PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

The Heterogeneity and Differential Expression of Protein Kinase C in Nervous Tissues [and Discussion]

U. Kikkawa, K. Ogita, M. S. Shearman, K. Ase, K. Sekiguchi, Z. Naor, M. Ido, Y. Nishizuka, N. Saito, C. Tanaka, Y. Ono, T. Fujii, K. Igarashi, M. R. Hanley, Y. Nishizuka, A. Spat and M. Castagna

Phil. Trans. R. Soc. Lond. B 1988 **320**, 313-324
doi: 10.1098/rstb.1988.0079

Phil. Trans. R. Soc. Lond. B **320**, 313–324 (1988)
Printed in Great Britain

The heterogeneity and differential expression of protein kinase C in nervous tissues

By U. Kikkawa[1], K. Ogita[1], M. S. Shearman[1], K. Ase[1], K. Sekiguchi[1], Z. Naor[1], M. Ido[1], Y. Nishizuka[1], N. Saito[2], C. Tanaka[2], Y. Ono[3], T. Fujii[3] and K. Igarashi[3]

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

[Plate 1]

Protein kinase C exists as a large family of multiple subspecies with subtle individual characteristics. This heterogeneity comes from different genes as well as from different splicings of a single RNA transcript. The members of this family have closely related structures with a high degree of homology. Biochemical studies have shown that their mode of activation and kinetic and catalytic properties differ slightly from one another. By using a combination of biochemical and immunocytochemical techniques, their differential regional and cellular expression have been shown in the nervous tissues. Each member of this enzyme family may have a specialized function in transducing various physiological and pathological signals into different cell types.

1. Introduction

The physiological importance of protein kinase C is now widely accepted and well documented (Nishizuka 1986). Although it was once thought to be a single entity, it is now clear that the enzyme consists of a large family with multiple subspecies having closely related structures. In the brain tissue, at least seven, and probably more, subspecies are distinguished, and their structures have been deduced by analysis of their complementary DNA (cDNA) sequences. The structure and genetic identity of most of these subspecies have been determined by comparison with the enzyme fractions which were resolved by biochemical procedures. The evidence currently available strongly suggests that the members of this enzyme family show slightly different individual characteristics. Biochemical and immunocytochemical studies have indicated their different patterns of regional and cellular expression. The structural heterogeneity, kinetic and catalytic properties, and differential expression of some members of the protein kinase C family will be briefly described in this paper.

2. Structural heterogeneity

Protein kinase C was purified to apparent homogeneity from brain tissue in many laboratories. Recent sequence analysis of the cDNAs isolated from the bovine (Parker *et al.* 1986; Coussens *et al.* 1986), rat (Knopf *et al.* 1986; Ono *et al.* 1986; Housey *et al.* 1987), rabbit (Ohno *et al.* 1987) and human (Coussens *et al.* 1986) brain libraries and human spleen library (Kubo *et al.* 1987) has predicted the complete primary sequences of four subspecies of protein

kinase C (α, βI, βII, and γ). This integrated nomenclature used in this article, and its explanation, are as given elsewhere (Ono *et al.* 1987*b*; Kikkawa *et al.* 1987*b*). Figure 1 summarizes the complete sequences of the four subspecies of rat brain protein kinase C. Partial genomic analysis has shown that βI- and βII-subspecies are derived from alternative splicing of a single RNA transcript (Ono *et al.* 1987*b*), and differ from each other only in a short sequence of about 50 amino acid residues in their carboxy-terminal regions. The sequences of each subspecies in different animals are remarkably similar. More recently, an additional three subspecies of this family, termed δ, ε, and ζ, have been predicted to occur in the rat brain tissue (Ono *et al.* 1987*a*).

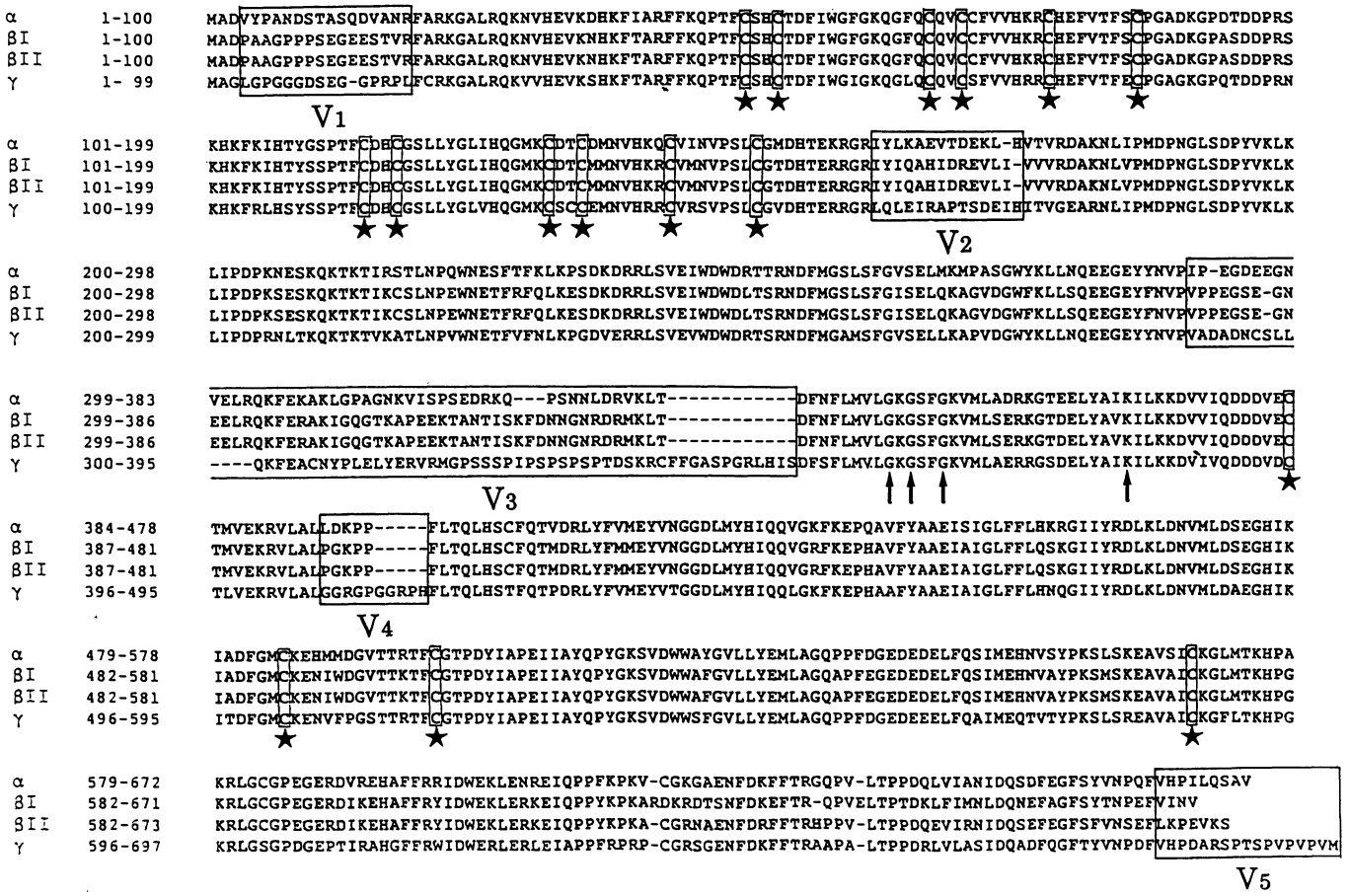

FIGURE 1. Complete primary structures of α-, βI-, βII-, and γ-subspecies of rat brain protein kinase C. Amino acids are shown by one-letter abbreviations. The sequences in the boxes indicate the variable regions. Stars indicate cysteine residues commonly present in the four subspecies. Arrows show an ATP-binding site. More details are described elsewhere (Kikkawa *et al.* 1987*a*).

