Localization of non-conventional protein kinase C isoforms in bovine brain cell nuclei

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Using Western blotting and immunofluorescence microscopy we detected the protein kinase C isoforms δ , ϵ and ζ in isolated cell nuclei from bovine cerebral cortex. Both protein kinase C (PKC) δ and PKC ϵ are present in higher concentrations in neuronal than in glial nuclei and are located inside the nucleus and at the nuclear envelope. There they give a punctate staining in immunofluorescence microscopy. PKC ζ is also present both in the nucleoplasm and at the nuclear envelope. PKC η could not be

detected in the cell nuclei and, even in the homogenate of cerebral cortex, this isoform is present only in very low concentrations. The antibody against PKC η bound strongly to a nucleoplasmic protein with an apparent molecular mass of 99 kDa. The localization of non-conventional PKC isoforms at the cell nucleus strongly indicates that these isoforms are directly involved in the regulation of nuclear processes.

INTRODUCTION

Protein kinase Cs (PKCs) are a family of serine/threonine kinases involved in a wide variety of cellular processes [for reviews, see Nishizuka (1992) and Hug and Sarre (1993)]. In mammals, 12 isoforms of PKC are known so far, with two of them (β_1 and β_{II}) being products of alternative splicing. The PKC isoforms can be divided into two main groups: the classical or conventional isoforms (α , β_1 , β_{II} and γ) can be activated by Ca^{2+} and phospholipids, whereas all other isoforms function independently of Ca^{2+} and are denoted as 'non-conventional' isoforms. This group comprises the novel or nPKCs (δ , ϵ , η , θ and μ) and the atypical or aPKCs (ζ , ι and λ) (Nishizuka, 1992; Dekker and Parker, 1994). The different properties and substrate specificities of the PKC isoforms are not fully understood, yet they are assumed to have specific functions in intracellular signalling (Dekker and Parker, 1994).

The PKC isoforms play an important role in the regulation of cell proliferation and differentiation [for a review, see Clemens et al. (1992)]. Although this has not been investigated for the non-conventional isoforms as extensively as for the classical isoforms, there are several reports which support the view that also the non-conventional isoforms are involved in nuclear processes: overexpression of PKC δ , for example, prevents cell division in Chinese-hamster ovary cells (Watanabe et al., 1992); PKC ϵ has been shown to be involved in the differentiation of neuroblastoma cells (Leli et al., 1992; Wada et al., 1989); PKC ζ is essential for mitogenic signal transduction in *Xenopus* oocytes and Swiss 3T3 fibroblasts (Berra et al., 1993).

Starting from these observations, it seemed worthwhile to investigate whether these isoforms can be localized at the cell nucleus. Such a localization would strongly indicate a direct role in the regulation of nuclear processes. A nuclear localization has been described for the classical isoforms [for a review, see Olson et al. (1993)], but has been reported in only a few cases for the non-conventional isoforms (Hagiwara et al., 1990; Greif et al., 1992; Knox et al., 1993; Beckmann et al., 1994).

After having shown that two of the classical isoforms (α and γ) are present at the nuclei isolated from bovine brain cortex (Buchner et al., 1992), we have extended this work with the analysis of the non-conventional isoforms and have demonstrated the presence of PKC δ , ϵ and ζ at bovine brain nuclei.

MATERIALS AND METHODS

Antibodies

The antisera against PKC δ , ζ and η were a gift from B. Schnurr, Institut für Molekulare Medizin und Naturstofforschung, Freiburg, Germany; the antiserum against PKC ϵ was a gift from K. Leach, Upjohn Laboratories, Kalamazoo, MI, U.S.A. All antibodies against PKC isoforms were raised against C-terminal peptides of the PKC isoforms. The monoclonal antibody against lamin B₂ was a gift from H. Hennekes, Institute Suisse de Recherches Experimentales sur le Cancer (ISREC), Epalinges, Switzerland. The monoclonal antibody against p62 (mab 414) was a gift from G. Blobel, Rockefeller University, New York, NY, U.S.A. All secondary antibodies were from Sigma. For the immunofluorescence experiments we used anti-mouse IgG coupled to fluorescein isothiocyanate (FITC) and anti-rabbit IgG coupled to tetraethylrhodamine (TRITC). For Western blotting we used anti-mouse IgG and anti-rabbit IgG, each coupled to alkaline phosphatase or horseradish peroxidase.

