained cells in the stratum granulosum of the cerebellum (Fig. 7 B), granulosa cells of the ovarian follicles, the spleen, heart, adrenal glands, and lung. Very light staining was observed in the testes, where the Sertoli cells and different cell types of the spermatogenic line showed light immunostaining. No immunostaining was seen in the liver or pituitary gland. Our Western blot and cytochemical findings for liver and spleen are in concert with *in situ* hybridization data (6). In addition, other investigators have observed the granular layer of the cerebellum to show β_1 immunoreactivity (2, 15, 52) and to express this RNA species (6).

PKC β_{II} antisera stained Sertoli cells and cells of the spermatogenic line in the testes (Fig. 4 C), cells in the stratum granulosum of the cerebellum (Fig. 7 C), peripheral cells of the islets of Langerhans, granulosa cells of the ovarian follicles, and cells in the lung, spleen, adrenal glands, and heart.

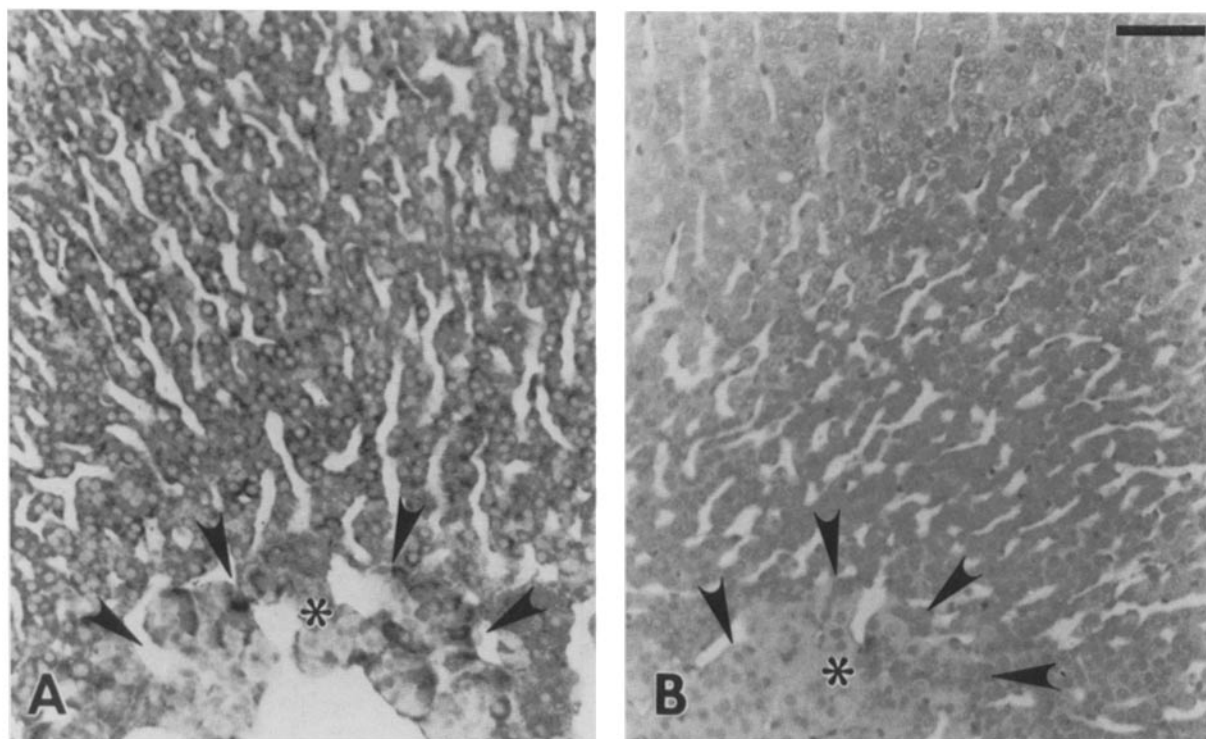


Figure 6. Immunostaining of rat adrenal gland with antiserum against PKC ζ (A). Both the cortex and the medulla contain PKC ζ -immunopositive cells. The border between the medulla and cortex is indicated by arrowheads. The medulla is shown by an asterisk. Following preabsorption of the antiserum with 10 μ g PKC ζ peptide per milliliter working dilution of the antiserum, the immunostaining was completely blocked (B). Paraffin sections were counterstained with toluidine blue. Bars, 100 μ m.