As schematically given in figure 2, α-, βI-, βII-, and γ-subspecies consist of a single polypeptide chain with four conserved (C_1–C_4) and five variable (V_1–V_5) regions. The two conserved regions C_3 and C_4, which are located in the carboxy-terminal half, appear to be essential for the catalytic activity, and the region C_3 contains an ATP-binding sequence, GXGXXG-----K, where G, K, and X represent glycine, lysine, and any amino acid, respectively. This sequence is commonly found in many protein kinases. On the other hand,

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

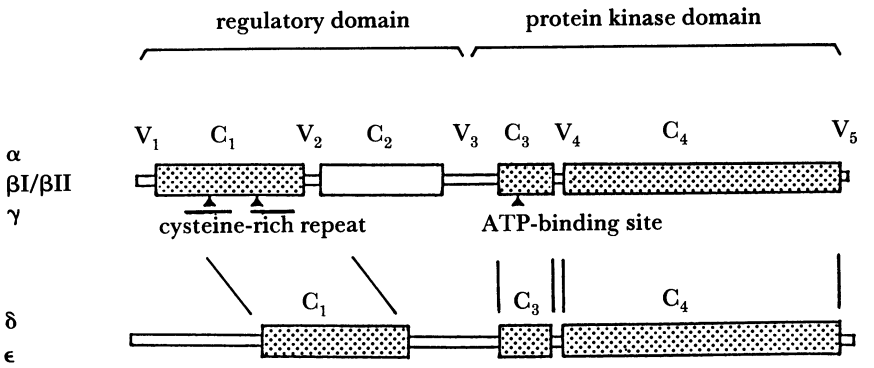

FIGURE 2. Schematic representation of subspecies of rat brain protein kinase C family. C_1–C_4 (thick boxes) and V_1–V_5 (thin boxes) indicate conserved and variable regions, respectively.

the conserved regions C_1 and C_2 in the amino-terminal half presumably compose the regulatory domain, that interacts with Ca^{2+}, phospholipid, and diacylglycerol. The first conserved region C_1 contains a tandem repeat of a cysteine-rich sequence, $CX_2CX_{13}CX_2CX_7CX_7C$, where C is cysteine and X represents any amino acid. This sequence agrees with the consensus of a 'cysteine–zinc DNA-binding finger', which is found in many metalloproteins and DNA-binding proteins that are related to transcriptional regulation (Berg 1986).

In comparison, the three newly added members of the protein kinase C family, the δ-, ε- and ζ-subspecies, show closely related but different structures (Ono *et al.* 1987*a*). The three subspecies also contain the characteristic cysteine-rich sequence, $CX_2CX_{13(14)}CX_2CX_7CX_7C$, in the amino-terminal half of the enzyme molecules, although the ζ-subspecies has only one such sequence. At present, no indication is available that any protein kinase C molecule binds to DNA under physiological conditions, but the conservation of this cysteine-rich sequence in all members of the protein kinase C family suggests their potential role in the control of gene expression.

It is worth noting that this second group of the enzyme family does not contain the conserved region C_2, but the sequences of all protein kinase domains are highly homologous to one another. The ATP-binding sequence, GXGXXG-----K, is commonly observed in these subspecies, except for ζ-subspecies in which one of these glycine residues is replaced by alanine. Table 1 summarizes the number of amino acid residues and molecular masses of these

TABLE 1. PROTEIN KINASE C SUBSPECIES OF RAT BRAIN DEDUCED FROM COMPLEMENTARY DNA SEQUENCE ANALYSIS

(Detailed experimental results are described elsewhere (Ono *et al.* 1987*a*, *b*; Kikkawa *et al.* 1987*a*, *b*).)

protein kinase C subspecies	amino acid residues	molecular mass / Da
α	672	76799
βI	671	76790
βII	673	76933
γ	697	78366
δ	673	77517
ε	737	83474
ζ[a]	>492	—

[a] The complete sequence has not been determined.

subspecies deduced from their cDNA sequence analysis. The primary amino acid sequences of some of these members of the protein kinase C family have been described (Kikkawa *et al.* 1987*a*; Ono *et al.* 1986). The cDNA for the ζ-subspecies that is currently available does not contain the complete reading frame: a part of the amino-terminal region is lacking. Nevertheless, the results so far obtained indicate that each subspecies is encoded by a different gene, except for the βI- and βII-subspecies, which result from alternative splicing of a single RNA transcript as mentioned above. The complete gene structure has not yet been determined for any of these subspecies. On the other hand, one species of *Drosophila* protein kinase C has been sequenced, which shows roughly 60% homology to the mammalian enzymes (Rosenthal *et al.* 1987).The gene of this insect enzyme spans about 20 kilobases (kb) and contains at least 14 exons.

3. Kinetic and catalytic heterogeneity

Direct enzymic analysis of each subspecies is a prerequisite essential to determine the physiological roles of this enzyme family. Recent analysis of this enzyme family by hydroxyapatite column chromatography has revealed that the apparently homogeneous enzyme previously purified from rat brain can be resolved into three subfractions, types I, II and III (Huang *et al.* 1986; Ono *et al.* 1987*b*; Kikkawa *et al.* 1987*b*). Comparison of these subfractions with the subspecies of the enzyme which have been individually expressed in COS 7 cells has allowed identification of the primary structure of each type isolated from the rat brain, as shown in figure 3. Type I corresponds to the γ-subspecies. Type II is an unequal mixture of βI- and βII-subspecies, which differ from each other only in a short range of the carboxy-terminal end regions. Type III corresponds to the α-subspecies, although this type is apparently heterogeneous. Thus far, βI- and βII-subspecies have not been separated by conventional enzyme fractionation procedures, but can be distinguished from each other by immunochemical methods (Shearman *et al.* 1987). These two subspecies separately expressed in COS 7 cells show identical kinetic properties (Ono *et al.* 1987*b*).

The positions in the elution profile from hydroxyapatite columns of the newly added members of the protein kinase C family, the δ- and ε-subspecies, remain unknown. Both subspecies, which were expressed in COS 7 cells, phosphorylate calf thymus H1 histone as a substrate protein (Ono *et al.* 1987*a*). As described in detail elsewhere (Kikkawa *et al.* 1986), protein kinase C in the rat brain shows major and minor peaks at an early step of purification. The major peak, which is normally employed for further purification, contains α-, βI-, βII-, and γ-subspecies. Further analysis is underway to isolate and identify the new subspecies of the enzyme family in the rat brain tissue.

