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KITANO, TATSURO

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# MONOCLONAL ANTIBODIES AGAINST RAT BRAIN PROTEIN KINASE C

TATSURO KITANO

Department of Biochemistry
Kobe University School of Medicine

## INDEXING WORDS

protein kinase C; monoclonal antibody; immunoprecipitation; Western blotting; immunocytochemistry

## SYNOPSIS

Protein kinase C has attracted great attention for the studies on the activation of cellular functions and proliferation, and the knowledge of possible roles of this enzyme in cell surface signal transduction is expanding rapidly. Under physiological conditions, protein kinase C is activated by diacylglycerol which may arise from receptor-mediated hydrolysis of inositol phospholipids. Three monoclonal antibodies were prepared against rat brain protein kinase C. Two of the antibodies, CKI-97 and CKII-90, showed weak binding to native protein kinase C. The third antibody, CKI-33 showed no binding. The mixture of these monoclonal antibodies exhibited much stronger binding activity to this protein kinase than any of the antibodies alone. None of these antibodies neutralized the enzymatic activity. These antibodies did not recognize cyclic AMP-dependent nor cyclic GMP-dependent protein kinase. In Purkinje cells, the immunoreactive material was

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Abbreviations used are: SDS, sodium dodecyl sulfate; EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; and ELISA, enzyme-linked immunosorbent assay.

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present throughout the cytoplasm including dendrites and axons, but pooly represented in the cell nucleus. These monoclonal antibodies may be useful for immunocytochemical studies of protein kinase C in the nervous system.

## INTRODUCTION

Various extracellular signals such as those from a group of neurotransmitters and hormones appear to initiate cellular responses through their receptor-mediated hydrolysis of inositol phospholipids. Diacylglycerol produced by the breakdown of inositol phospholipids activates protein kinase C.[14, 15] Kuo and collaborators,[6] and Rosen and collaborators[1] described polyclonal antibodies against protein kinase C that were prepared using pig brain and rat brain protein kinase C as antigen, respectively. As one of the approaches toward identifying the functional significance and structural feature of this enzyme, three monoclonal antibodies against rat brain protein kinase C were prepared. Western blot analysis indicated that these monoclonal antibodies specifically react with protein kinase C. Protein kinase C-positive immunoreaction was observed in many regions of the rat nervous tissue. The present paper will describe characterization of these monoclonal antibodies designated CKI-33, CKI-97, and CKII-90, and their application to immunocytochemical studies of this enzyme in some rat nervous tissues.

## MATERIALS AND METHODS

*Production of monoclonal antibodies against protein kinase C*

Protein kinase C purified from rat brain soluble fraction was used as antigen to prepare monoclonal antibodies. Protein kinase C was purified from rat brain soluble fraction as described previously.[10] Briefly, rat brain was homogenized in 0.25 M sucrose containing 20 mM Tris/HCl, 10 mM EGTA, 2 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride. The homogenate was centrifuged at 100,000 x g for 60 min. From the supernatant, protein kinase C was purified by DEAE-cellulose (DE-52, Whatman), followed by threonine-Sepharose, and phenyl-Sepharose CL-6B column chromatographies. Purified protein kinase C (50 μg) emulsified in an equal

volume of complete Freund's adjuvant (DIFCO) was injected intraperitoneally into male BALB/c mice. Two weeks after the primary immunization, the same amount of antigen in incomplete Freund's adjuvant (DIFCO) was administrated subcutaneously. This treatment was repeated ten to twelve times at two-week intervals. Three subsequent immunizations were performed at two to four-week intervals with purified enzyme in saline by intraperitoneal injections. During immunization, the mice were partially bled, and the sera were taken and used to detect antibodies against protein kinase C. Three days after final immunization, the splenocytes were isolated, mixed with 8-azaguanine-resistant parental cells (P3U1)[22] at a ratio of 2:1, and fused by a modification of the procedure described by Köhler and Milstein.[11] The fused cells were suspended at $2 \times 10^5$ tumor cells/ml in RPMI 1640 medium supplemented with $10^{-4}$M hypoxanthine, $4 \times 10^{-7}$ M aminopterin, $1.6 \times 10^{-5}$ M thymidine, and 10% fetal calf serum, and seeded in a volume of 1 ml to each well of Linbro 24-well microplates. The hybridomas were screened for anti-protein kinase C immunoglobulin secretion with the ELISA technique. The hybridomas in wells, that were positive for the antibody production, were cloned by limiting dilution method in the presence of thymocytes from BALB/c mice as feeder cells. The cloned hybridomas were grown in the culture media, and antibodies against protein kinase C were detected by immunoprecipitation and Western blot analysis using purified protein kinase C as antigen.

