Properties of protein kinase C associated with nuclear membranes

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To study signal transduction directed towards the cell nucleus and at the nuclear membranes, we investigated the association of protein kinase C (PKC) with nuclear membranes obtained from nuclei isolated from bovine brain. By use of phorbol-ester-binding assays, significant amounts of PKC could be demonstrated in nuclei and nuclear membranes. Nuclear membranes are shown to be able to activate purified PKC. The PKC endogenously present in nuclear membranes appears to be a so-called 'membrane-inserted' form: it is permanently active, still binds phorbol ester, but its activity is no longer dependent on Ca^{2+} and cannot be activated by phorbol ester. On the other hand, this form of PKC can be inhibited by specific PKC inhibitors. By using histone HIIIS and a specific peptide substrate, it could be shown that after extraction with Triton X-100 the PKC can be stimulated by phospholipid again. Immunoblot analysis with isoenzyme-specific antibodies revealed that the α - and γ -isoenzymes, but not the β -isoenzyme, are associated with membranes derived from brain nuclei.

INTRODUCTION

Activation of the Ca^{2+} - and phospholipid-dependent protein kinase (protein kinase C, PKC) is a central step in the transduction of a large variety of signals which involve receptor-mediated hydrolysis of inositol phospholipids into cells (Nishizuka, 1986). Concomitant with the activation of PKC, a translocation of the enzyme takes place from the cytosol to the membrane, especially to the plasma membrane (Kraft & Anderson, 1983). Once activated, PKC phosphorylates a large variety of proteins, among them receptors and ion channels, modulating their properties (for reviews see Nishizuka, 1986; Shearman *et al.*, 1989). As well as in such short-term regulatory events, PKC plays an important role in long-term events such as, e.g., proliferation and differentiation, which involve alterations in gene expression (Nishizuka, 1986). This is known mostly from experiments using tumour-promoting phorbol esters, which selectively activate PKC and lead to the alteration of the expression of quite a number of genes, including oncogenes such as *fos*, *jun* or *myc* and genes encoding enzymes such as ornithine decarboxylase or tyrosine hydroxylase (Nishizuka, 1986). However, little is known about the intermediate steps in the transduction of signals to the cell nucleus after the activation of PKC. Initially PKC was thought not to be involved in events at or in the nucleus, but more recently several groups presented evidence for PKC being associated with nuclei of different origins, e.g. liver (Capitani *et al.*, 1987; Masmoudi *et al.*, 1989; Rogue *et al.*, 1990) or brain (Girard *et al.*, 1985; Misra & Sahyoun, 1987). In the present study we investigated properties of PKC in nuclei, using a specific substrate for PKC (Alexander *et al.*, 1990) and new specific PKC inhibitors (Davis *et al.*, 1989), and we present evidence that the PKC associated with brain-derived nuclei may be a permanently active, so-called membrane-inserted (Bazzi & Nelsestuen, 1988), form of the enzyme.

MATERIALS AND METHODS

Materials

Histone HIIIS, L- α -phosphatidyl-L-serine (PtdSer) and other

chemicals were from Sigma. [^3H]Phorbol 12,13-dibutyrate and [γ - ^{32}P]ATP were from NEN. DEAE-cellulose (DE 52) and P81 ion-exchange-chromatography paper were from Whatman. PKC peptide substrate (peptide GS) and the pseudosubstrate inhibitor peptide were from Bachem, Heidelberg, Germany.

The PKC inhibitors Compound 2 (Ro 31-7549) and Compound 3 (Ro 31-8220) were generously given by Dr. P. D. Davis, Roche Products, Welwyn Garden City, Herts., U.K.

Antibodies

The specific antisera to the α , β and γ isoenzymes of PKC were produced by essentially the technique described by Makowske *et al.* (1988). Peptides that are specific to the α , β and γ isoenzymes of PKC were synthesized by Multiple Peptides Systems, San Diego, CA, U.S.A. The sequences, derived from the region of PKC that bridges the regulatory and catalytic domains, were as follows: α , AGNKVISPSDDRQ; β , GPKTPEEKTANTISKFD (this sequence is common to both β 1 and β 2 isoforms); γ , NYPLELYERVRTG. Each peptide was synthesized with a C-terminal cysteine for coupling to a carrier protein. The antisera were raised in rabbits by subcutaneous injection of the peptides coupled to keyhole-limpet haemocyanin. Characterization of the isoenzyme-specific antibodies obtained is described elsewhere (Beckmann *et al.*, 1990; Ganesan *et al.*, 1990). Antibody dilutions of 1:100 were used in the present study.

