Localization of Protein Kinase C \( \alpha \) Isoform Expression in the Human Gastrointestinal Tract

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Abstract
Protein kinase C (PKC) includes a family of related proteins which constitutes a major signal transduction pathway. The aim of this study was to determine the localization of the PKC-\( \alpha \) isoform throughout the human gastrointestinal tract. PKC-\( \alpha \) expression was also measured and compared between normal and neoplastic colorectal tissue. PKC-\( \alpha \) mRNA expression was detected in normal human gastrointestinal tract tissue using Northern blot analyses and \textit{in situ} hybridization. PKC-\( \alpha \) protein expression was detected in normal gastrointestinal tissue and colorectal neoplasia using Western blot and immunohistochemical analyses. PKC-\( \alpha \) was expressed throughout the human gastrointestinal tract. Distinct organ and cellular localization was characterized. PKC-\( \alpha \) mRNA and protein localization were most prevalent in the deep basal layer of the esophageal mucosa. In the stomach, PKC-\( \alpha \) expression was detected predominately in the cells of the deep glands and surface epithelial cells but less in the mucous neck cells of the gastric pits. In the duodenum and ileum, PKC-\( \alpha \) mRNA expression was greater in the deeper crypt cells than in the differentiated cells that line the villi. However, immunohistochemistry showed greater expression in the cells of the villi compared to crypt cells. In normal colonic tissue, PKC-\( \alpha \) mRNA and protein predominated in the cells of the upper crypt and surface epithelial cells. PKC-\( \alpha \) protein was also prominently expressed in the glands of colorectal adenocarcinoma. There was no quantitative difference in the level of PKC-\( \alpha \) protein expression between normal and neoplastic colorectal tissue. The specific organ and cellular expression of PKC-\( \alpha \) suggests separate and distinct functional roles for this PKC isoform throughout the gastrointestinal tissues.

Introduction
PKC\( ^3 \) is a serine/threonine-specific protein kinase which plays a key role in transmembrane signaling events (1–3). Originally identified in 1977 (1), this family of enzymes represents a major signal transduction pathway whereby extracellular signals influence multiple cellular processes, including cell growth and differentiation. Receptor-mediated hydrolysis of membrane phospholipids leads to the formation of 1,2-diacylglycerol, which activates PKC in the presence of physiological concentrations of calcium. A role for PKC has been implicated in a multitude of gastrointestinal functions including gastric (4) and pancreatic (5) secretions as well as gastric (6) and colonic malignant transformation (7–10).

PKC has been shown to include a family of at least 10 related proteins with different properties, organ specificity, and cellular localization (11–14). These isozymes are divided into three classes by analysis of their structure. All PKC isozymes contain certain conserved (C) regions involved in the function common to all PKC enzymes, specifically the kinase domain. In addition, there are less well-conserved variable (V) regions which allow for flexibility and specific properties of the individual isozymes. Class I includes four distinct calcium-dependent PKC isozymes (\( \alpha, \beta_\text{I}, \beta_\text{II}, \text{and } \gamma \)) derived from three distinct genes on chromosomes 17 (\( \alpha \)), 16 (\( \beta \)), and 19 (\( \gamma \); Ref. 12). \( \beta_\text{I} \) and \( \beta_\text{II} \) isozymes differ only in the sequence of the 50 most C-terminal amino acids and represent products of alternate gene splicing (15). Class II isozymes (\( \delta, \epsilon, \eta, \zeta \), and \( \theta \)) lack the \( \text{C}_2 \) domain and are characterized by their calcium independence. Class III isozymes (\( \xi \) and \( \lambda \)) lack the \( \text{C}_2 \) domain, are calcium independent, and contain only one cysteine-rich region in the C-1 region (14).

In this article, we report the expression and localization of PKC-\( \alpha \) in human gastrointestinal tract tissue. Expression of PKC-\( \alpha \) mRNA was detected using Northern blot analyses and ISHs. Specific monoclonal and polyclonal antibodies were utilized to localize PKC-\( \alpha \) peptide in human gastrointestinal tract tissue using Western blot analysis and immunocytochemistry. Comparison of PKC-\( \alpha \) protein expression was also measured and compared between normal colorectal tissue and adenocarcinoma. Distinct organ specificity and cellular localization along the crypt villous axis was detected for PKC-\( \alpha \).