Subcellular fractionation

Preparation of cell nuclei and nuclear envelopes

The isolation of cell nuclei and nuclear envelopes from bovine cerebral cortex was performed as described by Otto et al. (1992). In brief, grey matter was homogenized using a glass/Teflon Potter–Elvehjem homogenizer in a homogenization buffer containing 0.32 M sucrose, 20 mM Hepes, pH 7.4, 1 mM MgCl_2 , 47 mM 2-mercaptoethanol, 10 mg/l leupeptin, 10 mg/l aprotinin and 1 mM phenylmethanesulphonyl fluoride (PMSF). The homogenate was filtered through 125-mesh nylon gauze and

Abbreviations used: DAPI, 4',6-diamidino-2-phenylindole; FITC, fluorescein isothiocyanate; PKC, protein kinase C; PMSF, phenylmethanesulphonyl fluoride; TBS, Tris-buffered saline; TBST, TBS containing Tween-20; TRITC, tetraethylrhodamine isothiocyanate.

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centrifuged at 170 *g* for 15 min. The pellet was resuspended in homogenization buffer and re-centrifuged at 370 *g* for 20 min. The resulting pellet was adjusted to 1.85 M sucrose and centrifuged at 70000 *g* for 60 min. The nuclear pellet was carefully resuspended and adjusted to 2.15 M sucrose. This solution was applied on to a cushion containing 2.2 M sucrose and centrifuged at 70000 *g* for 60 min, yielding purified nuclei in the pellet. The isolated nuclei were resuspended in 0.32 M sucrose buffer and either frozen in liquid nitrogen or immediately used for preparing nuclear envelopes. For this the nuclei were quickly transferred into a large volume of hypotonic buffer [10 mM Tris (pH 7.4)/10 mM Na₂HPO₄/1 mM PMSF] containing DNAase I and heparin (0.2 and 5 mg/mg of nuclear protein respectively). The suspension was incubated 60 min at 4 °C and 15 min at room temperature, then centrifuged at 9500 *g* for 10 min. The pellet was washed and centrifuged again, yielding the nuclear-envelope fraction.

Preparation of particulate and cytosolic fractions

Particulate and cytosolic fractions were prepared using two different homogenization buffers: For particulate 1 (P1) and cytosol 1 (C1), a buffer containing 0.25 M sucrose, 2 mM EDTA and 0.5 mM EGTA was used. The homogenate was centrifuged 1 h at 100000 *g*, with the pellet yielding the particulate fraction and the supernatant yielding the cytosolic fraction. For particulate 2 (P2) and cytosol 2 (C2) a buffer containing 0.32 M sucrose and 1 mM MgCl₂ was used with the same centrifugation conditions. For cytosol 3 (C3) an aliquot of cytosol 2 was taken, adjusted to 0.11 M sucrose and centrifuged for another 3 h. All buffers contained additionally Hepes, 2-mercaptoethanol and proteinase inhibitors in the same concentrations as the homogenization buffer used for the preparation of nuclei.

For checking the enrichment of lamin B in nuclear fractions the supernatant of the 170 *g* centrifugation (postnuclear supernatant) was made up to 0.11 M sucrose and centrifuged for 12 h, with the supernatant giving the cytosolic, the pellet giving the particulate fraction.

Proteins of the cytosolic fractions were precipitated with 10 % (w/v) trichloroacetic acid prior to gel electrophoresis.

Western blotting

Subcellular fractions were subjected to SDS/10 %-(w/v)-PAGE (Laemmli, 1970) and transferred to nitrocellulose membranes (Sartorius). Proteins were stained with Ponceau Red to control the efficiency of blotting and equal loading of the gel. The membranes were then blocked for 2 h with a solution of 10 % (w/v) skimmed-milk powder in 20 mM Tris (pH 7.4)/150 mM NaCl, containing 0.1 % (v/v) Tween-20 (TBST). Incubation with the primary antibody was performed for 2 h at room temperature or at 4 °C overnight, followed by three washes with TBST and incubation with the secondary antibody. Finally blot membranes were washed again three times with TBST and once with TBS. Colour development with alkaline phosphatase-coupled secondary antibodies was performed using Nitroblue Tetrazolium and 5-bromo-4-chloroindol-3-yl phosphate (*p*-toluidine salt). Horseradish-peroxidase-coupled antibodies were revealed with the ECL ('enhanced chemiluminescence') system (Amersham) according to the supplier's instructions.