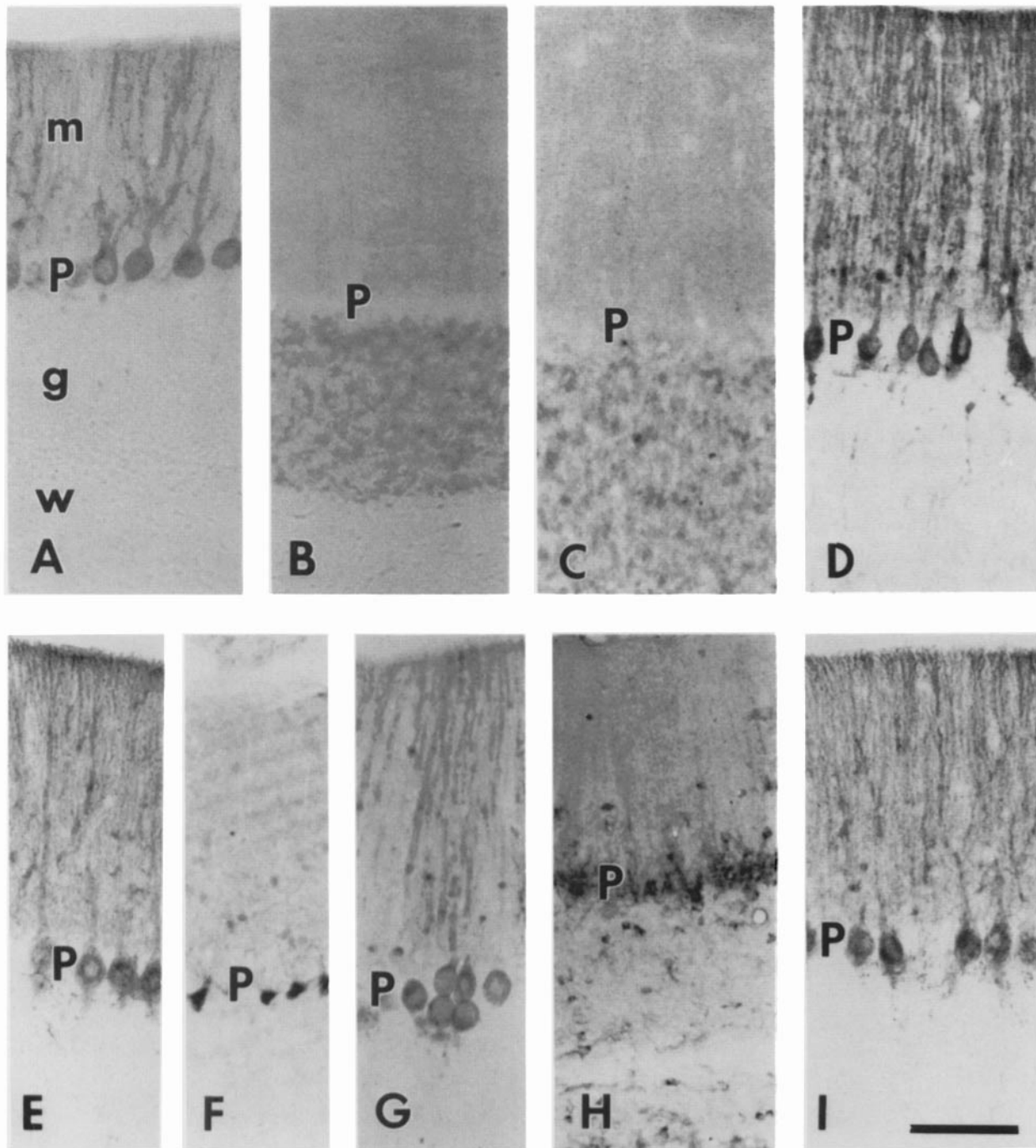


Figure 7. Immunostaining for different isoforms of PKC in the rat cerebellum. PKC α (A) is present in Purkinje cells (P). The dendrites of these cells can be followed as far as the top of the molecular layer (m). The granular layer (g) and the white matter (w) are not stained. To avoid crowding, the abbreviations for layers of the cerebellum are indicated only in (A); however, the layer of Purkinje cells (P) is indicated in each figure for orientation. PKC β_1 (B) and PKC β_{II} (C) are present only in cells of the granular layer. PKC γ is present in Purkinje cells and Bergmann glial cells in the molecular layer (D). Not only the perikarya but also the dendrites of Purkinje cells in the molecular layer and their axons in the granular layer are immunopositive. The antiserum for PKC δ stained Purkinje cells (E) and presently unidentified cells below the unstained Purkinje cells (F). The dorsally located folia contain mainly unstained Purkinje cells. Their axonal origin is surrounded by immunopositive cells. In the basal folia, the Purkinje cells are immunostained. PKC ϵ is present in Purkinje cells (G), whereas PKC ϵ' is present in cells in the molecular and granular layers and in the nerve fibers surrounding the unstained Purkinje cells (H). Antiserum against PKC ζ stained only Purkinje cells in the cerebellum (I). Bars, 100 μ m.

It did not stain cells in the liver or pituitary gland. Once again, our findings confirm RNA expression data in spleen (6) and immunologic findings in cerebellum (2, 52). Interestingly, PKC β_{II} immunostaining in cerebellum has been reported by the same group to be localized either to the molecular or granular layer and Bergmann fibers (2, 47). Another group has also observed PKC β_{II} immunoreactivity in the molecular and granular layers of the cerebellum (52).