Although the structural and genetic identities of the subspecies, and their correspondence to the available enzyme subfractions, have not yet been fully clarified, as discussed above, the biochemically fractionated enzyme types I, II and III from the rat brain do exhibit slightly different modes of activation, kinetic properties, and substrate specificities. For instance, 1-stearoyl-2-arachidonylglycerol, the major species of diacylglycerol derived from inositol phospholipids, is most active at low concentrations for type II (βI- and βII-subspecies) and type III (α-subspecies), whereas synthetic cell-permeable diacylglycerols, such as 1,2-didecanoylglycerol and 1,2-dioctanoylglycerol, are effective with the type I enzyme (γ-subspecies) but less effective with type II and III enzymes on an equivalent molar basis. Although less sensitive to 1-stearoyl-2-arachidonylglycerol, type I enzyme is significantly

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

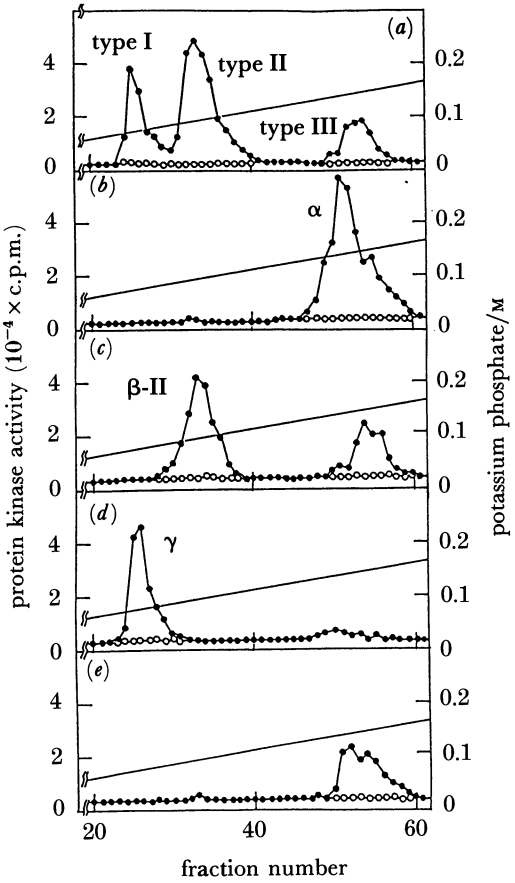

FIGURE 3. Expression of cDNAs of protein kinase C subspecies in COS 7 cells. Purified preparation of rat brain protein kinase C, and the enzymes newly expressed in COS 7 cells which were transfected with cDNA-containing plasmids, were extracted and applied to hydroxyapatite column chromatography. The enzymes were assayed with calf thymus H1 histone as phosphate acceptor in the presence of phosphatidylserine, diolein, and $CaCl_2$ under the conditions specified earlier (Kikkawa *et al.* 1986). (*a*) Rat brain enzyme; the enzyme was resolved into three subfractions, type I, II, and III. (*b*) The enzyme from the COS 7 cells transfected with an α-cDNA-containing plasmid. (*c*) The enzyme from the COS 7 cells transfected with βII-cDNA-containing plasmids. A similar profile was obtained with a βI-cDNA-containing plasmid. (*d*) The enzyme from the COS 7 cells transfected with a γ-cDNA-containing plasmid. (*e*) The enzyme from non-transfected (control) COS 7 cells. The cells normally contain type III enzyme, which corresponds to the α-subspecies. Filled circles, protein kinase activity assayed in the presence of phosphatidylserine, diolein, and $CaCl_2$; open circles, protein kinase activity assayed in the presence of EGTA instead of phosphatidylserine, diolein, and $CaCl_2$; solid line, potassium phosphate. The detailed experimental conditions are described elsewhere (Kikkawa *et al.* 1987*b*).

activated by low concentrations of free arachidonic acid, as shown in figure 4 (Sekiguchi *et al.* 1987). The type II enzyme exhibits substantial activity without elevated Ca^{2+} levels, and responds to some extent to arachidonic acid. In contrast, the type III enzyme shows nearly full activity in the presence of higher concentrations of arachidonic acid. The activation of protein kinase C by free arachidonic acid does not require phospholipids. Definitive evidence for this mode of activation under physiological conditions, however, is unavailable at present, even though this possibility was first pointed out by McPhail *et al.* (1984) and subsequently by Murakami & Routtenberg (1985). Some of the members of the protein kinase C family presumably exhibit enzymatic activity with the series of metabolic products of membrane phospholipids, such as diacylglycerol, arachidonic acid, and its metabolites such as lipoxin A

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

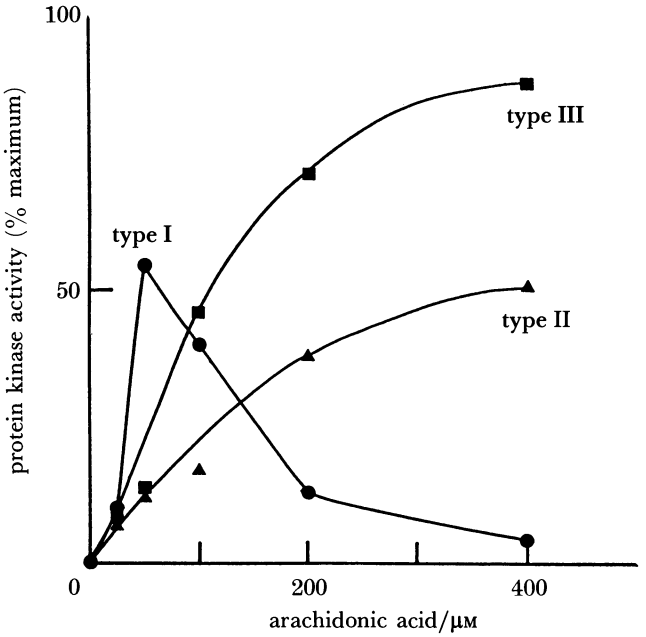

Figure 4. Activation of type I, II, and III protein kinase C by arachidonic acid. The enzyme subspecies were assayed in the presence of 0.3 mM $CaCl_2$ at various concentrations of free arachidonic acid. Neither phospholipid nor diacylglycerol was added. Results are normalized to the maximum activity obtained in the presence of phosphatidylserine, diolein, and $CaCl_2$. The detailed experimental conditions are described elsewhere (Sekiguchi *et al.* 1987).