All antibodies were diluted with TBS containing 5 % (w/v) skimmed-milk powder and, with the exception of horseradish-peroxidase-coupled antibodies, 0.02 % NaN₃.

Densitometry

Densitometric analysis was carried out with a videodensitometer (Fisher Biotech, Reiskirchen, Germany).

Electron microscopy

Isolated nuclei were centrifuged (500 *g*, 5 min), resuspended in PBS containing 2 mM MgCl₂ and centrifuged again. The resulting pellet was fixed in 1 % glutaraldehyde and 1 % tannic acid in 0.1 M phosphate buffer, pH 7.4. Subsequently, it was postfixed in a 2 % OsO₄ solution. After rinsing and dehydration in the ascending alcohol series, the preparations were embedded in Epon and sections were cut with an Ultracut E (Reichert) microtome. The ultrathin sections were contrasted with 2 % uranyl acetate and lead citrate and studied with a Zeiss EM10 transmission electron microscope.

Immunofluorescence microscopy

Isolated nuclei were diluted in homogenization buffer to give a final protein concentration of 0.2 mg/ml. A 15 µl portion of this suspension was loaded on single wells of microscope slides (Dunn, 15 wells) and nuclei were allowed to sediment for 15 min. The buffer was carefully removed and for fixation a solution of 3.7 % (w/v) paraformaldehyde in PBS was added for 30 min at room temperature. Nuclei were permeabilized by incubating them with 80 % methanol in PBS for 60 min at -20 °C. After three washes with PBS the incubation with primary antibody was done at room temperature for 1 h, followed by three washes with PBS and incubation with the secondary antibody under the same conditions. After another three washes with PBS, DNA was stained by adding 0.0004 % (w/v) 4',6-diamidino-2-phenylindole (DAPI) for 5 min, followed by a final wash with PBS. Cover slides were mounted with Mowiol (Polysciences) and allowed to dry overnight.

Protein determination

Protein determination was done with the BCA system (Pierce, Rockford, IL, U.S.A.) using BSA as a standard. The protein content of fractions containing 2-mercaptoethanol was determined as described by Bradford (1976).

RESULTS AND DISCUSSION

Purity and integrity of the nuclei prepared from bovine cerebral cortex were judged by phase-contrast microscopy, electron microscopy and by measuring the activity of marker enzymes. Marker enzyme activities were measured as described by Otto et al. (1992). The contamination of nuclei with other cell compartments was below 2 % for plasma membrane and below 1 % for cytosol (see Table 1). The nuclei appeared intact under the phase-contrast microscope, and only a few of them were aggregated (Figure 1a). The preparation contained two kinds of cell nuclei: neuronal nuclei with an average diameter of about 12 µm and glial nuclei with an average diameter of about 7 µm (Thompson, 1987). Whereas the neuronal nuclei contained one or two large nucleoli, the glial nuclei did not show any distinct internal structure under the phase-contrast microscope.

Electron microscopy confirmed the low degree of contamination with membranous material and revealed the intact double-membrane structure of the nuclear envelope (Figure 1b).

Since no marker enzyme for the nuclear envelope exists, characterization of the nuclear-envelope fraction was done by monitoring the enrichment of lamin B₂ by Western blotting.

Table 1 Characterization of subcellular fractions

Results are means $\pm$ S.D. For protein, DNA, lactate dehydrogenase and 5'-nucleotidase, $n \geq 3$. Values for PKC isoforms resulting from densitometric evaluation of enhanced-chemiluminescence films are expressed in arbitrary units and represent values obtained in one representative experiment. Abbreviation: n.d., not determined.