Antisera against PKC γ stained the CA1-CA3 region of the hippocampus (Fig. 4 D), cell bodies, and dendrites of Purkinje cells very intensely, and less intensely stained Bergmann glial cells and fibers in the white matter of the cerebellum (Fig. 7 D). No staining was observed in any other tissues examined except for adrenal cortex and pancreas, which stained very lightly. Our cytochemical findings in hippocampus and cerebellum are in agreement with the findings of others (2, 16, 18).

Antisera against PKC δ stained the thalamus, especially the nucleus posterolateralis (Fig. 5 A), Purkinje cells of the cerebellum (Fig. 7, E and F), granulosa cells of the ovarian follicles, and peripherally located cells in the islets of Langerhans. Staining was also observed in the lung, spleen, adrenal glands, and heart. No staining was seen in liver, testes, or pituitary gland.

The PKC ϵ antiserum stained cells in the heart (Fig. 5 B), Purkinje cells in the cerebellum (Fig. 7 G), cells in the islets of Langerhans, spleen, ovaries, adrenal glands, and lung. It also showed modest staining in Sertoli cells of the testes. No staining was seen in liver or pituitary gland.

Antisera against PKC ϵ' stained only nerve terminals around Purkinje cells and Golgi cells in the stratum granulosum of the cerebellum (Fig. 5 C and Fig. 7 H). Some staining was detected in adrenal glands, pancreas, and ovary. Faint immunoreactivity was seen in heart, spleen, and testes, whereas liver, lung, and pituitary gland showed no staining.

PKC ζ immunoreactivity was present in middle-sized and round cells of the pituitary gland (Fig. 5 D). The PKC ζ antisera stained Purkinje cells and processes of Bergmann glial cells in the cerebellum (Fig. 7 I). This antiserum stained cells in the zonae glomerulosa, fasciculata and reticularis of the adrenal cortex, and cells in the medulla (Fig. 6). Interestingly, not all the cells in these layers were stained. This antiserum also stained granulosa cells of the ovarian follicles, cells within the islets of Langerhans in the pancreas, and, very lightly, Sertoli cells and different cell types of the spermatogenetic process in the testes. Moderate immunoreactivity was detected in heart and pineal gland, whereas spleen and lung showed faint staining.

