Distinct Cellular Expression of β I- and β II-Subspecies of Protein Kinase C in Rat Cerebellum

Katsuhiko Ase,¹ Naoaki Saito,² Mark S. Shearman,¹ Ushio Kikkawa,¹ Yoshitaka Ono,³ Koichi Igarashi,³ Chikako Tanaka,² and Yasutomi Nishizuka¹

Departments of ¹Biochemistry and ²Pharmacology, Kobe University School of Medicine, Kobe 650, and ³Biotechnology Laboratories, Central Research Division, Takeda Chemical Industries, Osaka 532, Japan

Immunohistochemical and biochemical studies with sub-species-specific antibodies have revealed that β I- and β II-subspecies of protein kinase C, which result from alternative splicing of a single RNA transcript, show different regional expression in rat CNS. In the cerebellar cortex, β I-subspecies is localized mainly in the granular layer, whereas β II-subspecies is found predominantly in the molecular layer, most apparently in the presynaptic nerve endings that terminate at Purkinje cells. These distribution patterns are in sharp contrast to that of γ -subspecies, which is most abundant within the Purkinje cells. The different patterns of expression imply that the multiple subspecies of protein kinase C may each have a specific function in modulating the neuronal activity of particular cell types.

Protein kinase C is most abundant in nervous tissue and has been implicated in modulating many Ca^{2+} -dependent processes during signal transduction of various extracellular stimuli (Kikkawa and Nishizuka, 1986; Nishizuka, 1986). Although once thought to be a single entity, recent molecular cloning analysis in this and other laboratories (Coussens et al., 1986; Knopf et al., 1986; Makowske et al., 1986; Ono et al., 1986a, b; Parker et al., 1986; Housey et al., 1987; Kubo et al., 1987; Ohno et al., 1987) has revealed the enzyme to exist as a family of multiple subspecies. In the mammalian brain the complete sequences of at least 4 subspecies— α , β I, β II, and γ —have been clarified by complementary DNA (cDNA) clone analysis (Coussens et al., 1986; Knopf et al., 1986; Ono et al., 1986a, b; Parker et al., 1986; Ohno et al., 1987). This integrated nomenclature and its explanation are as given in preceding reports (Kikkawa et al., 1987a; Ono et al., 1987). Enzymologically, 3 major distinct types of protein kinase C—I, II, and III—can normally be resolved upon hydroxyapatite column chromatography (Huang et al.,

1986; Jaken and Kiley, 1987; Kikkawa et al., 1987a; Ono et al., 1987; Pelosin et al., 1987), and the structure and genetic identity of each type has been determined by comparison with the enzymes that were separately expressed in COS 7 cells transfected by the respective cDNA-containing plasmids (Kikkawa et al., 1987a; Ono et al., 1987). Type I protein kinase C corresponds to γ -subspecies, Type II protein kinase C is a mixture of β I- and β II-subspecies, and Type III protein kinase C corresponds to α -subspecies. Partial genomic analysis has provided evidence that β I- and β II-subspecies are derived from a single gene by alternative splicing of the RNA transcript (Ono et al., 1987). These 2 subspecies, which consist of 671 and 673 amino acids, respectively, differ from each other only in the short range of about 50 amino acid residues in their carboxyl-terminal end regions, but still show a considerable sequence homology. In fact, β I- and β II-subspecies separately expressed in COS 7 cells show similar physical and kinetic properties and are not separable by conventional enzyme purification procedures (Kikkawa et al., 1987a; Ono et al., 1987). The studies presented herein were conducted to develop specific antibodies that are able to distinguish these 2 subspecies and to investigate their expression in different areas and cell types of the rat CNS. Their expression patterns were found to be entirely different from that of the γ -subspecies of protein kinase C, which has been found only in CNS (Kikkawa et al., 1987b).