Fraction	Total protein (mg)	DNA (μ g/mg)	5'-Nucleotidase (munits/mg)	Lactate dehydrogenase (munits/mg)	Isoform ...	PKC (arbitrary units/mg)		
						δ	ϵ	ζ
Homogenate	8640 $\pm$ 2100	4.7 $\pm$ 2.1	66 $\pm$ 16	2240 $\pm$ 400		27.2	89.1	17.4
Nuclei	15.3 $\pm$ 6.0	390 $\pm$ 70	1.1 $\pm$ 0.5	< 30		11.5	14.9	5.4
Particulate 1	2700 $\pm$ 700	n.d.	87 $\pm$ 18	1070 $\pm$ 300		19.2	46.0	16.4
Cytosol 1	2130 $\pm$ 450	n.d.	11.4 $\pm$ 4.6	4730 $\pm$ 500		14.6	11.7	1.7

Figure 1 Isolated cell nuclei

(a) Phase-contrast picture. Two different kinds of nuclei can be distinguished: neuronal nuclei with an average diameter of about 12 μ m and one or two nucleoli ($\triangleright$), and glial nuclei with an average diameter of about 7 μ m and no visible internal structure ($\blacktriangleright$). The nuclei appear to be pure and are mostly not aggregated. The bar represents 20 μ m. (b) Electron-microscopic picture of isolated nuclei. The intact double membrane structure can clearly be seen. The bar represents 2 μ m.

Figure 2 Lamin B₂ in subcellular fractions

A portion (25 μ g of protein) of each fraction was subjected to SDS/PAGE, transferred to a nitrocellulose membrane and probed with a monoclonal antibody against lamin B₂. The strong enrichment of this nuclear-lamina protein in the nuclear-envelope fraction is evident, especially in comparison to the homogenate. Abbreviations: N, nuclei; NE, nuclear envelope; H, homogenate; P, particulate; and C, cytosolic fraction derived from the postnuclear supernatant; M, molecular mass.

Lamin B₂ was absent in the particulate and cytosolic fractions derived from the postnuclear supernatant and hardly detectable in the homogenate (Figure 2). The strong signal for lamin B₂, together with the low DNA content of the nuclear-envelope fraction (Beckmann et al., 1992), demonstrated the high enrichment of nuclear envelopes in this fraction. After the purity of both the nuclear and nuclear-envelope fraction was shown, both fractions were used for the analysis of non-conventional isoforms of PKC.

PKC δ

Using Western blotting, the presence of PKC δ in both nuclei and nuclear envelope could clearly be shown (Figure 3). The staining could be blocked by the addition of the peptide against which the antibody was raised. The bands obtained for nuclei and nuclear envelope were less intense than in the homogenate, particulate and cytosolic fractions, except C3. To estimate the relative PKC content in the various fractions, we tried to quantify the PKC signals. For this purpose the PKC bands were revealed by using a luminescence detection system. Subsequently the films were

Figure 3 Non-conventional PKC isoforms in subcellular fractions

A portion (25 μ g of protein) of each fraction was subjected to SDS/PAGE, transferred to nitrocellulose membranes, probed with specific antibodies against the PKC-isoforms δ , ϵ , ζ and η and complexes revealed with the alkaline phosphatase system. PKC δ , ϵ and ζ (arrow) are present in both the nuclear and the nuclear-envelope fractions. The distribution of PKC δ between particulate and cytosolic fractions was found to depend on presence or absence of chelators and on centrifugation conditions, whereas the distribution of PKC ϵ and PKC ζ was widely unaffected. The PKC ζ antibody recognized two bands with an apparent molecular mass of about 80 kDa (arrow). The lower band shows an even distribution between nuclear and nuclear-envelope fractions, the higher one being enriched in the nuclear-envelope fraction. The nature of the proteins underlying the bands at about 105 kDa is unknown. PKC η could not be detected in the nuclear or nuclear-envelope fraction. The identity of the nuclear protein with an apparent molecular mass (M) of 99 kDa (arrowhead), which strongly binds the antibody against PKC δ , is not known. Mouse lung homogenate (L) as a positive control gave the strongest signal. The antibodies against PKC δ and PKC η were used for immunofluorescence experiments. Abbreviations: N, nuclei; NE, nuclear envelope; H, homogenate; P1 and C1, particulate and cytosolic fractions prepared in the presence of EDTA and EGTA; P2 and C2, particulate and cytosolic fractions prepared in the presence of 1 mM $MgCl_2$; C3, like C2, after 4 h centrifugation in the presence of only 0.11 M sucrose.

evaluated by densitometry. Luminescence detection overall yielded results similar to those obtained with colour development, but in some cases the former resulted in larger intensity differences between single lanes than the colour-development method. As it was not possible for us to calibrate the films with purified PKC isoforms, no absolute values could be obtained. Nevertheless, they could be used for comparison of the fractions within one film; comparisons between different isoforms were not possible.