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3 The abbreviations used are: PKC, protein kinase C; DEPC, diethyl pyrocarbonate; ISH, \textit{in situ} hybridization; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
Results

Northern and Western Blot Analysis. Northern and Western blot analyses demonstrated that PKC-α mRNA and protein were expressed ubiquitously throughout the human gastrointestinal tract. In Northern blot analyses, the radiolabeled probe was specific for PKC-α of the correct molecular weight in rat brain and human gastrointestinal tissues and did not demonstrate background hybridization to other RNA species. The major transcript size in brain and gastrointestinal tissue of PKC mRNA was 8.5 kb (Fig. 1) with a minor transcript of 3.8 kb (data not shown), which is in agreement with previous determinations. This highly specific probe was also used in our ISH experiments. Relative to the expression of GAPDH, PKC mRNA expression was highest in esophageal, duodenal, and colonic tissues and least in stomach and ileal tissues. Western blot analyses using a monoclonal antibody (Upstate Biotechnology, Inc., Lake Placid, NY) or a polyclonal antibody (Life Technologies, Inc., Grand Island, NY) demonstrated a protein of appropriate size equal to that found in brain samples (16, 17). Similar quantitative and qualitative results for PKC-α protein expression were obtained with the two antibodies used in this study. α peptide expression was least in esophageal tissue (0.45) compared to stomach tissue (Fig. 2). Also demonstrated in Fig. 2 is the presence of the catalytic subunit of PKC-α (Mr ~45,000). Quantitatively, using densitometry, PKC-α protein was not different in the cytosol fraction of eight adenocarcinomas (4.68 ± 0.35) compared to adjacent normal mucosa (4.78 ± 0.91; Fig. 3). In six of eight adenocarcinomas, the membrane content of PKC-α was increased compared to adjacent normal tissue but the differences were not significant (3.79 ± 0.66 cancer versus 2.21 ± 0.66 normal mucosa).

Localization of PKC-α Expression. In parallel sections of tissues used for ISH studies for α mRNA expression, intense labeling for the radiolabeled antisense β-actin probe was seen. The labeling for β-actin and PKC-α probe was obliterated in tissues by pretreatment with RNase A. In immunohistochemistry experiments, no reaction product was noted on tissue sections incubated with the secondary antibody alone. In general, more precise localization of PKC-α expression was demonstrated with immunohistochemical staining. Immunostaining was noted equally well in paraffin-embedded and frozen tissue. In the ISH studies, a stronger signal was seen in frozen tissue than in paraffin-embedded tissue, which has approximately 25% less RNA (18). However, histology is less precise in frozen tissues than in paraffin-embedded tissue.

Analysis of PKC-α mRNA by ISH and peptide expression using immunohistochemistry demonstrated distinct tissue-specific expression. Distinct cellular expression was also noted in different organs. In the esophagus, PKC-α mRNA (Fig. 4A) and peptide expression (Fig. 4C) were highest in the basal cell layer of the epithelium, the zone of cell proliferation. PKC-α expression was also seen to an extent, albeit to a lesser extent in the polyhedral and squamous cell layer of the intermediate and outermost layer, respectively.
Analysis of PKC-α mRNA (Fig. 4D) and peptide expression (Fig. 4E) in the stomach revealed two distinct areas of increased localization. In both the body and antrum of the stomach, expression was highest in the cells of the surface epithelium which overlies the gastric pits (Fig. 4F). Expression was less in mucous neck cells that line the gastric pits. In the deeper glandular level below the foveolae, intense PKC-α mRNA and protein expression were notable. Particularly chief and parietal cells, which reside within the deep glands of the fundic mucosa and G cells of the antrum, demonstrated marked PKC-α antibody staining (Fig. 4E).

Distinct differences in PKC-α mRNA detected with ISH and PKC-α protein detected using immunohistochemistry were noted in duodenal and ileal samples. ISH showed intense labeling of cells in the intestinal glands. Labeling was also noted to a lesser extent on the columnar and goblet cells that line the villi (Fig. 4G). In contrast, PKC-α immunostaining was marked in cells of the villous tips and decreased toward the intestinal glands (Fig. 4H). This pattern was similar in duodenal and ileal samples.

ISH of normal colonic epithelium revealed diffuse staining along the entire crypt column, with increased expression in the upper crypt and surface epithelial cells (Fig. 4I). Immunohistochemistry demonstrated a more precise expression of PKC-α expression in normal colonic epithelium. Expression was limited to the differentiated surface columnar epithelium and goblet cells of the upper colonic crypts (Fig. 4J). Expression was minimal in cells that line the base and lower crypt column, which represents the area of cellular proliferation. In contrast, PKC-α immunostaining was noted throughout the neoplastic glands of adenocarcinomas of varying degrees of differentiation (Fig. 4L). Staining of dysplastic glands of adenocarcinomas was distinctly more intense than in the desmoplasia that accompanied the neoplasia.