All isoenzymes were detected in the cerebellum (Fig. 7). However, within the cerebellum, the PKC isoenzymes showed a differential regional, cellular, and subcellular distribution. In this brain region and in all other organs and tissues examined, immunostaining was always located in the cytoplasm of the immunostained cells. In the cerebellum, the staining was also located mainly in the cytoplasm of Purkinje cells or Golgi cells; however, some of the antisera also stained the processes of Purkinje cells, basket cells, and Bergmann glial cells in the cerebellum. Finally, it should be noted that our Western blot and immunocytochemical findings complement each other in all cases except for liver. Here, immunohistochemistry failed to detect any of the isoenzymes in liver, although PKC α , δ , and ζ were detected

by Western blot. In an effort to remove all exogenous cells or proteins from liver, this organ was perfused and then resubjected to Western blot analyses. The patterns of immunoblotting were identical to the nonperfused tissues. Hence, the nature of the discrepancy between the Western blot and immunocytochemical analyses is unclear at this time.

Discussion

Elucidation of the regulation and function of PKC in transmembrane signalling processes has been rendered more complex with the identification of multiple members of this family. The PKC isoenzymes can be grouped into two subfamilies depending on their requirement for calcium (36), which may impart a limited selectivity in the regulation of these isoenzymes. A major distinguishing feature that emerged from previous studies on the tissue distribution of the calcium-dependent subgroup of the PKC isoenzymes (PKC α , β_I , β_{II} , and γ) relates to their selective, but overlapping, distribution. This information has been obtained from limited Northern blot analysis of mRNA (6, 25, 38, 40) and from studies using Western blot analysis (27, 29, 46, 56). Various cytochemical studies have identified the calcium-dependent isoenzymes in specific tissues: brain shows differential distribution of the three isoenzymes (2, 15, 16, 18, 27, 52, 54); pancreas expresses the PKC α and β_{II} isoforms but not PKC γ or β_I (21, 43); adrenocortical and Sertoli cells contain PKC α , whereas Leydig cells express PKC α , β_I , and γ (46); human leukemia cells contain the PKC α and β subtypes (14, 48) and possibly the PKC γ subtype (31); and bovine aortic tissues express the type III (PKC α) isoenzyme (55).

By contrast, the absence of information on the tissue distribution of the calcium-independent subgroup of PKC isoenzymes (PKC δ , ϵ , ϵ' , and ζ) limits our understanding of their roles in normal and pathophysiological circumstances. Therefore, we examined the tissue distribution of PKC isoenzymes by Western blot studies as well as immunohistochemistry. To this end, we have developed nine antisera specific for each of the known PKC isoenzymes. (While this study was in progress, an additional PKC isoenzyme was cloned that shows highest homology to PKC ϵ [3, 45]). Characterization of these antisera demonstrated their high titer of reactivity, high specificity, and high binding affinity.

The specificity of these isoenzymes is emphasized by the following observations. First, the antisera show high affinities and specificities to the respective immunizing peptides. Second, these antisera detect the appropriate PKC isoenzymes from rat brain, which can be distinguished by their size on SDS-PAGE. In addition, this immunostaining in both Western blots and immunocytochemistry can be blocked by coincubation with the immunizing peptide. The isoenzymes with similar (80-kD) molecular mass can be separated on hydroxyapatite chromatography. PKC α , β_I , β_{II} , and γ can be resolved by this procedure, and these isoenzyme-specific antisera react specifically with each of the respective resolved peaks on hydroxyapatite. PKC δ , which also has a molecular mass of 80 kD, does not copurify with the calcium-dependent isoenzymes when a sequential separation scheme is applied. Hence, purification of the calcium-dependent isoforms is not hindered by other possible contaminating species.

An important observation emerged from Western blot analysis of hydroxyapatite-resolved PKC isoenzymes. These studies demonstrate that the calcium-independent isoenzymes do not copurify with the calcium-dependent ones when sequential chromatography is used; therefore, the former are not detected following hydroxyapatite chromatography. The calcium-independent isoenzymes are separated from the calcium-dependent isoforms during DEAE chromatography since they elute at higher salt concentrations (data not shown). This observation should permit the investigation of purified isoenzymes from various tissues.