Materials and Methods

Purification and assay of protein kinase C. A mixture of multiple forms of protein kinase C was purified from rat brain soluble fraction by DE-52 column chromatography, followed by threonine-Sepharose and TSK phenyl-SPW column chromatographies connected to high-performance liquid chromatography (HPLC), Pharmacia FPLC system, as described (Kikkawa et al., 1986). This apparently homogeneous preparation was further resolved into 3 forms—Type I, II, and III—upon chromatography on a hydroxyapatite column connected to the HPLC under the conditions specified elsewhere (Kikkawa et al., 1987a; Ono et al., 1987). The 3 forms each showed a single band upon SDS-PAGE. The α -, β I-, β II-, and γ -subspecies of protein kinase C employed here were obtained by separately transfecting each of the cDNA-containing plasmids into mammalian cells, followed by extraction and purification as described earlier (Kikkawa et al., 1987a; Ono et al., 1987). The enzyme was assayed with calf thymus HI histone as a phosphate acceptor as described (Kikkawa et al., 1986). Calf thymus HI histone was prepared as described previously (Hashimoto et al., 1976).

Preparation of antibodies. The antibodies specific to β I- and β II-subspecies were prepared with synthetic oligopeptides SYTNPEFVINV and SFVNSEFLKPEVKS, which correspond to the carboxyl-terminal end sequences of each subspecies (Fig. 1), and are designated hereafter as CKpV5 β I-a and CKpV5 β II-a, respectively. The oligopeptides were prepared with a solid-phase polypeptide synthesizer (Applied Biosystems, model 430A) and purified by HPLC using C18 reverse-phase column. The samples were coupled to bovine thyroglobulin with

Received Nov. 20, 1987; revised Feb. 3, 1988; accepted Feb. 5, 1988.

We thank Drs. Y. Sugino and A. Kakinuma for encouragement, Dr. O. Nishimura (Biotechnology Laboratories, Takeda Chemical Industries) for the kind donation of synthetic oligopeptides, and Sachiko Nishiyama and Yoko Kume for secretarial assistance. The work in the Department of Biochemistry, Kobe University School of Medicine, was supported in part by research grants from the Scientific Research Fund of Ministry of Education, Science and Culture, Japan (1986–1987); Muscular Dystrophy Association (1986–1987); Yamanouchi Foundation for Research on Metabolic Disorders (1986–1987); Merck Sharp & Dohme Research Laboratories (1986–1987); Biotechnology Laboratories of Takeda Chemical Industries (1986–1987); Ajinomoto Central Research Laboratories (1986–1987); and Meiji Institute of Health Sciences (1986–1987).

Data are taken in part from the dissertation that will be submitted by K. Ase to Kobe University School of Medicine in partial fulfillment of the requirement for the degree of Doctor of Medical Science.

Correspondence should be addressed to Yasutomi Nishizuka at the above address.

Copyright © 1988 Society for Neuroscience 0270-6474/88/103850-07\$02.00/0

β I	622-671	RDKRDTSNFDKEFTR-QPVELTPTDKLFIMNLDQNEFAGF	<u>SYTNPEFVINV</u>
		★ ★★ ★★ ★★ ★★ ★★ ★★ ★★ ★★ ★★ ★★	
β II	622-673	-CGRNAENFDRFFTRHPPV-LTPPDQEVIRNIDQSEFEGF	<u>SFVNSEFLKPEVKS</u>

Figure 1. Comparison of the amino acid sequences of the divergent regions of β I- and β II-subspecies of protein kinase C in their carboxyl-terminal ends. Gaps indicated by *hyphens* are introduced to optimize the homology between the β I- and β II-subspecies of protein kinase C. The polypeptide sequences employed to prepare specific antibodies against β I- and β II-subspecies of protein kinase C are shown in *boxes*. Stars indicate the amino acid residues that are common to the 2 sequences.

1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide hydrochloride (Erlanger, 1980). New Zealand White rabbits were injected subcutaneously with each oligopeptide (0.85 mg) coupled to bovine thyroglobulin (1 mg), which was emulsified in Freund's complete adjuvant (DIFCO). Two subsequent additional immunizations were performed at 2-week intervals with the same amount of the antigen in Freund's incomplete adjuvant (DIFCO). One week after the final immunization, the rabbits were bled, and each antibody was purified from the serum by affinity column chromatography on Sepharose CL-4B coupled with each oligopeptide. The monoclonal antibody CKmI γ -97 was raised originally against a mixture of Type I, II, and III protein kinase C (Kitano et al.,

1987). Subsequent analysis with these protein kinase C fractions has revealed that the antibody specifically reacts with Type I protein kinase C (γ -subspecies) (Hashimoto et al., 1987).