As shown in Table 1 for nuclei, the 'specific content', i.e. the ratio of PKC δ to protein, is about 40% that of the homogenate and about 60% and 78% for particulate and cytosolic fraction respectively. As the contamination of the nuclei with plasma membrane is below 2% and contamination with cytosol below 1%, the PKC δ found in the nuclei cannot be attributed to contamination. As can be calculated from Table 1, the total amount of PKC δ seems to be quite small. This value, however, would be misleading, as one has to consider the recovery of nuclei (about 15% on the basis of DNA determination) on the one hand and the low protein content of the nuclear fraction compared with the homogenate on the other. So although the nuclear content of PKC δ seems to be low at first sight, it nevertheless appears to be significant and therefore also of

potential functional importance. Furthermore, PKC δ in the nucleus is confined to small regions (see below), implicating high local concentrations. These considerations also apply to the other isoforms (see below).

The distribution of PKC δ between particulate and cytosolic fractions was found to strongly depend on the preparation method. In most reports studying translocation of PKC, buffers containing millimolar amounts of EDTA and EGTA, and sometimes additionally low concentrations of Triton X-100 (e.g. Ha and Exton, 1993) have been used. After homogenization with a buffer containing 2 mM EDTA and 0.5 mM EGTA we found somewhat more PKC δ in the particulate fraction (P1) than in the cytosolic fraction (C1) (Figure 3). Interestingly, the use of a homogenization buffer containing $MgCl_2$ to stabilize nuclei and no chelators resulted in appearance of the major part of PKC δ in the particulate fraction (P2). The amount found in the cytosolic fraction is higher after 1 h than after 4 h centrifugation (Figure 3) and extremely low after 12 h (results not shown), indicating that PKC δ may be associated with some high-molecular-mass structures. Western blotting revealed that short periods (1 h) of centrifuging the postnuclear supernatant are not sufficient to completely remove marker proteins of, e.g., the plasma mem-

Figure 4 PKC δ and p62 in isolated nuclei

A double staining of isolated nuclei with a polyclonal antibody against PKC δ (a) and a monoclonal antibody against the nuclear-pore-complex protein p62 (b) was performed. PKC δ is located at foci at the nuclear envelope, but the distribution appears different from the distribution of p62. The bars represent 10 μ m.

brane (results not shown). Thus the distribution of PKC δ between particulate and cytosolic fractions strongly depends on buffer composition and centrifugation times. This behaviour probably is the reason for the differences between the present findings and those of Szallasi et al. (1994), who found PKC δ mainly in the soluble fraction in resting cells.

The effect of EDTA and EGTA on the distribution of PKC δ may be due to their effect of not only chelating Ca^{2+} but also Mg^{2+} . According to a model established by Luo and Weinstein (1993), Ca^{2+} is bound with high affinity to a pocket formed by the C1 and C2 domains of the conventional PKC isoforms, with the C2 domain conferring high selectivity for Ca^{2+} binding when compared with the binding of Mg^{2+} . Mg^{2+} may satisfy the role of Ca^{2+} in the activation of non-conventional PKC isoforms which lack the C2 domain (Luo and Weinstein, 1993), and it is conceivable that removal of Mg^{2+} may have effects not only on the conformation of the non-conventional isoforms but also on their association with cellular structures.

Owing to the high specificity of the antibody against PKC δ , it was possible to use it for localization studies with immunofluorescence microscopy. With this method, PKC δ could be localized mainly at the nuclear envelope of both neuronal and glial nuclei, with the neuronal nuclei showing stronger signals (Figure 4a). The punctate pattern resembles nuclear-pore-complex staining (Davis and Blobel, 1986). To test a possible co-localization of PKC δ with nuclear-pore complexes, a double staining with the antiserum against PKC δ and a monoclonal antibody against the nuclear-pore-complex protein p62 was performed. As Figure 4 demonstrates, both antibodies stain the nuclear envelope, but with different patterns. A co-localization of PKC δ and p62 could not be observed with this method. Thus PKC δ probably is not located at the nuclear-pore complex, although it is present at the nuclear envelope at distinct foci. Immunocytochemical electron microscopy will be necessary for a precise localization of PKC δ at the nuclear envelope.

The immunofluorescence pictures suggested that PKC δ is present inside the nucleus as well, although conclusive evidence cannot be obtained with conventional immunofluorescence microscopy. However, strong evidence for a localization of PKC δ in the nucleoplasm came from the fact that there was no enrichment within the nuclear envelope fraction compared with the nuclear fraction (Figure 3).