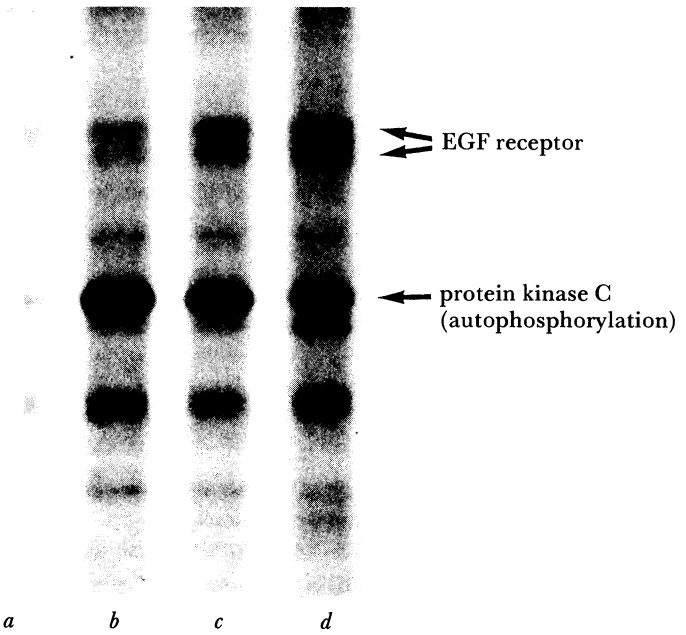

Figure 5. Phosphorylation of the EGF receptor of A431 cells by three types of protein kinase C. The membrane of A431 cells was prepared and phosphorylated *in vitro* by three types of rat brain protein kinase C. The membrane proteins were separated on sodium dodecyl sulphate–polyacrylamide gel electrophoresis, and the radioactive proteins were visualized by autoradiography. (*a*) Results without added protein kinase C; (*b*) with type I protein kinase C; (*c*) with type II protein kinase C; (*d*) with type III protein kinase C. The 170 kDa and 150 kDa proteins are the EGF receptor proteins: the latter is probably a proteolytic product of the former. The autophosphorylation of the enzyme molecule itself is much faster for the type I enzyme. The detailed experimental conditions are described elsewhere (Ido *et al.* 1987). Some difference between the three enzyme subfractions in their pattern of autophosphorylation has also been reported recently by Huang *et al.* (1986) and by Jaken & Kiley (1987).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

(Hansson *et al.* 1986), that appear successively after receptor stimulation. The various members of the protein kinase C family are also sensitive to the spectrum of Ca^{2+} levels that may result from several signal-induced mechanisms. The heterogeneity of the protein kinase C family is consistent with the early observation made by Blumberg and his colleagues (Dunn *et al.* 1985) who reported heterogeneity of the tumour-promoting phorbol ester-binding protein.

It is a general problem in protein phosphorylation research, that following disruption of the cell most protein kinases show activity and phosphorylate many physiological and non-physiological substrate proteins. Although a number of proteins, including cell surface receptors, enzymes and other proteins, have been reported to serve as the phosphate acceptors of protein kinase C in cell-free systems, it is difficult to assess the physiological significance of these proposed reactions (for review, see Nishizuka 1986). In addition, most earlier efforts to clarify the target proteins of this enzyme family have been made by using a mixture of several protein kinase C subspecies. Preliminary studies suggest that several protein kinase C subspecies each show preferences for different substrate proteins. For instance, the EGF receptor of A431 epidermoid carcinoma cells is phosphorylated most rapidly by the type III enzyme that is found in many tissues and cell types, but very slowly by the type I enzyme that is found only in central nervous tissues, as shown in figure 5 (Ido *et al.* 1987). Further enzymic analysis of these subspecies is needed to clarify the precise physiological role of each member of this enzyme family.

4. Differential expression

Studies by Worley *et al.* (1986), using autoradiographic procedures with tritiated phorbol-12,13-dibutyrate, have shown an uneven distribution of protein kinase C in the brain with high concentrations in the hippocampus, the substantia nigra, and the cerebellar and cerebral cortices. Northern blot analysis with specific oligonucleotide probes suggested tissue-specific expression of some subspecies of this protein kinase family (Coussens *et al.* 1986; Knopf *et al.* 1986; Ohno *et al.* 1987). More recently, using *in situ* hybridization histochemistry with oligonucleotide probes, Brandt *et al.* (1987) have shown a distinct distribution of the transcripts of the enzyme subspecies in the rat brain and spleen. On the other hand, immunohistochemical staining with antibodies was first applied to identify protein kinase C in the brain tissue by Wood *et al.* (1986). This study has been extended by Mochly-Rosen *et al.* (1987), who have shown, by using three monoclonal antibodies, different regional distributions of apparently distinct protein kinase C subspecies in the rat brain.

By means of a combination of biochemical, immunological, and cytochemical techniques with subspecies-specific antibodies, the relative activity as well as the individual pattern of expression of multiple subspecies of the enzyme has been extensively assessed in our laboratories (Kikkawa *et al.* 1987*a*, *b*; Kitano *et al.* 1987; Shearman *et al.* 1987; Saito *et al.* 1988). The cerebrum contains a large quantity of type II enzyme, particularly βII-subspecies, whereas the cerebellum contained a marked activity of type I enzyme (γ-subspecies). Type II is an unequal mixture of βI- and βII-subspecies as determined by immunochemical procedures with subspecies-specific antibodies (Shearman *et al.* 1987). Table 2 shows the regional acitivity of the four subspecies in the rat brain soluble fractions.

The type I enzyme with a γ-sequence is expressed postnatally (figure 6). This subspecies is found only in the brain and spinal cord, but not in other tissues so far examined, including peripheral nerves (Shearman *et al.* 1987; Hashimoto *et al.* 1988). The highest specific activity

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

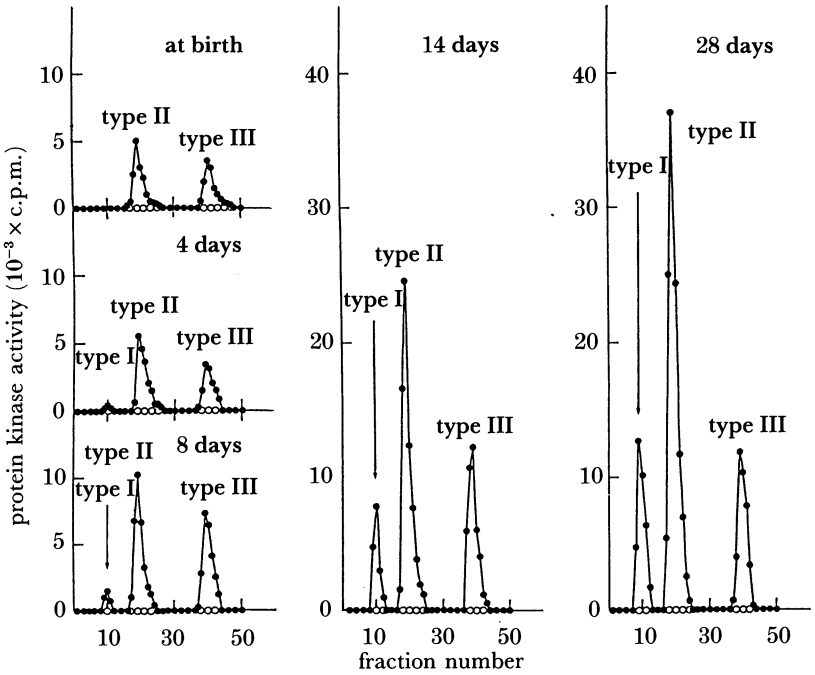

Figure 6. Postnatal development of several types of protein kinase C in rat brain. The enzyme was assayed with calf thymus H1 histone as phosphate acceptor; the activity is given as the value equivalent to milligrams of protein in the crude extract of developing rat brain. The enzyme was fractionated by chromatography on a hydroxyapatite column which was connected to a high performance liquid chromatography system as described by Kikkawa *et al.* (1987*b*). (Symbols as for figure 3.) Other detailed experimental conditions are as described elsewhere (Hashimoto *et al.* 1988).