*Screening assay for antibodies*

As a screening procedure to detect antibodies against protein kinase C in the sera of immunized mice, and those in the culture supernatant of hybridomas, the ELISA technique was employed. Purified protein kinase C was immobilized in the wells of a 96-well microtiter plate (NUNC InterMED) by incubating with 100 µl of the enzyme solution (3 µg/ml in 10 mM sodium phosphate buffer at pH 8.0 containing 10 mM sodium chloride) for 24 hr at 4C. After the solution was removed by suction, the wells were incubated with 150 µl each of 2% (w/v) bovine serum albumin in a phosphate-buffered saline at least overnight until use at 4C to block the remaining binding sites. Then, the albumin solution was discarded. Subsequently, 100 µl of an antibody solution to be tested was

added to each well, and the reaction was allowed to proceed for 3 hr at room temperature or overnight at 4C. Antibody solutions either of serum, ascites fluid, or culture supernatant of hybrid cells, were diluted to appropriate concentrations with a phosphate-buffered saline containing 1% (w/v) bovine serum albumin. After removing the antibody solution, each well was washed five times with 0.15 M sodium chloride, and then 100 µl of 1:5000 dilution of peroxidase-conjugated goat antiserum to mouse immunoglobulins (Cappel Laboratories) in a phosphate-buffered saline containing 1% (w/v) bovine serum albumin was added. The incubation was made for 3 hr at room temperature. After washing extensively again as before, 100 µl of 100 mM sodium citrate at pH 5.5 containing 0.2% (w/v) orthophenylenediamine and 0.02% $H_2O_2$ was added. The wells containing antibodies against protein kinase C could be visually detected after 3 to 5 min at room temperature, and peroxidase reaction was stopped by adding 100 µl of 4 N $H_2SO_4$. The optical density at 490 nm of each sample was read with an automatic microwell plate colorimeter (ImmunoReader NJ-2000, Nippon InterMed).

*Purification and properties of monoclonal antibodies*

BALB/c mice were pretreated with 0.5 ml of 2,6,10,14- *tetra*-methylpentadecane (Pristane, Aldrich) a week before, hybridomas ($2 \times 10^6$ cells) were injected intraperitoneally into them. Most mice developed ascites within 2 to 3 weeks. By Protein A-Sepharose CL-4B (Pharmacia) column chromatography, monoclonal antibodies were purified from ascites fluids as described Ey et al.[5] Briefly, ascites fluids were applied to the column equilibrated with 0.1 M sodium phosphate at pH 8.0, and the antibodies were eluted successively with 0.1 M sodium citrate at pH 6.0 (elutes $IgG_1$ subclass antibody), pH 4.5 (elutes $IgG_{2a}$ subclass antibody), and pH 3.0 (elutes $IgG_{2b}$ subclass antibodies). The classes and subclasses of the antibodies were confirmed by immunodiffusion method (Ouchterlony, 1953)[18] using class-specific goat antiserum to mouse IgM and subclass-specific rabbit antiserum to mouse IgG (Miles Laboratories). Non-immune mouse IgG fraction was obtained from normal mouse sera by similar procedures.

*Immunoprecipitation of protein kinase C with monoclonal antibodies and protein kinase C-neutralizing activity of monoclonal antibodies*