Discussion
A limited number of studies are available which investigated the presence of PKC isoforms in peripheral rodent tissues (19–24). Distinct organ and cellular localization of PKC isoforms in different tissues has been detected by previous studies using primarily Northern blot and immunoblot analyses. To our knowledge, this study is the first to demonstrate the localization of PKC-α expression in the human gastrointestinal tract. In agreement with most other studies, our study demonstrated that the PKC-α isoform is expressed ubiquitously throughout the human gastrointestinal tract. In a study of mouse epithelium by Osada et al. (23), PKC-α mRNA showed equivalent expression in the stomach, small intestine, and large intestine compared to the brain. Similarly, Wetsel et al. (22) and Kosaka et al. (20) demonstrated the presence of PKC-α in all peripheral rat tissues tested. Jiang et al. (24) demonstrated the presence of PKC-α, -δ, -ε, and -ζ mRNA and protein in the rat colon. In agreement with our findings in human colon, PKC-α mRNA was expressed throughout the crypt column, whereas PKC-α protein was concentrated in cells of the upper crypt and surface epithelium.

Our study demonstrated selective expression of the PKC-α transcript and protein within certain cells throughout the human gastrointestinal tract. Although the goal of the current study was not to define the function of PKC-α, one can speculate by its localization within selective cell types. The proliferating zone of gastrointestinal tissue has been well established. PKC-α mRNA and protein were detected throughout the esophageal tissues, but particularly in the proliferating basal cell layer. In contrast, the PKC-α transcript and protein were more prominent in surface epithelial cells and differentiated cells of the deep glands compared to the proliferating mucous necks of the stomach. In duodenal and ileal tissues, a different pattern of PKC-α mRNA and protein expression was detected. The failure to detect a protein in the intestinal glands more likely represents a failure to expose or preserve the relevant epitope detected by the antibodies used in this study. The two antibodies used in this study showed a similar pattern of expression. An alternative explanation is that there are differences in PKC-α posttranscriptional events between cells of the deep crypts and cells that line the intestinal villous.

Colonic expression of PKC-α protein was limited to surface epithelial cells and goblet cells of the upper colonic crypts. Minimal detection of α protein was noted in the proliferative zones contained within the lower half of the colonic crypts. Given the restriction of PKC-α protein expression to differentiated cells of the normal colonic mucosa, the diffuse expression noted in neoplastic colorectal glands was surprising. Our Western blotting results are in agreement with most other investigators who demonstrated no quantitative difference in PKC-α expression between normal and neoplastic tissues (25–27). The immunohistochemical staining performed in the current study clearly demonstrated that PKC-α is present in neoplastic glands and not in the desmoplasia that often accompanies large bowel cancer. These results also demonstrate the complementary data gained by using histochemical staining in conjunction with analysis of protein production by Western blots of whole-tissue homogenates.

Limited studies are available that have investigated a role for PKC isoforms in gastrointestinal tissue. Analysis of PKC isoforms using immunoblot was investigated by Berghe et al. (28) in a differentiated colon cancer cell line that demonstrated ion secretion under cholinerigic control. A role for PKC-α but not -β or -γ was suggested by the translocation of PKC-α to the membrane fraction with activation of ion transport by carbachol. Musch et al. (29) isolated mature chick enterocytes from the upper third of the ileal villi. Activation of PKC by phorbol esters resulted in the stimulation of anion secretion and inhibition of the apically located sodium hydrogen pump. The localization of PKC-α protein in the present study to mature surface cells of the ileal villi and surface epithelial cells of the colon supports a role for PKC-α in the regulation of ion transport. In the current study, prominent expression of PKC-α was noted in surface epithelial cells of gastric folds and differentiated cells of the deep gastric glands. In a study of isolated parietal cells by Anderson and Hanson (4), a role for PKC in the intracellular control of acid secretion was identified. The methods used in the
Fig. 4. ISH and immunohistochemical detection of PKC-α in human gastrointestinal tissues. A, ISH localization of PKC-α mRNA in esophageal tissue showed greater labeling in the basal cell layer compared to superficial cells. H&E, ×400. B, negative control for ISH of PKC-α mRNA in normal human esophagus. Tissue section was pretreated with RNase (20 μg/ml) for 30 min at room temperature prior to ISH. An absence of black grains is noted. H&E, ×400. C, corresponding immunohistochemical staining of human esophagus with monoclonal antibody to PKC-α (United Biomedical, Inc.) also showed...
current study allow for the localization of different PKC isoforms in normal and pathological gastrointestinal tissue. By approximation, the intense expression of PKC-α in differentiated epithelial cells in the human stomach, ileum, and colon in the current study suggests a possible role for PKC-α in epithelial absorptive function.