TABLE 2. REGIONAL DISTRIBUTION OF PROTEIN KINASE C SUBSPECIES IN RAT BRAIN

(The protein kinase C subspecies (types I, II, and III) were separated by chromatography on a hydroxyapatite column, which was connected to a high performance liquid chromatography system. The relative ratio of βI- and βII-subspecies in the type II fraction was estimated by immunochemical procedures with subspecies-specific antibodies. The detailed procedures have been described elsewhere (Shearman *et al.* 1987). Specific activity is defined as picomoles of radioactive phosphate from [γ-^{32}P]ATP into calf thymus H1 histone per minute per milligram wet mass of tissue under the standard conditions specified (Kikkawa *et al.* 1987*a*, *b*).)

	specific activity			
nervous tissue	α	βI	βII	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

of this subspecies is found in the cerebellar cortex, particularly in the cell bodies, dendrites and axons of Purkinje cells as well as in the pyramidal cells of the hippocampus. Presumably this subspecies plays a role in modulating some specialized neuronal functions, such as long-term potentiation and depression (Lovinger *et al.* 1986).

Normally, the activity of βII-subspecies far exceeds that of βI-subspecies, and the activity of the latter in the cerebrum and hippocampus is very low. Immunocytochemical analysis has indicated that βI- and βII-subspecies show a clearly different cellular expression (Ase *et al.* 1988). In the rat cerebellar cortex, for instance, βI-subspecies is localized mainly in the granular layer, whereas βII-subspecies is found predominantly in the molecular layer, most

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

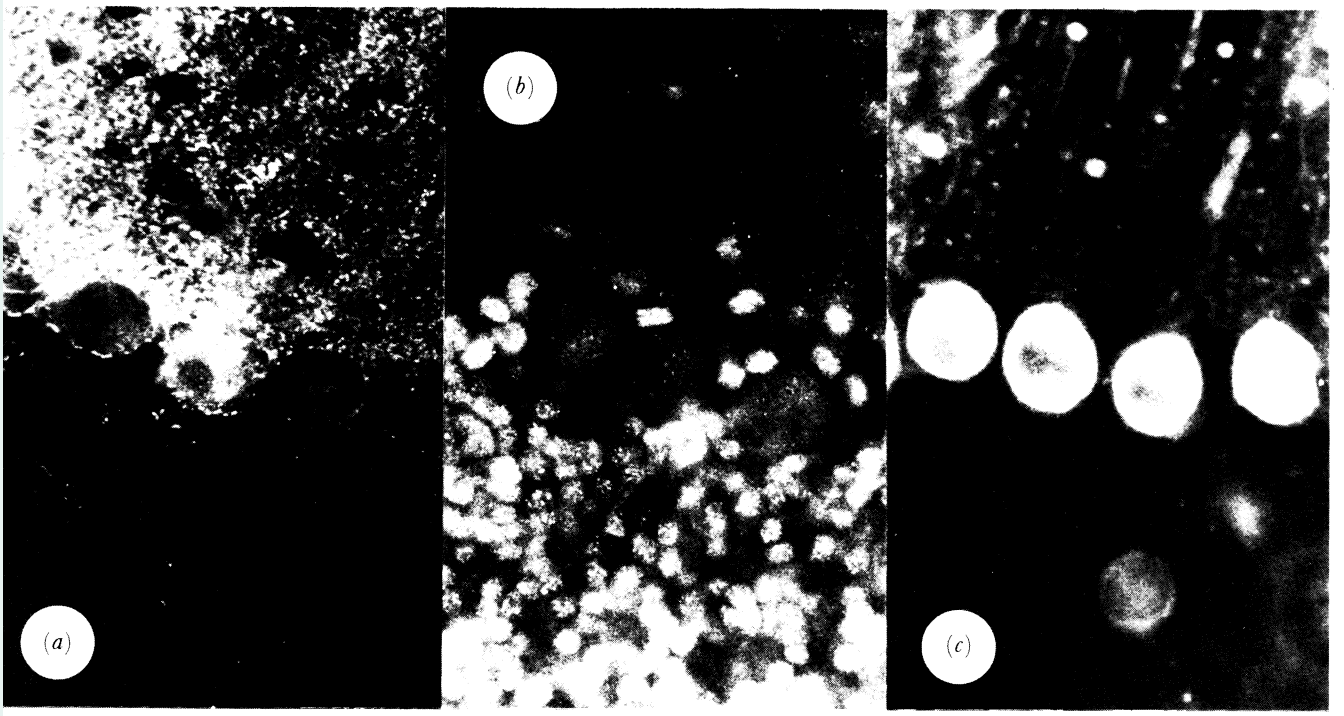

Figure 7. Fluorescent micrographs of the rat cerebellar cortex stained with the specific antibodies against protein kinase C subspecies. The upper portion illustrates the molecular layer, the middle portion represents Purkinje cells, and the lower portion shows the granular layer. (*a*) Cortex stained with antibodies against βII-subspecies. Terminal-like structures are observed in the molecular layer and surrounding Purkinje cells. (*b*) Stained with antibodies against βI-subspecies. Granule cells are heavily stained but no immunoreaction is seen in the molecular layer or Purkinje cells. (*c*) Stained with antibodies against γ-subspecies. Perikarya, dendrites, and axons of Purkinje cells are heavily stained. The detailed experimental conditions are described elsewhere (Ase *et al.* 1988).

(*Facing p.* 320)

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

apparently in the presynaptic nerve endings which terminate at Purkinje cells as shown in figure 7 (plate 1). These distribution patterns are in sharp contrast to that of γ-subspecies which is most abundant within Purkinje cells as mentioned above.

On the other hand, protein kinase C with the α-sequence (type III) is found in many tissues and cell types so far examined (Ido *et al.* 1987; Kikkawa *et al.* 1987*b*; McCaffrey *et al.* 1987; Pelosin *et al.* 1987). Although the precise patterns of expression of other subspecies have not yet been clarified, the results thus far obtained suggest that the multiple subspecies of this enzyme family each play specific roles in modulating the physiological functions of particular cell types.

5. Conclusions

Early studies in this laboratory have proposed that protein kinase C activation interacts synergistically with Ca^{2+} mobilization to induce a variety of cellular responses to external stimuli that provoke the hydrolysis of inositol phospholipids (Nishizuka 1984). The biochemical basis of this synergistic action, however, is not fully understood. Nevertheless, by using direct activators of protein kinase C, such as cell-permeable diacylglycerol and tumour-promoting phorbol esters, several physiological functions have been assigned to this enzyme, including involvement in secretion and exocytosis, modulation of ion conductance, interaction and down-regulation of various receptors, smooth muscle contraction, gene expression and cell proliferation (for review, see Nishizuka 1986).

On the other hand, protein kinase C appears to show a dual action, and exerts feedback control on the Ca^{2+}-signalling pathway. For instance, protein kinase C may inhibit the receptor-mediated breakdown of inositol phospholipids. The enzyme has been proposed to stimulate inositol trisphosphatase which degrades inositol 1,4,5-trisphosphate, thus removing the second messenger responsible for mobilization of Ca^{2+} from its internal store (Connolly *et al.* 1986). A number of reports have appeared to suggest that this enzyme also activates the Ca^{2+}-transport ATPase and Na^{+}–Ca^{2+} exchanger which lower cytosolic Ca^{2+} concentrations (for review, see Nishizuka 1986). Several lines of evidence suggest that protein kinase C may have crucial roles in modulating, both positively and negatively, many Ca^{2+}-dependent cellular processes. The structural heterogeneity and differential regional expression of this enzyme family open a new vista in the research of signal transduction. Our preliminary results indicate that the protein kinase C family consists of a much larger number of subspecies than those described above. Their functional roles in physiological and pathological responses will be clarified in molecular terms by further investigations.