Western blot analysis. Each type of protein kinase C, which was obtained by hydroxyapatite column chromatography from rat brain or transfected COS 7 cells, was applied to SDS-PAGE and transferred to a nitrocellulose filter as described (Kitano et al., 1987). After reaction with each antibody, the nitrocellulose filter was incubated with biotinylated second antibody and subsequently with avidin-biotinylated HRP complex (Vectastain, Vector Laboratories). The color reaction was developed using diaminobenzidine tetrahydrochloride and H_2O_2 .

Figure 2. Specificity of polyclonal antibodies against β I- and β II-subspecies of protein kinase C. Specificity of polyclonal antibodies was analyzed by immunoblot procedures using the standard preparations of α -, β I-, β -II and γ -subspecies of protein kinase C. *A* and *B*, Western blot analysis with the antibodies against β I- and β II-subspecies of protein kinase C (2 μ g/ml), respectively. The molecular sizes in kilodalton of the standards are indicated by *arrows*. Lane *a*, α -subspecies; lane *b*, β I-subspecies; lane *c*, β II-subspecies; lane *d*, γ -subspecies. Molecular-weight markers: 98 kDa, phosphorylase *b*; 66 kDa, BSA; 45 kDa, ovalbumin.

Figure 3. Western blot analysis of biochemically fractionated and nonfractionated rat brain protein kinase C. The purified protein kinase C preparations and crude DE-52 fraction were applied to SDS-PAGE. The gel was stained with Coomassie brilliant blue or subjected to Western blot analysis as described under Materials and Methods. *A*, Protein staining. *B* and *C*, Western blot analysis with the antibodies against β I- and β II-subspecies of protein kinase C (2 μ g/ml), respectively. The molecular sizes of the standards are indicated by arrows as described in the legend to Figure 2. Lane *a*, Type I; lane *b*, Type II; lane *c*, Type III; lane *d*, DE-52 fraction.

Immunohistochemical procedures. Immunohistochemical studies were carried out as described (Kitano et al., 1987; Saito et al., 1988). Anesthetized rats were perfused through the left ventricle with 200 ml of periodate-lysine-paraformaldehyde fixative (McLean and Nakane, 1974), and the brain was removed. The postfixed cerebellum, in the same fixative, was cut on a cryostat at 20 μ m. The adjacent cerebellar sections were stained by the peroxidase-antiperoxidase method or the immunofluorescent method with either antibody CKmI γ -97, CKpV5 β I-a, or CKpV5 β II-a.

Results

The specificity of the antibodies against β I- and β II-subspecies of protein kinase C was examined using several protein kinase C subspecies, which were separately expressed in COS 7 cells transfected with each cDNA-containing plasmid. The antibodies, CKpV5 β I-a and CKpV5 β II-a, reacted specifically with β I- and β II-subspecies of protein kinase C, respectively, and were not cross-reactive on Western blot analysis. Neither α - nor γ -subspecies was reacted with these antibodies (Fig. 2). To examine further the specificities of these antibodies, another set of experiments was carried out with both crude and purified rat brain enzyme preparations. The 3 types of protein kinase C employed here were each purified to near homogeneity as assessed by the presence of a single major band upon SDS-PAGE as shown in Figure 3. The specific activities of the 3 types of protein kinase C were comparable to one another when calf

thymus H1 histone was used as a common phosphate acceptor.⁴ The 2 antibodies described above reacted with only Type II protein kinase C but not with the others (Fig. 3). The results also show that, under the given conditions, β I- and β II-subspecies of protein kinase C were the sole proteins in the crude extract that were reactive with the respective antibodies, CKpV5 β I-a and CKpV5 β II-a. Neither of these antibodies, however, could neutralize the catalytic activities of the protein kinase C subspecies.