Purified protein kinase C (0.1 μg) was incubated for 4 hr at 10C with monoclonal antibodies in 100 μl of 20 mM Tris/HCl at pH 8.0 containing 4 mg/ml bovine serum albumin, 2 mM EGTA, 2 mM EDTA, 10 mM 2-mercaptoethanol, and 10% (w/v) glycerol. After addition of 100 μl of 10% (w/v) *Staphylococcus aureus* (Cowan 1), which was obtained from Calbiochem and suspended in the same buffer solution, the incubation was continued overnight at 4C. The *S. aureus* cells were removed by centrifugation for 2 min at 16,000 x g, and an aliquot of the supernatant was assayed for protein kinase C activity. Protein kinase C was assayed by measuring the incorporation of $^{32}$Pi into H1 histone from [$\gamma$-$^{32}$P]ATP as described previously.[10] The reaction mixture (0.25 ml) contained 20 mM Tris/HCl at pH 7.5, 200 μg/ml H1 histone, 10 μM [$\gamma$-$^{32}$P]ATP (200 to 400 cpm/pmol, Amersham), 5 mM magnesium acetate, 0.3 mM $CaCl_2$, 8 μg/ml phosphatidylserine, 0.8 μg/ml diolein, and enzyme fraction. After incubation for 30 min at 30C, the reaction was stopped by the addition of 25% (w/v) trichloroacetic acid, and the acid-precipitable materials were collected on a Toyo-Roshi membrane filter (pore size, 0.45 μm). The radioactivity was determined using a Packard Tri-Carb liquid scintillation spectrometer, Model 4640. Calf thymus H1 histone was prepared as described previously.[7] When protein kinase C-neutralizing activity of the monoclonal antibodies was assayed, purified protein kinase C (0.1 μg) was incubated with the antibodies in the absence of *S. aureus*, and protein kinase C activity was measured as described above.

*Western blot analysis*

Purified protein kinase C, cyclic AMP-dependent protein kinase, and cyclic GMP-dependent protein kinase were subjected to SDS-polyacrylamide gel electrophoresis with a 1.0-mm thick 8.5% polyacrylamide slab gel by the method of Laemmli.[12] Catalytic subunit of cyclic AMP-dependent protein kinase was partially purified from rat heart by the method of Reimann and Beham.[19] Cyclic GMP-dependent protein kinase was prepared from bovine lung as described by Lincoln.[13] Proteins on the gel were electrophoretically transferred to a nitrocellulose paper at 11.4 V/cm electrode distance for 3 hr at 4C by the method of Towbin et al.

21) After transfer, the nitrocellulose paper was rinsed with Tris-NaCl buffer (10 mM Tris/HCl at pH 7.5 containing 150 mM sodium chloride) containing 0.05% Tween 20, and was then incubated overnight at 4C with a phosphate-buffered saline containing 5% (w/v) bovine serum albumin and 20% (v/v) horse serum to block remaining binding sites.

Subsequent procedures were carried out at room temperature. The nitrocellulose paper was incubated for 30 min with monoclonal antibodies in Tris-NaCl buffer containing 3% (w/v) bovine serum albumin. After washing with Tris-NaCl buffer containing 0.05% Tween 20, the nitrocellulose was incubated for 30 min with biotinylated anti-mouse IgG (Vectastain, Vector Laboratories; 1:2200 dilution in 50 mM Tris/HCl at pH 7.5). The nitrocellulose paper was washed as before, and incubated for 15 min with avidin-biotinylated horseradish peroxidase complex. After washing with Tris-NaCl buffer containing 0.05% Tween 20, the paper was incubated with 0.1% (w/v) diaminobenzidine tetrahydrochloride and 0.02% $H_2O_2$. Development of the orange color was stopped by rinsing with distilled water.

*Immunocytochemical procedures*

Immunocytochemical studies were carried out as described previously.[20] Briefly, the fixed cerebellum of Wistar rats was cut into sections (20 μm) which were dipped directly in 0.1 M phosphate-buffered saline containing 0.3% (w/v) Triton X-100. The sections were washed with the same buffer, and incubated for 18 hr at 4C with monoclonal antibodies. Then, the sections were washed with a phosphate-buffered saline containing Triton X-100, and incubated for 2 hr in fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Miles Laboratories) diluted 1:250. After rinsing, the sections were mounted in buffered glycerol for fluorescent microscopic observation.

*Protein determination*

Protein was determined by the method of Bensadoun and Weinstein (1976)[2] with bovin serum albumin as a standard.