The investigations were supported in part by research grants from the Research Fund of the Ministry of Education, Science and Culture, Japan; Muscular Dystrophy Association; Yamanouchi Foundation for Research on Metabolic Disorders; Merck Sharp & Dohme Research Laboratories; and Meiji Institute of Health Sciences.

References

Ase, K., Saito, N., Shearman, M. S., Kikkawa, U., Ono, Y., Igarashi, K., Tanaka, C. & Nishizuka, Y. 1988 Distinct cellular expression of βI- and βII-subspecies of protein kinase C in rat cerebellum. *J. Neurosci.* (In the press.)

Berg, J. M. 1986 Potential metal-binding domains in nucleic acid binding proteins. *Science, Wash.* **232**, 485–487.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Brandt, S. J., Niedel, J. E., Bell, R. M. & Young, W. S. III 1987 Distinct patterns of expression of different protein kinase C mRNAs in rat tissues. *Cell* **49**, 57–63.

Connolly, T. M., Lawing W. J. Jr. & Majerus, P. W. 1986 Protein kinase C phosphorylates human platelet inositol trisphosphate 5′-phosphomonoesterase, increasing the phosphatase activity. *Cell* **46**, 951–958.

Coussens, L., Parker, P. J., Rhee, L., Yang-Feng, T. L., Chen, E., Waterfield, M. D., Francke, U. & Ullrich, A. 1986 Multiple, distinct forms of bovine and human protein kinase C suggest diversity in cellular signaling pathways. *Science, Wash.* **233**, 859–866.

Dunn, J. A., Jeng, A. Y., Yuspa, S. H. & Blumberg, P. M. 1985 Heterogeneity of [^{3}H]phorbol 12,13-dibutyrate binding in primary mouse keratinocytes at different stages of maturation. *Cancer Res.* **45**, 5540–5546.

Hansson, A., Serhan, C. N., Haeggström, J., Ingelman-Sundberg, M. & Samuelsson, B. 1986 Activation of protein kinase C by lipoxin and other eicosanoids. Intracellular action of oxygenation products of arachidonic acid. *Biochem. biophys. Res. Commun.* **134**, 1215–1222.

Hashimoto, T., Ase, K., Sawamura, S., Kikkawa, U., Saito, N., Tanaka, C. & Nishizuka, Y. 1988 Postnatal development of a brain specific protein kinase C in rat. *J. Neurosci.* (In the press.)

Housey, G. M., O'Brian, C. A., Johnson, M. D., Kirschmeier, P. & Weinstein, I. B. 1987 Isolation of cDNA clones encoding protein kinase C: evidence for a protein kinase C-related gene family. *Proc. natn. Acad. Sci. U.S.A.* **84**, 1065–1069.

Huang, K.-P., Nakabayashi, H. & Huang, F. L. 1986 Isozymic forms of rat brain Ca^{2+}-activated and phospholipid-dependent protein kinase. *Proc. natn. Acad. Sci. U.S.A.* **83**, 8535–8539.

Ido, M., Sekiguchi, K., Kikkawa, U. & Nishizuka, Y. 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. & Kiley, S. C. 1987 Purification and characterization of three types of protein kinase C from rabbit brain cytosol. *Proc. natn. Acad. Sci. U.S.A.* **84**, 4418–4422.

Kikkawa, U., Go, M., Koumoto, J. & Nishizuka, Y. 1986 Rapid purification of protein kinase C by high performance liquid chromatography. *Biochem. biophys. Res. Commun.* **135**, 636–643.

Kikkawa, U., Ogita, K., Ono, Y., Asaoka, Y., Shearman, M. S., Fujii, T., Ase, K., Sekiguchi, K., Igarashi, K. & Nishizuka, Y. 1987*a* The common structure and activities of four subspecies of rat brain protein kinase C. *FEBS Lett.* **223**, 212–216.

Kikkawa, U., Ono, Y., Ogita, K., Fujii, T., Asaoka, Y., Sekiguchi, K., Kosaka, Y., Igarashi, K. & Nishizuka, Y. 1987*b* Identification of the structures of multiple subspecies of protein kinase C expressed in rat brain. *FEBS Lett.* **217**, 227–231.

Kitano, T., Hashimoto, T., Kikkawa, U., Ase, K., Saito, N., Tanaka, C., Ichimori, Y., Tsukamoto, K. & Nishizuka, Y. 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., Lee, M.-H., Sultzman, L. A., Kriz, R. W., Loomis, C. R., Hewick, R. M. & Bell, R. M. 1986 Cloning and expression of multiple protein kinase C cDNAs. *Cell* **46**, 491–502.

Kubo, K., Ohno, S. & Suzuki, K. 1987 Primary structure of human protein kinase C βI and βII differ only in their C-terminal sequences. *FEBS Lett.* **223**, 138–142.

Lovinger, D. M., Colley, P. A., Aker, R. F., Nelson, R. B. & Routtenberg, A. 1986 Direct relation of long-term synaptic potentiation to phosphorylation of membrane protein F_1, a substrate for membrane protein kinase C. *Brain Res.* **399**, 205–211.

McCaffrey, P. G., Rosner, M. R., Kikkawa, U., Sekiguchi, K., Ogita, K., Ase, K. & Nishizuka, Y. 1987 Characterization of protein kinase C from normal and transformed cultured murine fibroblasts. *Biochem. biophys. Res. Commun.* **146**, 140–146.

McPhail, L. C., Clayton, C. C. & Snyderman, R. 1984 A potential second messenger role for unsaturated fatty acids: activation of Ca^{2+}-dependent protein kinase. *Science, Wash.* **224**, 622–625.

Mochly-Rosen, D., Basbaum, A. I. & Koshland D. E. Jr. 1987 Distinct cellular and regional localization of immunoreactive protein kinase C in rat brain. *Proc. natn. Acad. Sci. U.S.A.* **84**, 4660–4664.

Murakami, K. & Routtenberg, A. 1985 Direct activation of purified protein kinase C by unsaturated fatty acids (oleate and arachidonate) in the absence of phospholipids and Ca^{2+}. *FEBS Lett.* **192**, 189–193.

Nishizuka, Y. 1984 The role of protein kinase C in cell surface signal transduction and tumour promotion. *Nature, Lond.* **308**, 693–697.

Nishizuka, Y. 1986 Studies and perspectives of protein kinase C. *Science, Wash.* **233**, 305–312.

Ohno, S., Kawasaki, H., Imajoh, S., Suzuki, K., Inagaki, M., Yokokura, H., Sakoh, T. & Hidaka, H. 1987 Tissue-specific expression of three distinct types of rabbit protein kinase C. *Nature, Lond.* **325**, 161–166.

Ono, Y., Fujii, T., Ogita, K., Kikkawa, U., Igarashi, K. & Nishizuka, Y. 1987*a* Identification of three additional members of rat protein kinase C family: δ-, ε-, and ζ-subspecies. *FEBS Lett.* **266**, 125–128.

Ono, Y., Kikkawa, U., Ogita, K., Fujii, T., Kurokawa, T., Asaoka, Y., Sekiguchi, K., Ase, K., Igarashi, K. & Nishizuka, Y. 1987*b* Expression and properties of two types of protein kinase C: alternative splicing from a single gene. *Science, Wash.* **236**, 1116–1120.