Recent neuroanatomical work showed the presence of PKC δ in nuclei of thalamic neurons and spinal-cord motoneurons (Merchenthaler et al., 1993). In the present work we extended these observations to nuclei derived from cortical neurons. Apparently PKC δ is located at the cell nucleus of neurons in several areas of the central nervous system. Furthermore, we can show that, to an lower extent, PKC δ is also present in glial nuclei.

Interestingly, PKC δ could also be found in the nuclei of cultured cells of neuronal origin, but in this case it was located in a different nuclear compartment, namely the nucleoli (Beckmann et al., 1994).

PKC ϵ

The presence of PKC ϵ in nuclei and nuclear envelopes could clearly be shown by Western blotting, with the signal being more intense for the nuclear envelope (Figure 3). Estimation of the 'specific content' of PKC ϵ in nuclei and comparison with homogenate, particulate and cytosolic fractions again made contamination with plasma membrane and cytosol as a source of nuclear PKC ϵ very unlikely (Table 1). The considerations concerning the amount of nuclear PKC δ also apply to PKC ϵ and to PKC ζ (see below).

The distribution of PKC ϵ between particulate and cytosolic fractions for unknown reasons showed differences from that of PKC δ . PKC ϵ appeared mostly in the particulate fraction no matter whether buffers containing chelators were used or not, and the amount of PKC ϵ within the cytosolic fractions was not sensitive to centrifugation time (Figure 3).

Immunofluorescence microscopy showed a strong nuclear-envelope staining with the antibody against PKC ϵ (Figure 5a). The signal was much stronger for neuronal than for glial nuclei, as can be seen when comparing the immunofluorescence staining for PKC ϵ with the DNA-staining using DAPI (Figure 5). The distribution of PKC ϵ at foci of high concentration resembled the distribution of PKC δ . As in the case of PKC δ , double staining with a monoclonal antibody against the nuclear-pore-complex protein p62 and the antibody against PKC ϵ showed that the structures stained with the latter antibody probably are not nuclear-pore complexes (results not shown). Whether PKC ϵ is located at the same nuclear-envelope structures as PKC δ

Figure 5 PKC ϵ in isolated nuclei and DNA stain

Isolated nuclei were incubated with an antibody against PKC ϵ (a). DNA was stained using DAPI (b). PKC ϵ shows a punctate staining, mainly of the nuclear envelope. The higher concentration of PKC ϵ in neuronal nuclei compared with glial nuclei is evident when comparing the PKC ϵ staining with the DAPI staining. The bars represent 10 μ m.

could not be clarified, because a double staining with the two polyclonal rabbit antibodies was not possible.

As it was the case for PKC δ , PKC ϵ was also reported to be present at nuclei of neuroblastoma $\times$ glioma hybrid cells (Beckmann et al., 1994). In this case, however, PKC ϵ could be colocalized with the nuclear-pore-complex protein p62.

PKC ζ

Western-blotting with an antiserum against PKC ζ exhibited two bands with an apparent molecular mass of about 80 kDa. The appearance of both bands could be blocked by the addition of the peptide against which the antibody was raised (results not shown). The lower band shows an even distribution between the nuclear and nuclear-envelope fractions; the higher one is enriched in the nuclear-envelope fraction. The two bands may reflect different phosphorylation states, as was demonstrated for other isoforms by treatment with phosphatases (e.g. Koide et al., 1992). The nature of the proteins underlying the bands at about 105 kDa, which appeared in the homogenate, the particulate and the cytosolic fractions, is unknown.

Our finding that PKC ζ is present at brain nuclei is in agreement with a report by Hagiwara et al. (1990), who found PKC ζ associated with rabbit brain nuclei. In rat hippocampus, PKC ζ seems to play a role in long-term potentiation. A constitutively active 51 kDa form (PKM) of PKC ζ was found to be increased in the cytosol during the maintenance phase of long-term potentiation (Sacktor et al., 1993). We found no indications for such an active form of PKC ζ in bovine brain-cortex cytosol. There was a weak staining of some lower-molecular-mass proteins in the nuclear and nuclear-envelope fraction, but it remains to be elucidated whether these proteins are of physiological significance.