The eight PKC isoenzymes examined in this study show distinctive tissue distribution. PKC α was detected in all tissues examined, including spleen and liver. These findings are supported by a limited Northern blot study in which PKC α mRNA was detected in brain, muscle, lung, and kidney (38). Also, Kosaka et al. (27) detected PKC α immunoreactivity in all peripheral organs examined. On the other hand, Yoshida et al. (56) detected PKC α immunoreactivity in brain, pineal gland, retina, and spleen, but not in pituitary gland, thymus, liver, muscle, heart, lung, or intestine. Our results are more in line with the former studies, because we confirm the ubiquitous presence of PKC α in all tissues.

Compared to PKC α , PKC β_1 and $-\beta_{II}$ were more restricted in their distribution because they were localized primarily in the brain and spleen. This result is consistent with Northern blot studies (25) and in situ hybridization (6). By Western blot, we found PKC γ to be restricted to brain, as previously seen with other Northern and Western blot analyses (25, 27, 56). By immunohistochemistry, however, we did detect modest reactivity in adrenal gland. Our results suggest that PKC γ may be selectively expressed in cells derived from the neural crest.

The tissue distribution of the calcium-independent PKC isoenzymes (PKC δ , $-\epsilon$, and $-\zeta$) has not been previously determined. In this study, we detected PKC δ in a number of tissues, primarily brain, ovary, lung, and spleen. PKC ϵ shows reactivity in brain by both Western blot and immunocytochemical analyses. Using this latter approach, we also identified additional staining in other peripheral tissues. PKC ϵ' was found predominantly in the brain, with very weak staining in other peripheral tissues. Interestingly, PKC ζ was found by Western blot to be present in many tissues. This isoenzyme was predominantly localized in endocrine and blood-forming organs such as the pituitary and adrenal glands, pancreas, brain, ovaries, testes, lung, liver, and spleen.

The selective organ distribution of these various PKC isoenzymes provides a first approximation of their functional role. On the other hand, the presence of multiple isoenzymes in the same tissue and even within the same cell type suggests that individual isoenzymes may mediate distinct cellular functions. This distribution raises important questions as to how these isoenzymes could be selectively regulated and how their phosphorylated substrates could selectively modulate these cellular events under various physiologic and pathologic conditions. These isoenzyme-specific antisera should permit a detailed examination not only of the subcellular localization and/or translocation of individual isoenzymes within the same cell type, but they should also serve as an additional biochemical tool to examine the potential for

selective regulation or substrate phosphorylation by these isoenzymes.

We thank Dr. John Hong at the NIEHS, Dr. Lina M. Obeid, and Gerry Blobe for careful review of this manuscript, and Marsha Haigood for expert secretarial assistance. We also thank James Clark and Page Myers for assistance in boosting and bleeding the rabbits.

This work was supported in part by grants HL-43707 from the National Institutes of Health, CA 47741 from the National Cancer Institute, and by the NIEHS Intramural Program.

Received for publication 24 April 1991 and in revised form 9 December 1991.