## RESULTS

After fifth to tenth immunization, the titer of the sera of the mice which have contained monoclonal antibodies against protein kinase C slightly increased. Finally, three clones were prepared and designated CKI-33, CKI-97, and CKII-90. These antibodies bound to *S. aureus* cells directly and also reacted with biotinylated anti-mouse IgG antibodies by Western blot analysis, and so they were classified as IgG. The chromatographic behaviours of CKI-33, CKI-97, and CKII-90 on a Protein A-Sepharose column were consist with their tentative assignment to the subclass of $IgG_{2b}$, $IgG_{2b}$, and $IgG_1$ respectively. Ouchterlony immunodiffusion analysis confirmed the classes and subclasses of CKI-33, CKI-97, and CKII-90 as shown in Table 1.

The binding activity of these monoclonal antibodies to protein kinase C was determined using *S. aureus* cells. Fig. 1A shows that CKI-97 and CKII-90 have binding activity to purified protein kinase C, while CKI-33 has a very little binding activity to the native enzyme. The mixture of these three antibodies exhibited much stronger binding activity to this protein kinase. Thus, the affinity of these antibodies to protein kinase C seems to be different from one another, and probably these antibodies may recognize the different epitopes in this enzyme molecule. However, none of these monoclonal antibodies affected the catalytic activity of protein kinase C under the conditions where these antibodies bind to the enzyme as shown in Fig. 1B.

Fig. 2 shows the the results of Western blot analysis. *A* indicates protein staining with Coomassie brilliant blue. *B* and *C* show the results of Western blot analysis using CKI-97 and CKII-90, respectively. *Lane 1* indicates purified protein kinase C which shows approximate molecular weight of 82 kDa.[9] *Lane 2* and *Lane 3* indicate catalytic subunit of cyclic AMP-dependent protein kinase and cyclic GMP-dependent protein kinase, respectively. These cyclic nucleotide-dependent protien kinases were not reactive with these antibodies, and protein kinase C was specifically detected by these monoclonal antibodies. Similar Western blot profile was also obtained with CKI-33. When crude supernatant of rat brain was analyzed, a protein band with a molecular weight of 82 kDa, which corresponds to protein kinase C, was specifically detected by these monoclonal antibodies. Thus, it

Table 1 The classes and subclasses of monoclonal antibodies against protein kinase C. The classes and subclasses of monoclonal antibodies were determined by immunodiffusion analysis as described under "Materials and Methods."

| MONOCLONAL ANTIBODY | SUBCLASS |
|---|---|
| CKI-33 | $IgG_{2b}$ |
| CKI-97 | $IgG_{2b}$ |
| CKII-90 | $IgG_1$ |

was concluded that all of the three monoclonal antibodies CKI-33, CKI-97, and CKII-90 can react with protein kinase C specifically.

The antibodies defined above can be used for immunocytochemical studies of protein kinase C in many regions of rat nervous tissues. For example, in cerebellar cortex a unique feature of Purkinje cells was stained. This enzyme was present throughout the cytoplasm including dendrites and axons, and appeared to be absent or poorly represented in the nucleus (Fig. 3A). The immunoreaction was absorbed by the preincubation of monoclonal antibodies with purified protein kinase C (Fig. 3B).

## DISCUSSION

Biochemical fractionation of the brain tissues revealed that protein kinase C was distributed not only in the soluble fraction but also in membranes.[8, 9] At present, no obvious difference is observed in the catalytic and kinetic properties of protein kinase C from different subcellular fractions, and these antibodies appear to also react with the membrane associated protein kinase C.

Studies of amino acid sequencing and molecular cloning of this enzyme have shown some heterogeneity of this enzyme, and the purified protein kinase C occasionally reveals a duplex band upon SDS-polyacrylamide gel electrophoresis.[10] These antibodies CKI-33, CKI-97, and CKII-90, all recognized both of the protein of the duplex band. Recent analysis of complementary DNA clones has shown that more than four, probably multiple subspecies of protein

kinase C having closely similar structures may exist in rat brain [16, 17] and in bovine and human brains.[3] It remains unknown whether the antibodies described above can recognize all of these heterogeneous components of protein kinase C. Protein kinase C has amino acid sequences in its molecule which are commonly present in these protein kinase subspecies.[3, 16, 17] However, Western blot analysis indicated that these antibodies does not react with catalytic subunit of cyclic AMP-dependent protein kinase from rat heart, cyclic GMP-dependent protein kinase from bovine lung as shown in Fig. 2, nor with any subunit of glycogen phosphorylase b kinase from rabbit skeletal muscle (data not shown). These results indicate that the monoclonal antibodies described above react specifically with protein kinase C even though there might be some heterogeneity of this enzyme.