Biochemical analysis reveals that the relative distribution of activity among the 3 protein kinase C types varies greatly within different regions of rat CNS (Fig. 4). The cerebrum demonstrated high Type II activity, particularly β II-subspecies, as shown below, whereas the cerebellum contained marked activity of Type I protein kinase C. Type II protein kinase C was found to be an unequal mixture of β I- and β II-subspecies as judged by Western blotting with the 2 antibodies, followed by densitometric tracing analysis. The antibodies, CKpV5 β I-a and CKpV5 β II-a,

⁴ The specific activities of Type I, II, and III protein kinase C were 1.3, 1.7, and 1.3 units/mg protein respectively. One unit of protein kinase C is defined as the amount of enzyme that catalyzes the incorporation of 1 μ mol of ³²P from γ -³²P-ATP into calf thymus H1 histone per minute under the conditions described earlier (Kikkawa et al., 1986). Since the 3 types of protein kinase C phosphorylated the same seryl and threonyl residues in the histone molecule, the specific activities of the 3 types may be directly compared with one another.

Figure 4. Distinct patterns of 3 fractions of protein kinase C in rat cerebrum and cerebellum upon hydroxyapatite column chromatography. Rat cerebral and cerebellar tissues (1 gm each) were homogenized and centrifuged at $100,000 \times g$ for 60 min. The supernatant was applied to a DE-52 column, and the eluate from the DE-52 column was applied to a hydroxyapatite column. The protein kinase C fractions were then eluted from the column by linear concentration gradient of potassium phosphate, and the fractions were assayed as described under Materials and Methods. *A* and *B*, Protein kinase C from rat cerebrum and cerebellum, respectively. Symbols: ●, protein kinase activity in the presence of 8 $\mu\text{g}/\text{ml}$ phosphatidylserine, 0.8 $\mu\text{g}/\text{ml}$ diolein, and 0.5 mM CaCl_2 ; ○, protein kinase activity in the presence of EGTA instead of phosphatidylserine, diolein, and CaCl_2 ; —, potassium phosphate.

apparently showed identical affinities for the respective oligopeptides of each subspecies when assessed by enzyme-linked immunoadsorbent assay, making it possible to determine semi-quantitatively the relative activities of βI - and βII -subspecies in the fraction of Type II protein kinase C. Using a combination of such biochemical and immunological procedures, the specific activities of the 4 subspecies of protein kinase C in the brain tissues could be roughly quantitated (Table 1). It is clear that a distinct regional pattern of each subspecies is evident and that the activity of βII -subspecies far exceeds that of βI -subspecies in many regions. The activity of βI -subspecies appears to be almost negligible in the cerebrum and hippocampus.

The antibodies described above can be used for immunohistochemical studies of the protein kinase C subspecies in many regions of neuronal and non-neuronal tissues. In the rat brain, unique features of the distribution of βI - and βII -subspecies were visualized, and normal rabbit serum did not cross-react with the sections of rat brain under these conditions. For instance, in the cerebellar cortex, where significant quantities of both of these subspecies are expressed, the regions heavily stained by CKpV5 βI -a and CKpV5 βII -a antibodies appeared to be complementary (Fig. 5). βI -subspecies of protein kinase C was present in the granular layer but not the molecular or Purkinje cell layer. The highest level of βII -subspecies immunoreactivity was in the molecular layer, and moderately dense immunoreactivity was present in the granular layer. Pyramidal tract also showed

Figure 5. Image analysis of immunoreactivity in the cerebellar sections with the antibodies against γ -, βI -, and βII -subspecies of protein kinase C. The adjacent frontal cerebellar sections of rat were stained by the peroxidase-antiperoxidase method with either the antibody CKm γI -97, CKpV5 βI -a, or CKpV5 βII -a (1 $\mu\text{g}/\text{ml}$) as described under Materials and Methods. The immunoreactivity in the sections was analyzed using an IBAS II image analyzer (Zeiss). *A–C*, Immunostaining with CKm γI -97, CKpV5 βI -a, and CKpV5 βII -a, respectively.