References

- Akita, Y., S. Ohno, Y. Konno, A. Yano, and K. Suzuki. 1990. Expression and properties of two distinct classes of the phorbol ester receptor family, four conventional protein kinase C types, and a novel protein kinase C. *J. Biol. Chem.* 265:354-362.
- Ase, K., N. Saito, M. S. Shearman, U. Kikkawa, Y. Ono, K. Igarashi, C. Tanaka, and Y. Nishizuka. 1988. Distinct cellular expression of bI- and bII- subspecies of protein kinase C in rat cerebellum. *J. Neurosci.* 8: 3850-3856.
- Bacher, N., Y. Zisman, E. Berent, and E. Livneh. 1991. Isolation and characterization of PKC-L, a new member of the protein kinase C-related gene family specifically expressed in lung, skin, and heart. *Mol. Cell. Biol.* 11:126-133.
- Bell, R. M. 1986. Protein kinase C activation by diacylglycerol second messengers. *Cell.* 45:631-632.
- Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* 72:248-254.
- Brandt, S. J., J. E. Niedel, R. M. Bell, and W. S. Young III. 1987. Distinct pattern of expression of different protein kinase C mRNAs in rat tissues. *Cell.* 49:57-63.
- Burns, D. J., J. Bloomenthal, M.-H. Lee, and R. M. Bell. 1990. Expression of the a, bII, and gamma protein kinase C isozymes in the baculovirus-insect cell expression system. Purification and characterization of the individual isoforms. *J. Biol. Chem.* 265:12044-12051.
- Castagna, M., Y. Takai, K. Kaibuchi, K. Sano, U. Kikkawa, and Y. Nishizuka. 1982. Direct activation of calcium-activated, phospholipid-dependent protein kinase by tumor-promoting phorbol esters. *J. Biol. Chem.* 257:7847-7851.
- Coussens, L., P. J. Parker, L. Rhee, T. L. Yang-Feng, E. Chen, M. D. Waterfield, U. Francke, and A. Ullrich. 1986. Multiple, distinct forms of bovine and human protein kinase C suggest diversity in cellular signaling pathways. *Science (Wash. DC)*. 233:859-866.
- Culler, M. D., and A. Negro-Vilar. 1986. Development of specific antisera and a radioimmunoassay procedure for the gonadotropin-releasing hormone associated peptide (GAP) of the LHRH prohormone. *Brain Res. Bull.* 17:219-223.
- Finley, J. C. W., and P. Petrusz. 1982. The use of proteolytic enzymes for improving localization of tissue antigens with immunocytochemistry. In *Techniques in Immunocytochemistry*. Vol. 1. G. R. Bullock and P. Petrusz, editors. Academic Press, New York. 239-249.
- Hannun, Y. A., C. R. Loomis, and R. M. Bell. 1985. Activation of protein kinase C by Triton X-100 mixed micelles containing diacylglycerol and phosphatidylserine. *J. Biol. Chem.* 260:10039-10043.
- Harlow, E., and D. Lane. 1988. *Antibodies: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 79-85.
- Hashimoto, K., A. Kishimoto, H. Aihara, I. Yasuda, K. Mikawa, and Y. Nishizuka. 1990. Protein kinase C during differentiation of human promyelocytic leukemia cell line, HL-60. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 263:31-34.
- Hidaka, H., T. Tanaka, K. Onoda, M. Hayiwara, M. Watanabe, H. Okta, Y. Ito, M. Tsurudome, and T. Yoshida. 1988. Cell type-specific expression of protein kinase C isoenzymes in the rabbit cerebellum. *J. Biol. Chem.* 263:4523-4526.
- Huang, F. L., Y. Yoshida, H. Nakabayashi, and K. P. Huang. 1987. Differential distribution of protein kinase C isoenzymes in the various regions of the brain. *J. Biol. Chem.* 262:15714-15720.
- Huang, F. L., Y. Yoshida, H. Nakabayashi, J. L. Knopf, and W. S. Young. 1987. Immunocytochemical identification of protein kinase C isoenzymes as products of distinct genes. *Biochem. Biophys. Res. Commun.* 149:946-952.
- Huang, F. L., Y. Yoshida, H. Nakabayashi, W. S. Young III, and K. P. Huang. 1988. Immunocytochemical localization of protein kinase C isozymes in rat brain. *J. Neurosci.* 8:4734-4744.
- Huang, K. P., H. Nakabayashi, and F. L. Huang. 1986. Isozymic forms of rat brain Ca²⁺-activated and phospholipid-dependent protein kinase.