PKC η

Immunoblot analysis of PKC η revealed that this isoform is present in bovine brain cortex only in very small amounts. In the molecular-mass range of about 80 kDa, where a staining from PKC η would be expected, only two faint bands could be detected in the homogenate (Figure 3). Mouse lung homogenate

was used as a control and gave the strongest signal (Figure 3). These findings are in accordance with the notion that PKC η is located predominantly in lung, skin and heart (Nishizuka, 1992). In human epidermal carcinoma A431 cells, PKC η was localized in the cell nucleus (Greif et al., 1992), whereas others reported that it was not present in nuclei but enriched in the endoplasmic reticulum (Chida et al., 1994).

In the nuclear and nuclear-envelope fraction from bovine brain cortex, no immunoreactivity could be observed in the 80 kDa region, suggesting that PKC η is not present at bovine brain nuclei. However, strong immunoreactivity appeared at 99 kDa (Figure 3). Recently, in rat hippocampus a protein with an apparent molecular mass of 97 kDa reacting with an antibody against PKC η was observed. This protein was described as having properties of PKC and designated 'PKC(HMW)', 'HMW' meaning 'high molecular weight' (Sublette et al., 1993). In their work this protein was described as being associated with a postnuclear membrane fraction, whereas we find the immunoreactivity almost exclusively in the nuclear fraction (Figure 3). It remains to be clarified whether the two proteins are different or whether the varying distribution reflects differences in the methods of subcellular fractionation.

The finding that the 99 kDa protein was present in the nuclear-envelope fraction only to a very small degree suggests that this protein is located in the nucleoplasm. This was also confirmed by immunofluorescence microscopy, where the application of the antibody against PKC η gave an even staining within the nuclei, very probably reflecting the distribution of the 99 kDa protein (results not shown).

Conclusion

The PKC isoforms δ , ϵ and ζ were shown to be located in nuclei isolated from bovine brain cortex. In addition, a nuclear-specific 99 kDa protein, probably related to PKC η , was found. A striking feature of the two PKC isoforms, which could be localized with immunofluorescence microscopy, is their predominant location in neuronal nuclei. These findings reinforce the notion that non-conventional PKC isoforms can be directly involved in the regulation of nuclear processes, particularly in neuronal cells.

In a previous study we demonstrated the presence of PKC α and γ at bovine brain nuclei (Buchner et al., 1992). Activity measurements showed that PKC was present in a permanently active, so-called 'membrane-inserted' form (Buchner et al., 1992). Nothing is known up to the present concerning the activity of the nucleus-associated non-conventional PKC isoforms. Since these isoforms show enzymic properties and substrate specificities distinct from those of the classical isoforms (histone, for example, is a poor substrate for PKC ϵ and δ) (Liyanage et al., 1992; Dekker et al., 1992), the activity of these isoforms very probably contributed to only a very small extent to the PKC activity measured in our earlier study. Since isoform-specific inhibitors are not yet available, reliable activity measurements with nuclei or nuclear-envelope preparations, specific for the single non-conventional PKC isoforms, presently appear to be impossible.

The differences in the nuclear location of PKC isoforms between bovine brain and, e.g., neuroblastoma cells, extends the current view that each cell type expresses a defined set of PKC isoforms which are differentially located within the cell (Asaoka et al., 1992). Apparently, PKC isoforms can even be differentially distributed between subnuclear compartments. To achieve this, a variety of proteins may be present that bind PKC isoforms and direct them to specific compartments.

One example for such a PKC-binding protein is the receptor for activated C-kinase (RACK) (Ron et al., 1994). However, the isoform specificity of RACK is unknown. To achieve the complex pattern of distribution, one must postulate the existence of at least several isoform-specific proteins, which, furthermore, may be different for each cell type.

Little is known about the specific functions of the various nucleus-associated PKC isoforms. There are several nuclear proteins known to be phosphorylated by PKC [for a review, see Olson et al. (1993)], but to our knowledge up to the present, only one case of preferential phosphorylation by one isoform has been reported (Hocevar et al., 1993). In order to unravel the function of nuclear PKC isoforms, more must be learned about such specific phosphorylations caused by the specific localizations. In the context of neuronal nuclei, it is tempting to speculate on a role of nuclear PKC isoforms in long-term neuronal processes such as maintenance of full differentiation of neuronal cells or participation in processes of long-term plasticity such as learning and memory formation.

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