Figure 6. Microphotographs and fluorescent microphotographs of the cerebellar cortex stained with the antibodies against γ -, β I-, and β II-subspecies of protein kinase C. The cerebellar sections were prepared and immunostained as described in Materials and Methods and the legend to Figure 5. *A–C*, Peroxidase-antiperoxidase staining. *D–F*, Immunofluorescent staining. *A* and *D*, CKm γ L-97 staining. *B* and *E*, CKpV β I-a staining. *C* and *F*, CKpV β II-a staining. *CC*, cerebellar cortex; *ML*, molecular layer; *PL*, Purkinje cell layer; *GL*, granular layer. Scale bars: *A–C*, 250 μ m; *D–F*, 30 μ m.

Table 1. Distribution of the 4 subspecies of protein kinase C in rat CNS

Tissue	Specific activity (nmol/min/gm wet weight tissue)			
	Type III α	Type II β I	β II	Type I γ
Whole brain	4.9	1.2	8.5	5.2
Cerebrum	2.8	0.3	10.3	3.2
Cerebellum	6.0	3.2	10.3	21.5
Hippocampus	10.8	0.3	12.9	8.3
Spinal cord	3.2	1.1	2.4	0.2

Protein kinase C was separated into Type I, II, and III fractions by hydroxyapatite column chromatography and assayed as described in Materials and Methods. The specific activities of β I- and β II-subspecies in the fraction of Type II were estimated as described in the text.

high immunoreactivity. Regions with high β II-subspecies immunoreactivity did not appear to contain β I-subspecies and vice versa. At this level of resolution, the pattern for β II-subspecies was apparently similar to that of γ -subspecies. The monoclonal antibody CKmI γ -97, previously prepared (Kitano et al., 1987), specifically recognizes Type I protein kinase C (γ -subspecies) as described (Hashimoto et al., 1988). The γ -subspecies was present in the molecular layer and Purkinje cell layer, though no immunoreactivity is observed in the granular layer or white matter of the cerebellar cortex. Moderately low immunoreactivity was also seen in the deep cerebellar nuclei. Higher magnification revealed that β I-subspecies was associated mainly with the granular layer, particularly granule cell bodies, but no immunoreaction was seen in the molecular layer or Purkinje cells. Whereas β II-subspecies was found predominantly in the molecular layer (Fig. 6). More precisely, the materials reactive with the antibody, CKpV5 β II-a, surrounded the Purkinje cell bodies as well as their dendrites, most likely representing the numerous nerve terminals synapsing with Purkinje cells. This distribution pattern can now be seen to contrast markedly with that of γ -subspecies, which is present in the cell bodies, dendrites, and axons of Purkinje cells (Hashimoto et al., 1988; Saito et al., 1987).

Discussion

Several attempts have been made to explore the tissue- or cell-specific expression of protein kinase C. Autoradiographic procedures developed by Snyder and colleagues (Worley et al., 1986a-c, 1987) using tritiated phorbol-12,13-dibutyrate (3 H-PDBu) showed that protein kinase C is distributed unevenly in the brain with high concentrations in the hippocampus, substantia nigra, and the molecular layers of the cerebellar and cerebral cortices. This procedure, however, is unable to distinguish multiple subspecies of the enzyme. Early studies of Northern blotting using specific polynucleotide probes (Coussens et al., 1986; Knopf et al., 1986; Parker et al., 1986; Ohno et al., 1987) first suggested tissue-specific expression of protein kinase C subspecies. More recently, using *in situ* hybridization histochemistry with oligonucleotide probes complementary to 3 cDNA sequences of rat brain protein kinase C, Brandt et al. (1987) have shown distinct distribution of the transcripts of protein kinase C subspecies within the brain and spleen. These workers have proposed that in the rat brain the transcripts of their PKC-III and PKC-II, which correspond to our β I- and

β II-subspecies, colocalize in some brain regions such as the granular layer of the cerebellar cortex and the pyramidal cells in the CA1 area of the hippocampus. In the brain stem and spinal cord, however, they reported that some cells appeared to contain only PKC-III (β I-subspecies) mRNA. The transcript of PKC-I, which corresponds to γ -subspecies in this paper, has been shown to localize in Purkinje cells, as well as in the pyramidal and granule cells of the hippocampus.