20. Huang, K. P., F. L. Huang, H. Nakabayashi, and Y. Yoshida. 1988. Biochemical characterization of rat brain protein kinase C isozymes. *J. Biol. Chem.* 263:14839-14845.
21. Ito, A., N. Saito, H. Taniguchi, T. Chiba, U. Kikkawa, Y. Saitoh, and C. Tanaka. 1989. Localization of bII subspecies of protein kinase C in b-cells. *Diabetes.* 38:1005-1011.
22. Kikkawa, U., O. Kouji, Y. Ono, Y. Asaoka, M. S. Shearman, T. Fujii, K. Ase, K. Sekiguchi, K. Igarashi, and Y. Nishizuka. 1987. The common structure and activities of four subspecies of rat brain protein kinase C family. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 223:212-216.
23. Kiley, S., D. Schaap, P. Parker, L.-L. Hsieh, and S. Jaken. 1990. Protein kinase C heterogeneity in GH₄C₁ rat pituitary cells. Characterization of a Ca²⁺-independent phorbol ester receptor. *J. Biol. Chem.* 265:15704-15712.
24. Kitano, T., M. Go, U. Kikkawa, and Y. Nishizuka. 1986. Assay and purification of protein kinase C. *Methods Enzymol.* 124:349-352.
25. Knopf, J., M.-H. Lee, L. A. Sultzman, R. W. Kritz, C. R. Loomis, R. M. Hewick, and R. M. Bell. 1986. Cloning and expression of multiple protein kinase C cDNAs. *Cell.* 46:491-502.
26. Konno, Y., S. Ohno, Y. Akita, H. Kawasaki, and K. Suzuki. 1989. Enzymatic properties of a novel phorbol ester receptor/protein kinase, nPKC. *J. Biochem. (Tokyo).* 106:673-678.
27. Kosaka, Y., K. Ogita, K. Ase, H. Nomura, U. Kikkawa, and Y. Nishizuka. 1988. The heterogeneity of protein kinase C in various rat tissues. *Biochem. Biophys. Res. Commun.* 151:973-981.
28. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the bacteriophage T4. *Nature (Lond.)*. 227:680-685.
29. Leach, K. L., E. A. Powers, J. C. McGuire, L. Dong, S. C. Kiley, and S. Jaken. 1988. Monoclonal antibodies specific for type 3 protein kinase C recognize distinct domains of protein kinase C and inhibit *in vitro* functional activity. *J. Biol. Chem.* 263:13223-13230.
30. Lowenthal, J. W., D. W. Ballard, H. Bogerd, E. Böhnlein, and W. C. Greene. 1989. Tumor necrosis factor- α activation of the IL-2 receptor- α gene involves the induction of kappa B-specific DNA binding proteins. *J. Immunol.* 142:3121-3128.
31. Makowske, M., R. Ballester, Y. Cayre, and O. M. Rosen. 1988. Immunocytochemical evidence that three protein kinase C isozymes increase in abundance during HL-60 differentiation induced by dimethyl sulfoxide and retinoic acid. *J. Biol. Chem.* 263:3402-3410.
32. Mavis, R. D., R. M. Bell, and P. R. Vagelos. 1972. Effect of phospholipase C hydrolysis of membrane phospholipids on membranous enzymes. *J. Biol. Chem.* 247:2835-2847.
33. Merchenthaler, I., S. Vigh, P. Petrusz, and A. V. Schally. 1982. Immunocytochemical localization of corticotropin releasing factor (CRF) in the rat brain. *Am. J. Anat.* 165:385-396.
34. Niedel, J. E., L. J. Kuhn, and G. R. Vandenbark. 1983. Phorbol diester receptor copurifies with protein kinase C. *Proc. Natl. Acad. Sci. USA.* 80:36-40.
35. Nishizuka, Y. 1986. Studies and perspectives of protein kinase C. *Science (Wash. DC)*. 233:305-312.
36. Nishizuka, Y. 1988. The molecular heterogeneity of protein kinase C and its implications for cellular regulation. *Nature (Lond.)*. 334:661-665.
37. Nishizuka, Y. 1989. Studies and perspectives of the protein kinase C family for cellular regulation. *Cancer.* 63:1892-1903.
38. Ohno, S., H. Kawasaki, S. Imajoh, and K. Suzuki. 1987. Tissue-specific expression of three distinct types of rabbit protein kinase C. *Nature (Lond.)*. 325:161-166.