Materials and Methods

Human Tissue. Biopsy samples of normal gastrointestinal tissue were obtained from patients undergoing elective endoscopy. Patients undergoing colonoscopy were prepared with a lavage containing balanced salt solution containing polyethylene glycol. No enemas were given prior to colonoscopy. Four to six forceps pinch biopsies for Northern and Western blot analysis were obtained and immediately placed in liquid nitrogen and stored at -70°C until analysis. Tissue for histology studies was placed on a clean glass slide to prevent curling and immediately frozen in either an isopentane slush or fixed in 10% neutral buffered formalin. Informed written consent was obtained from all patients. Additional human samples were obtained from surgically resected adenocarcinomas and adjacent normal mucosa of the large intestine at least 10 cm from the gross tumor (n = 8). The protocol had prior approval of the Clinical Investigation Committee of the University Hospital, Pennsylvania State University.

Northern Blot Analysis. Total RNA was prepared from tissue samples using the acid guanidinium-thiocyanate method of Chomczynski and Sacchi (30) and Cathala et al. (31).

Twenty μg total RNA/sample were denatured and separated using electrophoresis in a 1% formaldehyde-containing agarose gel and then transferred to a nitrocellulose membrane by capillary action. Rat brain total RNA served as a positive control. The RNA was baked onto the nitrocellulose membranes at 80°C in a vacuum oven for 1 h. A maximally divergent sequence for human PKC-α was identified which coded for bp 1993-2032 (32). A cognate antisense DNA oligonucleotide (40-mer) was synthesized using a MillGen/Biosearch 7200 DNA synthesizer in the Macromolecular Core Facility, Milton S. Hershey Medical Center (Hershey, PA). The DNA oligonucleotide probe was labeled with [-32P]ATP by the T4 polynucleotide kinase method (33). Unincorporated ATP was removed using spin column chromatography. The radiolabeled antisense DNA probe was added to the hybridization buffer containing 1.0 μM NaCl, 50 mM Tris-HCl (pH 7.5), 10% dextran sulfate, 1% SDS, and 100 μg/ml denatured nonhomologous DNA (salmon sperm). Hybridization was carried out at 65°C for 18 h. Following hybridization, the blots were washed four times in 2 x SSC/0.1% SDS at room temperature for 30 min, 1 time in 2 x SSC/0.1% SDS at 65°C for 30 min, 2 x SSC/0.1% SDS at room temperature for 5 min, and then rinsed briefly in 2 x SSC at room temperature. The blots were air dried and exposed to Kodak XAR X-ray film at -70°C for 72 h. The RNA data obtained from different organs was normalized to the expression of the housekeeping gene GAPDH (a kind gift from Susan LaJeune, Oxford, United Kingdom). The cDNA for GAPDH was labeled with [-32P]dCTP using the random priming technique (Ref. 34; Prime-a-Gene labeling system; Promega, Madison, WI). The resultant autoradiographs were analyzed with a scanning laser densitometer and quantitated on the basis of μg of RNA applied versus relative radiographic signal. Sizes of the transcripts were determined by using the RNA standard ladder (Life Technologies, Inc.).