Using immunohistochemical procedures, Kuo and colleagues (Girard et al., 1985; Wood et al., 1986) have shown that, in general, protein kinase C is present in presynaptic terminals and the periphery of the nucleus of many neuronal cell types. Mochly-Rosen et al. (1987) have raised 3 monoclonal antibodies and showed different immunostaining patterns of these antibodies in rat brain, although their correspondence to the structurally defined protein kinase C subspecies described herein remains unknown. The pattern stained by one of the antibodies, designated CK1.12, is similar to that stained by CKmI γ -97, which recognizes γ -subspecies. With more defined and specific antibodies, the present paper demonstrates distinctly different localization of the β I-, β II-, and γ -subspecies in certain areas of the CNS. The patterns of cellular localization of the 3 protein kinase C subspecies clearly differ from one another in the cerebellar cortex.

The dissimilarities of the results described here and those obtained by *in situ* hybridization may be attributable to several causes. First, the oligonucleotide probes that Brandt et al. (1987) employed to detect the PKC-III (β I-subspecies) and PKC-II (β II-subspecies) transcripts did not effectively distinguish the 2 protein kinase C mRNAs, since they are generated by alternative splicing of a single common primary transcript, and the probe for PKC-II (β II-subspecies) hybridizes only with the inserted sequence and does not recognize the mRNA encoding PKC-III (β I-subspecies). Whereas the probe they used for PKC-III (β I-subspecies) is complementary with a part of the sequences commonly present in both mRNAs and, therefore, could detect both subspecies (Brandt et al., 1987; Kikkawa et al., 1987b; Kubo et al., 1987; Ono et al., 1987). Second, there is not necessarily a direct relationship between the mRNA levels and those of the enzyme within a particular cell type. Third, it seems possible that additional subspecies of protein kinase C may exist in the brain which do or do not react cross-wise with the oligonucleotide probes or antibodies employed for these studies. A partial sequence of a cDNA clone isolated from the rat brain library (Housey et al., 1987) is apparently similar to, but clearly different from any of the 4 subspecies described above.

On the other hand, the α -subspecies appears to be ubiquitous and has been found in many tissues and cell types (McCaffrey et al., 1987). Although the present studies do not provide information as to the localization of the α -subspecies,⁵ a combination of biochemical and immunochemical procedures seems to indicate clear distinct regional and cellular patterns of expression of the several protein kinase C subspecies so far identified in the brain tissue.

Recent enzymological studies have indicated that these subspecies exhibit subtle individual characteristics, with slightly different modes of activation, kinetic properties, and substrate

⁵ The polyclonal antibody against the amino-terminal end sequence of α -subspecies is available. This antibody specifically recognizes α -subspecies of protein kinase C on immunoblot analysis, although a clear immunostaining pattern has not yet been obtained.

specificities (Huang et al., 1986; Ido et al., 1987; Jaken and Kiley, 1987; Sekiguchi et al., 1987). It is attractive to surmise, then, that, by virtue of their individual enzymological properties and differential expression in particular areas and cell types of the brain, each member of the protein kinase C family may have a defined function in processing and modulating the relay of sensory and motor information within the complex neuronal circuits. Determination of the precise physiological role of protein kinase C subspecies in the brain and in peripheral tissues, however, awaits identification of their true endogenous substrates.