39. Ohno, S., Y. Akita, Y. Konno, I. Shinobu, and K. Suzuki. 1988. A novel phorbol ester receptor/protein kinase, nPKC, distantly related to the protein kinase C family. *Cell.* 53:731-741.
40. Ono, Y., T. Fujii, K. Ogita, U. Kikkawa, K. Igarashi, and Y. Nishizuka. 1988. The structure, expression, and properties of additional members of the protein kinase C family. *J. Biol. Chem.* 263:6927-6932.
41. Ono, Y., T. Fujii, K. Igarashi, T. Kuno, C. Tanaka, U. Kikkawa, and Y. Nishizuka. 1989. Phorbol ester binding to protein kinase C requires a cysteine-rich zinc-finger-like sequence. *Proc. Natl. Acad. Sci. USA.* 86:4868-4871.
42. Ono, Y., T. Fujii, K. Ogita, U. Kikkawa, K. Igarashi, and Y. Nishizuka. 1989. Protein kinase C zeta subspecies from rat brain: its structure, expression, and properties. *Proc. Natl. Acad. Sci. USA.* 86:3099-3103.
43. Onoda, K., M. Hagiwara, T. Hachiya, N. Usuda, T. Nagata, and H. Hidaka. 1990. Different expression of protein kinase C isozymes in pancreatic islet cells. *Endocrinology.* 126:1235-1240.
44. Ordronneau, P., P. B. M. Lindstrom, and P. Petrusz. 1981. Four unlabeled antibody bridge techniques: a comparison. *J. Histochem. Cytochem.* 29:1397-1404.
45. Osada, S., K. Mizuno, T. C. Saido, Y. Akita, K. Suzuki, T. Kuroki, and S. Ohno. 1990. A phorbol ester receptor/protein kinase, nPKC ϵ , a new member of the protein kinase C family predominantly expressed in lung and skin. *J. Biol. Chem.* 265:22434-22440.
46. Pelosin, J.-M., A. Ricouart, C. Sergheraert, M. Benahmed, and E. M. Chambaz. 1991. Expression of protein kinase C isoforms in various steroidogenic cell types. *Mol. Cell. Endocrinol.* 75:149-155.
47. Saito, N., A. Kose, A. Ito, K. Hosoda, M. Mori, M. Hirata, K. Ogita, U. Kikkawa, Y. Ono, K. Igarashi, Y. Nishizuka, and C. Tanaka. 1989. Immunocytochemical localization of b_{II} subspecies of protein kinase C in rat brain. *Proc. Natl. Acad. Sci. USA.* 86:3409-3413.
48. Sawamura, S., K. Ase, N. Berry, U. Kikkawa, P. G. McCaffrey, J. Minowada, and Y. Nishizuka. 1989. Expression of protein kinase C subspecies in human leukemia-lymphoma cell lines. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 247:353-357.
49. Schaap, D., P. J. Parker, L. L. Hsieh, and S. Jaken. 1989. Unique substrate specificity and regulatory properties of PKC- ϵ : a rationale for diversity. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 243:351-357.
50. Schaap, D., J. Hsuan, N. Totty, and P. J. Parker. 1990. Proteolytic activation of protein kinase C- ϵ . *Eur. J. Biochem.* 191:431-435.
51. Shearman, M. S., Z. Naor, U. Kikkawa, and Y. Nishizuka. 1987. Differential expression of multiple protein kinase C subspecies in rat central nervous system. *Biochem. Biophys. Res. Commun.* 147:911-919.
52. Shimohama, S., T. Saitoh, and F. H. Gage. 1990. Differential expression of protein kinase C isoenzymes in rat cerebellum. *J. Chem. Neuroanat.* 3:367-375.
53. Towbin, H., T. Staethlin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets. *Proc. Natl. Acad. Sci. USA.* 76:4350-4354.
54. Tsujino, T., A. Kose, N. Saito, and C. Tanaka. 1990. Light and electron microscopic localization of bI-, bII, and gamma-subspecies of protein kinase C in rat cerebral neocortex. *J. Neurosci.* 10:870-884.
55. Watanabe, M., T. Hachiya, M. Hagiwara, and H. Hidaka. 1989. Identification of type III protein kinase C in bovine aortic tissue. *Arch. Biochem. Biophys.* 273:165-169.
56. Yoshida, Y., F. L. Huang, H. Nakabayashi, and K. P. Huang. 1988. Tissue distribution and developmental expression of protein kinase C isozymes. *J. Biol. Chem.* 263:9868-9873.