ISH. Fresh tissue that had been frozen in isopentane slush was placed in OCT media. Ten-μm sections were thaw mounted onto SuperFrost/Plus microscope slides (Fisher Scientific). Tissues fixed in 10% neutral buffered formalin were embedded in paraffin. Paraflin-embedded tissue sections were dewaxed in fresh xylene plus 0.1% DEPC, then rehydrated through 100%, 90%, 80%, and 30% ethanol containing 0.1% DEPC, and washed in PBS. Slides were fixed for 10 min in fresh 4% paraformaldehyde. Sodium phosphate (pH 7.4) at 4°C, then washed for 5 min in 0.5× SSC at room temperature. The slides were then permealized with proteinase K (Sigma Co., St. Louis, MO) solution (2 μg/ml) in RNase buffer (500 mM NaCl, 10 mM Tris, pH 8.0) for 10 min at room temperature, then washed for 10 min in 0.5× SSC at room temperature. Paraflin-embedded tissues were acetylated with 0.25% acetic anhydride containing 0.1 M triethanolamine (pH 8.0). These slides were then washed in PBS three times for 5 min each at room temperature. The paraflin-embedded tissue was then dehydrated through graded alcohols from 30% to 100% containing 0.1% DEPC prior to hybridization. The hybridization conditions included 10× DTT, 1× Denhardt’s, 5× SSC, 100 μg/ml single-stranded DNA, 100 μg/ml tRNA, 10% dextran sulfate, and 20% formamide (18, 35). The 40-mer oligonucleotide specific for PKC-α was labeled with [-32P]dATP (<1300 Ci/mmol, New England Nuclear Research Products/DuPont, Boston, MA) at the 3′ end by terminal deoxynucleotidyl transferase which uses cobalt as a cofactor (36). To prevent nonspecific binding of Co2+–SO42- complexes to oxidative enzymes present in the lamina propria of gastrointestinal tissue, 1 μg 2.3-dimercaptopropanol solution (BAL; Sigma) was added to the hybridization buffer and probe prior to placing the tissue on the slide (37). A riboprobe of human β-actin (450 bp) linearized by Dru restriction digestion was generated using T3 RNA polymerase (Ref. 38; Promega). β-actin is expressed ubiquitously and was used as a positive control to document the presence of intact RNA in the tissue samples. Human β-actin was a generous gift from Richard Poulsom (Imperial Cancer Research Fund, London, United Kingdom). To each slide, 2 μl probe (500,000 cpm/μl and 1 μl tRNA were added together and heated for 3 min at 95°C. Immediately, 17 μl ice-cold hybridization buffer/slide were added; the mixture was vortexed and placed on ice. Twenty μl of this mixture were added to the slide. The slides were incubated and sealed in air-tight boxes (Nalgene utility boxes: Baxter Health Care Products) containing filter paper saturated with 4× SSC and 50% formamide for 18 h at 55°C. The slides are then washed twice for 10 min in 2× SSC with 10 μg/ml β-mercaptoethanol and 1 mM EDTA at room temperature. The slides were then immersed in RNase solution (20 μg/ml) for 30 min at room temperature. The slides are washed for 2 h with 4 liters 0.1× SSC plus 10 mM β-mercaptoethanol and 1 mM EDTA at 55°C and then for 3 min in 0.5× SSC at room temperature. The slides were dehydrated for 2 min each in 50, 70, and 90% ethanol containing 0.3 M ammonium acetate. The slides were dried and then dipped in Kodak NTB2 emulsion diluted 1:2 with water at 42°C. The slides were dried for 2 h and exposed in the dark at 4°C for 2 to 6 weeks. Slides were developed for 3 min at 15°C in Kodak D19 diluted 1:2 with water, 20 s in waterstop, and 3 min in Kodak fixer. The slides were washed three times in water and then stained with H&E. Specificity of mRNA hybridization was measured by removal of the highest expression in the basal cell layer. Diffuse staining is also noted in superficial and superficial layers. Immunoreactivity is demonstrated by a brown diaminobenzidine reaction product. Methyl green counterstaining is seen as a faint blue-green. All immunohistochemical staining was performed in a similar manner in this study. Incubation of secondary antibody alone also revealed no brown reaction product. Methyl green, ×400. D, ISH of PKC-α mRNA in human antrum showed diffuse labeling which was more pronounced in superficial mucous cells and deeper glandular cells than in mucous neck cells lining the gastric pits. A similar pattern was seen in fundic tissue. H&E, ×200. E, immunohistochemistry using PKC-α monoclonal antibody of fundic mucosa detected intense staining for a protein in superficial mucous cells and deep glandular cells with less intense staining of mucous neck cells. A similar staining pattern was noted in tissue from the stomach antrum. Methyl green, ×200. F, superficial mucous cells of gastric antral folds demonstrated intense staining for PKC-α monoclonal antibody. The apical membrane stained strongly for the PKC-α antibody as well as the theca of the surface epithelial cells. Methyl green, ×400. G, localization of PKC-α mRNA in human duodenum by ISH demonstrated predominant expression in the crypt cells and a lesser extent in the mature epithelial cells that line the villi. H&E, ×200. H, immunohistochemical staining with PKC-α monoclonal antibody was confined to mature enterocytes of the duodenal villi. Little staining was noted in the crypt cells. A similar pattern of staining was noted in the ileal samples. Methyl green, ×200. I, ISH of PKC-α mRNA in human colon showed diffuse labeling along the crypt column with accentuation in the upper crypt cells and surface epithelial cells, ×200. J, immunostaining for PKC-α in human large bowel was concentrated to surface epithelial cells and goblet cells of the upper crypt. Staining of cells at the base and portion of the crypt portion of the crypt is well defined and negative control for immunohistochemistry of normal colonic crypts. The diaminobenzidine brown reaction product is absent. The methyl green counterstain is seen as a blue-green. Methyl green, ×200. K, immunohistochemistry of well-differentiated large bowel adenocarcinoma showed intense staining for PKC-α throughout the neoplastic glands, which is decreased in the intervening desmoplasia which often accompanies tumor formation. Methyl green, ×200.
signal following preigestion of tissue sections with RNase (20 μg/ml) for 30 min at room temperature.