Fig. 3 shows the results of immunocytochemical studies of protein kinase C using these antibodies. Protein kinase C-positive immunoreaction was observed in many regions of rat nervous tissues. In cerebellar cortex, a unique feature of Purkinje cells was obtained, and the immunoreaction was present throughout the cytoplasm of the cell. The immunoreaction was absorbed by the preincubation of these antibodies with purified protein kinase C, indicating that the immunoreaction shows the localization of protein kinase C. Thus, the antibodies obtained in the present studies are useful for the Western blot and immunocytochemical studies of protein kinase C, although they do not affect the enzymatic activity.

Kuo and collaborators showed immunocytochemical localization of this enzyme in some tissues using their polyclonal antibodies.[6] They observed little dendritic staining but prominent nuclear staining highly restricted to the periphery of the nucleus of Purkinje cells. Their antibodies were revealed to recognize 80 and 67 kDa protein bands, but such a 67 kDa protein was not detected with the monoclonal antibodies as shown in Fig. 2B and Fig. 2C. The two sets of antibodies might be recognizing the heterogeneous components of protein kinase C, or it may be possible to explain the different results of immunocytochemical studies by some methodological differences of fixation and staining. However, the precise reason of the different results of immunocytochemical studies is not clear at present.

In rat cerebellum, the localization of protein kinase C

detected by these monoclonal antibodies is apparently very similar to that of cyclic GMP-dependent protein kinase which has been reported by De Camilli et al.[4)]

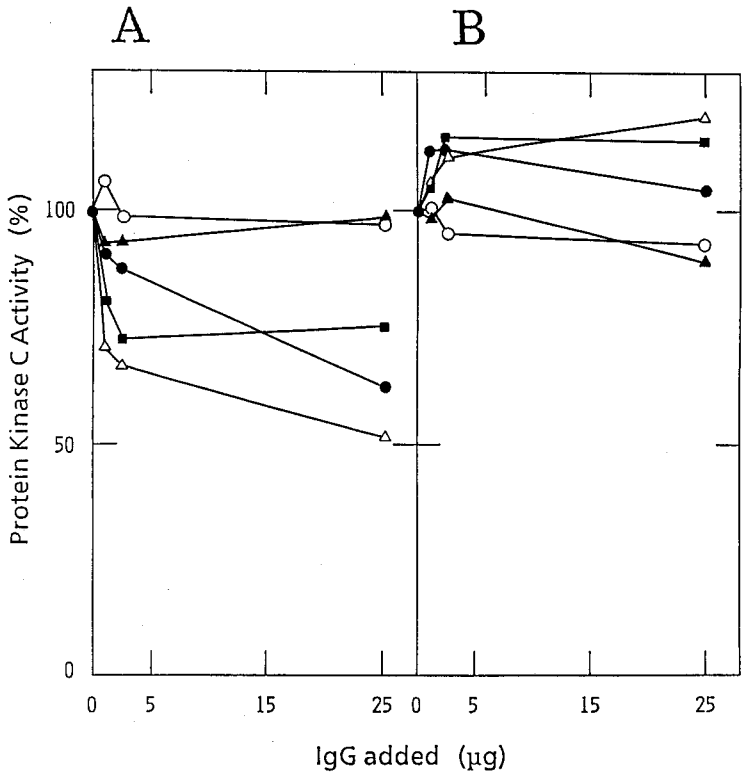


Fig. 1 The effects of monoclonal antibodies on protein kinase C. Purified protein kinase C (0.1 μg) was incubated for 4 hr at 10C with various amounts of monoclonal antibodies in 100 μl of 20 mM Tris/HCl at pH 8.0 containing 4 mg/ml bovine serum albumin, 2 mM EGTA, 2 mM EDTA, 10 mM 2-mercaptoethanol, and 10% (w/v) glycerol. After addition of 100 μl of 10% (w/v) *S. aureus*, the immunoprecipitation of protein kinase C by monoclonal antibodies was assayed as described under "Materials and Methods." Protein kinase C-neutralizing activity of the antibodies was assayed in the absence of *S. aureus*. *A*, immunoprecipitation of protein kinase C by monoclonal antibodies. *B*, protein kinase C-neutralizing activity of monoclonal antibodies. (■-■), with CKI-97; (●-●), with CKII-90; (▲-▲), with CKI-33; (△-△), with a mixture of equal amounts of CKI-97, and CKII-90, and CKI-33; (○-○), with normal mouse IgG fraction.