Ono, Y., Kurokawa, T., Fujii, T., Kawahara, K., Igarashi, K., Kikkawa, U., Ogita, K. & Nishizuka, Y. 1986 Two types of complementary DNAs of rat brain protein kinase C, heterogeneity determined by alternative splicing. *FEBS Lett.* **206**, 347–352.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

Parker, P. J., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M. D. & Ullrich, A. 1986 The complete primary structure of protein kinase C-the major phorbol ester receptor. *Science, Wash* **233**, 853–859.

Pelosin, J.-M., Vilgrain, I. & Chambaz, E. M. 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.

Rosenthal, A., Rhee, L., Yadegari, R., Paro, R., Ullrich, A. & Goeddel, D. V. 1987 Structure and nucleotide sequence of a *Drosophila melanogaster* protein kinase C gene. *EMBO J.* **6**, 433–441.

Saito, N., Kikkawa, U., Nishizuka, Y. & Tanaka, C. 1988 Distribution of protein kinase C-like immunoreactive neurons in rat brain. *J. Neurosci.* **8**, 369–382.

Sekiguchi, K., Tsukuda, M., Ogita, K., Kikkawa, U. & Nishizuka, Y. 1987 Three distinct forms of rat brain protein kinase C: differential response to unsaturated fatty acids. *Biochem. biophys. Res. Commun.* **145**, 797–802.

Shearman, M. S., Naor, Z., Kikkawa, U. & Nishizuka, Y. 1987 Differential expression of multiple protein kinase C subspecies in rat central nervous tissue. *Biochem. biophys. Res. Commun.* **147**, 911–919.

Wood, J. G., Girard, P. R., Mazzei, G. J. & Kuo, J. F. 1986 Immunocytochemical localization of protein kinase C in identified neuronal compartments of rat brain. *J. Neurosci.* **6**, 2571–2577.

Worley, P. F., Baraban, J. M. & Snyder, S. H. 1986 Heterogeneous localization of protein kinase C in rat brain: autoradiographic analysis of phorbol ester receptor binding. *J. Neurosci.* **6**, 199–207.

Discussion

M. R. Hanley (*University of Cambridge Medical School, Cambridge, U.K.*). I should like to offer the observation that we have a cell line model of the function and regulation of the γ-species of protein kinase C. The NG115-401L neural line (see Hanley *et al.*, this symposium) has been analysed for protein kinase C expression by Northern blots of poly(A^+) RNA with a consensus probe for protein kinase C species derived from the N-terminal region. From using this probe (M. Dreher & M. Hanley, unpublished observations), it appears that the 401L line makes a major transcript of 3.5 kb and a minor transcript of 2.9 kb, which are 'protein kinase C-like'. Compared with the transcript patterns of identified protein kinase C species, the simplest interpretation is that this line expresses the γ-species and not the α- or β-species of protein kinase C (see Coussens *et al.* 1986). This compels special attention because this is the enzymic species that Professor Nishizuka has suggested may not be regulated by diglyceride, but can be activated by arachidonic acid. Our evidence supports the idea that this form is not physiologically regulated by diglyceride in that we can find no biological activities of a variety of synthetic and natural diacylglycerol analogues, whereas phorbol diesters and structurally-unrelated tumour promoters have several of the expected activities (e.g. activation of the sodium-proton antiporter). Nevertheless, 401L cell stimulation by bradykinin has functional consequences which can be mimicked selectively by tumour promoters, such as activation of the sodium-proton antiporter, which implies that there is some mode of coupling between inositol lipid breakdown events and the gamma form of protein kinase C. It remains to be seen whether this coupling is provided by arachidonic acid or a related metabolite.

Y. Nishizuka. I thank Dr Hanley for his very interesting information. We have not checked the protein kinase C expression pattern of NG115-401L cells, but our direct enzyme analysis, which is a more reliable index than Northern blots, has shown that the NIE-115 (neuroblastoma) cell-line contains both β- and α-subspecies but not γ-subspecies. So far we have not found any cultured cell lines which express γ-subspecies.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

A. Spät (*Semmelweis University Medical School, Budapest, Hungary*). In view of the possibility that translocation of protein kinase C from the cytoplasm to the membrane is an artefact, what kind of approach does Professor Nishizuka recommend to estimate the effect of a hormone on protein kinase C enzyme activity?

Y. Nishizuka. This question is very important and serious, but we have no definitive answer. Even if the translocation of protein kinase C from the soluble cytosol to membranes is a physiological artefact, this phenomenon may still provide a practical indication for the protein kinase C activation. But the translocation *per se* may not be the proof. Ideally, I think, phosphorylation of some specific proteins, for instance 47 kDa protein in platelets, should be measured.

M. Castagna (*Institut de Recherches Scientifiques sur le Cancer, Villejuif Cedex, France*). We have shown that, like arachidonic acid, retinoic acid was able to activate the protein kinase C α form.

Because β_1 and β_2 forms do not appear to have a nuclear localization, and because the γ form occurs only in neural tissue, could Professor Nishizuka say that the α form is responsible for effects on gene expression?

Y. Nishizuka. We do not know the answer. Our biochemical analysis indicates that the protein kinase C α subspecies appears to be expressed commonly in tissues and cell types, and the EGF receptor is more preferentially phosphorylated by this subspecies. The α subspecies is most likely to be involved in the control of cell proliferation and gene expression.