References

- Brandt, S. J., J. E. Nidel, R. M. Bell, and W. S. Young, III (1987) Distinct patterns of expression of different protein kinase C mRNAs in rat tissues. *Cell* 49: 57–63.
- 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* 233: 859–866.
- Erlanger, B. F. (1980) The preparation of antigenic hapten-carrier conjugates: A survey. *Methods Enzymol.* 70: 85–104.
- Girard, P. R., G. J. Mazzei, J. G. Wood, and J. F. Kuo (1985) Polyclonal antibodies to phospholipid/ Ca^{2+} -dependent protein kinase and immunocytochemical localization of the enzyme in rat brain. *Proc. Natl. Acad. Sci. USA* 82: 3030–3034.
- Hashimoto, E., M. Takeda, Y. Nishizuka, K. Hamana, and K. Iwai (1976) Studies on the sites in histones phosphorylated by adenosine 3':5'-monophosphate-dependent protein kinases. *J. Biol. Chem.* 251: 6287–6293.
- Hashimoto, T., K. Ase, S. Sawamura, U. Kikkawa, N. Saito, C. Tanaka, and Y. Nishizuka (1988) Postnatal development of a brain-specific subspecies of protein kinase C in rat. *J. Neurosci.* 8: 1678–1683.
- Housey, G. M., C. A. O'Brian, M. D. Johnson, P. Kirschmeier, and I. B. Weinstein (1987) Isolation of cDNA clones encoding protein kinase C: Evidence for a protein kinase C-related gene family. *Proc. Natl. Acad. Sci. USA* 84: 1065–1069.
- Huang, K.-P., H. Nakabayashi, and F. L. Huang (1986) Isozymic forms of rat brain Ca^{2+} -activated and phospholipid-dependent protein kinase, *Proc. Natl. Acad. Sci. USA* 83: 8535–8539.
- Ido, M., K. Sekiguchi, U. Kikkawa, and Y. Nishizuka (1987) Phosphorylation of the EGF receptor from A431 epidermoid carcinoma cells by three distinct types of protein kinase C. *FEBS Lett.* 219: 215–218.
- Jaken, S., and S. C. Kiley (1987) Purification and characterization of three types of protein kinase C from rabbit brain cytosol. *Proc. Natl. Acad. Sci. USA* 84: 4418–4422.
- Kikkawa, U., and Y. Nishizuka (1986) The role of protein kinase C in transmembrane signalling. *Annu. Rev. Cell Biol.* 2: 149–178.
- Kikkawa, U., M. Go, J. Koumoto, and Y. Nishizuka (1986) Rapid purification of protein kinase C by high performance liquid chromatography. *Biochem. Biophys. Res. Commun.* 135: 636–643.
- Kikkawa, U., Y. Ono, K. Ogita, T. Fujii, Y. Asaoka, K. Sekiguchi, Y. Kosaka, K. Igarashi, and Y. Nishizuka (1987a) Identification of the structures of multiple subspecies of protein kinase C expressed in rat brain. *FEBS Lett.* 217: 227–231.
- Kikkawa, U., K. Ogita, Y. Ono, Y. Asaoka, M. S. Shearman, T. Fujii, K. Ase, K. Sekiguchi, K. Igarashi, and Y. Nishizuka (1987b) The common structure and activities of four subspecies of rat brain protein kinase C. *FEBS Lett.* 223: 212–216.
- Kitano, T., T. Hashimoto, U. Kikkawa, K. Ase, N. Saito, C. Tanaka, Y. Ichimori, K. Tsukamoto, and Y. Nishizuka (1987) Monoclonal antibodies against rat brain protein kinase C and their application to immunocytochemistry in nervous tissues. *J. Neurosci.* 7: 1520–1525.
- Knopf, J. L., M.-H. Lee, L. A. Sultzman, R. W. Kriz, C. R. Loomis, R. M. Hewick, and R. M. Bell (1986) Cloning and expression of multiple protein kinase C cDNAs. *Cell* 46: 491–502.
- Kubo, K., S. Ohno, and K. Suzuki (1987) Primary structure of human protein kinase C β I and β II differ only in their C-terminal sequences. *FEBS Lett.* 223: 138–142.
- Makowske, M., M. J. Birnbaum, R. Ballester, and O. M. Rosen (1986) A cDNA encoding protein kinase C identifies two species of mRNA in brain and GH₃ cells. *J. Biol. Chem.* 261: 13389–13392.