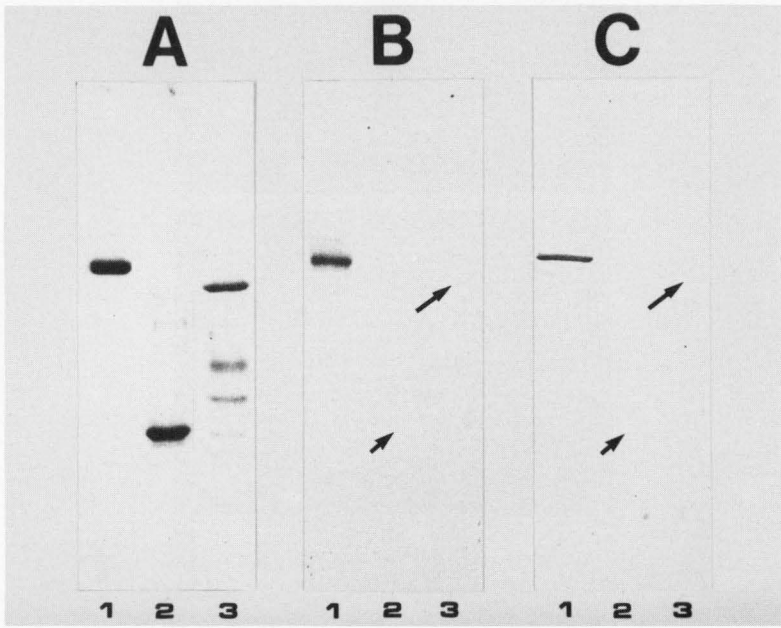


Fig. 2 Specificity of monoclonal antibodies to protein kinase C by Western blot analysis. Purified protein kinase C, cyclic AMP-dependent protein kinase, and cyclic GMP-dependent protein kinase were subjected to SDS-polyacrylamide gel electrophoresis, and were electrophoretically transferred to a nitrocellulose paper, and were reacted with monoclonal antibodies as described under "Materials and Methods." *A*, protein staining with Coomassie brilliant blue. *B* and *C*, the result of Western blot analysis using CKI-97 and CKII-90, respectively. *Lane 1* indicates purified protein kinase C. *Lane 2* and *Lane 3* indicate catalytic subunit of cyclic AMP-dependent protein kinase and cyclic GMP-dependent protein kinase, respectively. The arrow in *Lane 2* shows the position of catalytic subunit of cyclic AMP-dependent protein kinase. The arrow in *Lane 3* shows the position of cyclic GMP-dependent protein kinase.

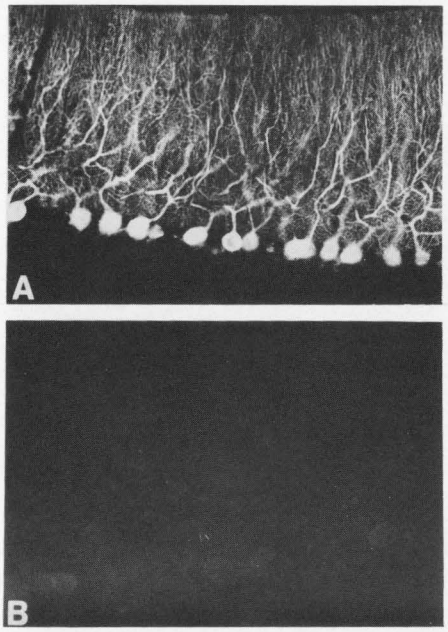


Fig. 3 Immunocytochemical localization of protein kinase C in rat cerebellum. Sections of rat cerebellum were incubated with a mixture (1 μg/ml of each) of monoclonal antibodies CKI-33, CKI-97, and CKII-90, and were stained by immunofluorescence as described under "Materials and Methods." *A*, immunostaining. *B*, absorption test of monoclonal antibody with purified protein kinase C.

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