**Western Blot Analysis.** Gastrointestinal tract samples were homogenized in 10 mM HEPES, 1 mM EDTA, 1 mM EGTA, 100 mM NaCl, and 100 μM leupeptin (buffer A) at 4°C. Homogenates were centrifuged at 1000 rpm for 10 min, and the pellet was discarded. Protein was determined on the supernatant using the method of Lowry et al. (39). Western blot analyses were also performed and compared between the adenocarcinoma and adjacent normal large bowel mucosa. The homogenate was separated into cytosol and membrane fractions by centrifugation at 100,000 × g for 1 h. The membrane pellet was resuspended in buffer A plus 0.2% Triton X-100 and centrifuged at 100,000 × g for 1 h.

One-dimensional SDS-PAGE was carried out. One hundred μg of sample protein were loaded into each lane. Prestained molecular weight markers were loaded onto each gel. Gels were then electroblotted onto nitrocellulose. The nitrocellulose was then stained with Ponceau S to confirm complete transfer and loading efficiency. Following destaining of the Ponceau S, the nitrocellulose blot was incubated in a blocking solution for 30 min [5% nonfat dry milk, 0.5 mM NaCl, 0.02% Tris (pH 7.4), and 0.1% Tween 20]. After washing, the blot was incubated with a PKC-α antibody for 1 h, either with monoclonal PKC-α (United Biomedical, Inc.) or polyclonal PKC-α (Life Technologies, Inc.) antibody, the specificity of which has been demonstrated previously (16, 17). The blots were then washed and incubated with the appropriate secondary antibody for 2 h. The presence of PKC-α antibody was visualized using the ECL system (Amersham, Arlington Heights, IL). Specificity of antibody binding was determined by preincubating the antibody with excess antigen prior to incubation on the blot.

**Immunohistochemistry.** Four-μm sections of paraaffin-embedded tissue were processed for immunohistochemistry. Sections were dewaxed in freshly prepared xylene twice for 5 min, then rehydrated through graded alcohols from 100% to 30%. Endogenous peroxidase activity is quenched by incubating the sections for 10 min in 400 ml methanol containing 2.4 ml of 30% H₂O₂. The sections were treated with 0.04% protease (Sigma) at 37°C for 0.5 h and washed in water. Sections were then incubated in normal swine serum diluted 1:25 with PBS for polyclonal antibody or normal rabbit serum diluted 1:25 with PBS for monoclonal antibodies for 15 min. The slides were then incubated with the primary antibody, either a polyclonal PKC-α diluted 1:40 or monoclonal PKC-α diluted 1:100 with PBS overnight in an air-tight system at 4°C. Sections were washed twice for 5 min in PBS, then incubated for 30 min at room temperature with biotinylated swine anti-rabbit (1:500) and with normal human serum added (1:25) for polyclonal antibodies (Life Technologies, Inc.) or 1:300 biotinylated rabbit anti-mouse plus 1:5 normal human serum for monoclonal antibodies (United Biomedical, Inc.). The slides were then washed twice in PBS and incubated for 30 min in a streptavidin peroxidase diluted 1:500 with PBS. Sections were washed twice in PBS for 5 min each. Fresh peroxidase substrate, 3,3′-diaminobenzidine (Sigma), was added: Slides were then washed in water and counterstained in methyl green for 15 min or hematoxylin for 3 min. The diaminobenzidine brown reaction product was observed using light microscopy. To determine the specificity of immunoreactivity, tissue sections were incubated with the secondary antibody alone as a negative control. Paraaffin-embedded rat brain sections were used as a positive control.

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