- McCaffrey, P. G., M. R. Rosner, U. Kikkawa, K. Sekiguchi, K., Ogita, K. Ase, and Y. Nishizuka (1987) Characterization of protein kinase C from normal and transformed cultured murine fibroblasts. *Biochem. Biophys. Res. Commun.* 146: 140–146.
- McLean, I. W., and P. K. Nakane (1974) Periodate-lysine-paraformaldehyde fixative, a new fixative for immunoelectron microscopy. *J. Histochem. Cytochem.* 22: 1077–1083.
- Mochly-Rosen, D., A. I. Basbaum, and D. E. Koshland, Jr. (1987) Distinct cellular and regional localization of immunoreactive protein kinase C in rat brain. *Proc. Natl. Acad. Sci. USA* 84: 4660–4664.
- Nishizuka, Y. (1986) Studies and perspectives of protein kinase C. *Science* 233: 305–312.
- Ohno, S., H. Kawasaki, S. Imajoh, K. Suzuki, M. Inagaki, H. Yokokura, T. Sakoh, and H. Hidaka (1987) Tissue-specific expression of three distinct types of rabbit protein kinase C. *Nature* 325: 161–166.
- Ono, Y., T. Kurokawa, K. Kawahara, O. Nishimura, R. Marumoto, K. Igarashi, Y. Sugino, U. Kikkawa, K. Ogita, and Y. Nishizuka (1986a) Cloning of rat brain protein kinase C complementary DNA. *FEBS Lett.* 203: 111–115.
- Ono, Y., T. Kurokawa, T. Fujii, K. Kawahara, K. Igarashi, U. Kikkawa, K. Ogita, and Y. Nishizuka (1986b) Two types of complementary DNAs of rat brain protein kinase C, heterogeneity determined by alternative splicing. *FEBS Lett.* 206: 347–352.
- Ono, Y., U. Kikkawa, K. Ogita, T. Fujii, T. Kurokawa, Y. Asaoka, K. Sekiguchi, K. Ase, K. Igarashi, and Y. Nishizuka (1987) Expression and properties of two types of protein kinase C: Alternative splicing from a single gene. *Science* 236: 1116–1120.
- Parker, P. J., L. Coussens, N. Totty, L. Rhee, S. Young, E. Chen, S. Stabel, M. D. Waterfield, and A. Ullrich (1986) The complete primary structure of protein kinase C—the major phorbol ester receptor. *Science* 233: 853–859.
- Pelosin, J.-M., I. Vilgrain, and E. M. Chambaz (1987) A single form of protein kinase C is expressed in bovine adrenocortical tissue, as compared to four chromatographically resolved isozymes in rat brain. *Biochem. Biophys. Res. Commun.* 147: 382–391.
- Saito, N., U. Kikkawa, Y. Nishizuka, and C. Tanaka (1988) Distribution of protein kinase C-like immunoreactive neurons in rat brain. *J. Neurosci.* 8: 369–382.
- Sekiguchi, K., M. Tsukuda, K. Ogita, U. Kikkawa, and Y. Nishizuka (1987) Three distinct forms of rat brain protein kinase C: Differential response to unsaturated fatty acids. *Biochem. Biophys. Res. Commun.* 145: 797–802.
- Wood, J. G., P. R. Girard, G. J. Mazzei, and J. F. Kuo (1986) Immunocytochemical localization of protein kinase C in identified neuronal compartments of rat brain. *J. Neurosci.* 6: 2571–2577.
- Worley, P. F., J. M. Baraban, and S. H. Snyder (1986a) Heterogeneous localization of protein kinase C in rat brain: Autoradiographic analysis of phorbol ester receptor binding. *J. Neurosci.* 6: 199–207.
- Worley, P. F., J. M. Baraban, E. B. De Souza, and S. H. Snyder (1986b) Mapping second messenger systems in the brain: Differential localizations of adenylate cyclase and protein kinase C. *Proc. Natl. Acad. Sci. USA* 83: 4053–4057.
- Worley, P. F., J. M. Baraban, C. Van Dop, E. J. Neer, and S. H. Snyder (1986c) G_o , a guanine nucleotide-binding protein: Immunocytochemical localization in rat brain resembles distribution of second messenger systems. *Proc. Natl. Acad. Sci. USA* 83: 4561–4565.
- Worley, P. F., J. M. Baraban, J. S. Colvin, and S. H. Snyder (1987) Inositol trisphosphate receptor localization in brain: Variable stoichiometry with protein kinase C. *Nature* 325: 159–161.