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

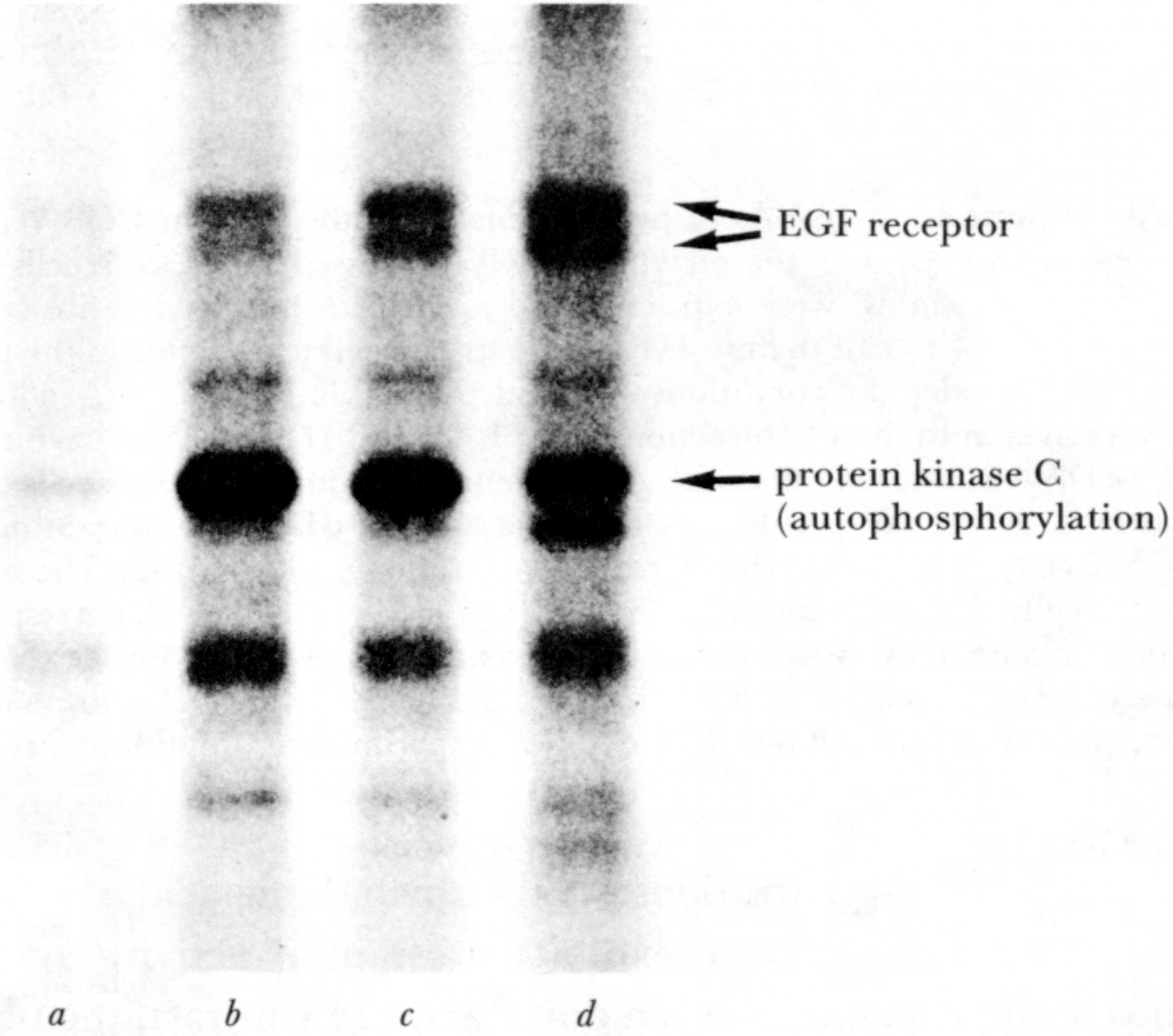

Figure 5. Phosphorylation of the EGF receptor of A431 cells by three types of protein kinase C. The membrane of A431 cells was prepared and phosphorylated *in vitro* by three types of rat brain protein kinase C. The membrane proteins were separated on sodium dodecyl sulphate–polyacrylamide gel electrophoresis, and the radioactive proteins were visualized by autoradiography. (*a*) Results without added protein kinase C; (*b*) with type I protein kinase C; (*c*) with type II protein kinase C; (*d*) with type III protein kinase C. The 170 kDa and 150 kDa proteins are the EGF receptor proteins: the latter is probably a proteolytic product of the former. The autophosphorylation of the enzyme molecule itself is much faster for the type I enzyme. The detailed experimental conditions are described elsewhere (Ido *et al.* 1987). Some difference between the three enzyme subfractions in their pattern of autophosphorylation has also been reported recently by Huang *et al.* (1986) and by Jaken & Kiley (1987).

PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

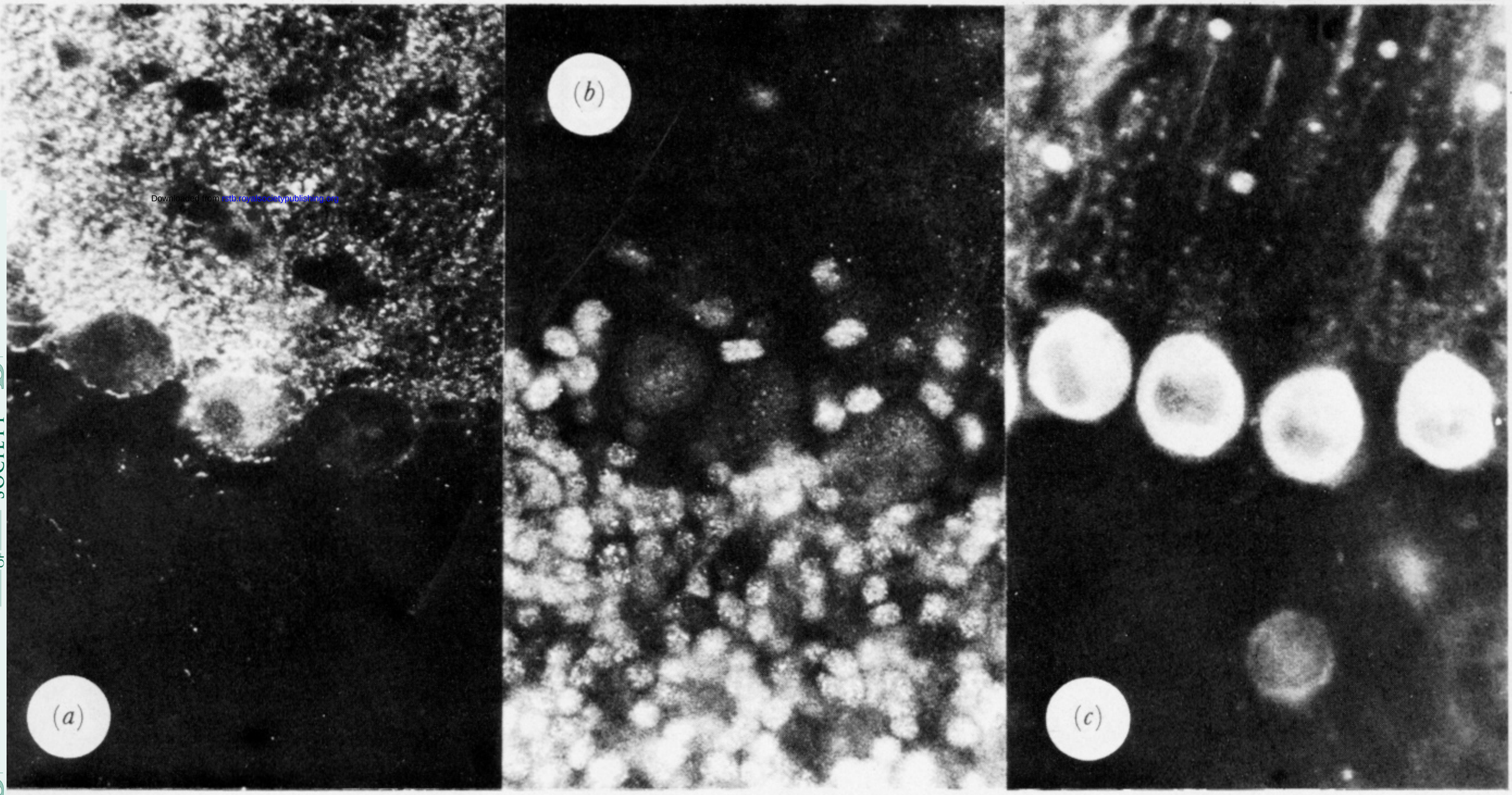

Figure 7. Fluorescent micrographs of the rat cerebellar cortex stained with the specific antibodies against protein kinase C subspecies. The upper portion illustrates the molecular layer, the middle portion represents Purkinje cells, and the lower portion shows the granular layer. (*a*) Cortex stained with antibodies against βII-subspecies. Terminal-like structures are observed in the molecular layer and surrounding Purkinje cells. (*b*) Stained with antibodies against βI-subspecies. Granule cells are heavily stained but no immunoreaction is seen in the molecular layer or Purkinje cells. (*c*) Stained with antibodies against γ-subspecies. Perikarya, dendrites, and axons of Purkinje cells are heavily stained. The detailed experimental conditions are described elsewhere (Ase *et al.* 1988).