Isolation of nuclei

Nuclei were prepared from bovine brain cortex by a modified procedure as described by Thompson (1987). Brain was obtained freshly from the slaughterhouse and transported to the laboratory on ice. All steps were carried out at 4 °C. After removing white matter, meninges and arteries, cortex was cut into small pieces and homogenized in NP 0.32 [20 mM-Hepes, pH 7.4, 1 mM-MgCl₂, 0.32 M-sucrose, 1 mM-phenylmethanesulphonyl fluoride (PMSF), 10 μg of leupeptin/ml, 10 μg of aprotinin/ml and 42.8 mM- β -mercaptoethanol] by five complete up-and-down strokes with a Teflon/glass homogenizer at 700 rev./min. After

Abbreviations used: PKC, protein kinase C; PMSF, phenylmethanesulphonyl fluoride; PtdSer, phosphatidylserine; DTE, dithioerythritol; PVDF, poly(vinylidene difluoride); PMA, phorbol 12-myristate 13-acetate.

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filtration through 110-mesh nylon gaze, the homogenate was made up to 15 % (w/v) cortex.

The homogenate was centrifuged for 20 min at 4000 *g*. The resulting pellet was resuspended in NP 0.32 and again centrifuged for 20 min at 16000 *g*. The pellet was resuspended in a small amount of NP 0.32. Then NP 2.4 (20 mM-Hepes, pH 7.4, 1 mM-MgCl₂, 2.4 M-sucrose) was added to a final concentration of 2.0 M-sucrose. This suspension was centrifuged for 50 min at 67000 *g* in a swinging-bucket rotor (TST 28.38). After the supernatant was discarded, the pellet was carefully resuspended in a small volume of NP 0.32. NP 2.4 was added to give a concentration of 2.28 M-sucrose. This suspension was layered between small volumes of NP 2.3 (2.3 M-sucrose) and NP 2.1 (2.1 M-sucrose).

After centrifugation for 40 min at 67000 *g*, fraction N1 was collected from the interface between 2.1 M- and 2.28 M-sucrose. The pellet of this centrifugation step was resuspended in NP 0.32, to obtain the N2 fraction. Both fractions were diluted with NP 0.32 and again centrifuged for 20 min at about 1000 *g*.

The white nuclear pellets were resuspended in small volumes of NP 0.32. Integrity and purity of the nuclei were judged by phase-contrast microscopy and by assaying 5'-nucleotidase (Kai *et al.*, 1966) as a plasma-membrane marker.

Preparation of nuclear envelopes

Nuclear envelopes were prepared by using heparin and DNAase I by a modification of the method described by Smith & Wells (1983). Briefly, nuclei were washed once in 20 mM-Hepes (pH 7.5)/25 mM-KCl/5 mM-MgCl₂, resuspended in 20 mM-Hepes (pH 7.5)/25 mM-KCl and adjusted to 5×10^7 nuclei/ml. After addition of heparin (final concn. 0.6 mg/10⁸ nuclei) and DNAase I (final concn. 0.5 mg/10⁸ nuclei), the preparation was gently stirred for 60 min at 4 °C. After centrifugation for 20 min at 12000 *g* (4 °C), the pellet was resuspended in NP 0.32, centrifuged as described above and the final pellet resuspended in a small volume of NP 0.32.

Phorbol-ester-binding assays

Phorbol-ester-binding assays were performed as described by Uchida & Filburn (1984).

Measurement of PKC activity

To measure PKC activity in the presence of Triton X-100, we used a mixed-micelle assay as described by Bell *et al.* (1986). Variable amounts of protein (about 20 µg) were incubated in a total volume of 50 µl, containing 20 mM-triethanolamine/HCl, pH 7.4, 10 mM-magnesium acetate, 100 µM-CaCl₂, 50 mM-β-mercaptoethanol, 50 µM peptide GS (see below) or 400 µg of histone H11S/ml, 20 µM-[γ-³²P]ATP (about 0.1 µCi) and up to 0.6 % (w/v) Triton X-100. Under activating conditions 8 mol% of PtdSer and 2 mol% of diolein were added. Phosphorylation was started by adding 10 µl of a reaction mixture of triethanolamine, magnesium acetate, CaCl₂, β-mercaptoethanol, substrate and ATP to 40 µl of protein, Triton X-100, PtdSer, diolein and other effector substances. Incubation was 10 min at 30 °C unless stated otherwise. When histone was used as substrate, the reaction was terminated by spotting 40 µl of the reaction mixture on to P81 filters. The filters were washed three times with 0.5 % H₃PO₄. Radioactivity was determined by measuring Čerenkov radiation in water.

In most experiments peptide GS was used as substrate. This peptide is a modification of a peptide derived from glycogen synthase and shown to be useful as a specific substrate for PKC (Alexander *et al.*, 1990). When this peptide was used, assays were stopped by addition of 50 µl of ice-cold 25 % trichloroacetic acid. After 15 min on ice, the samples were centrifuged for

10 min in an Eppendorf Microfuge. Portions (90 µl) of the supernatants were spotted on P81 filters, which filters were processed as described above.

In assays where no Triton X-100 was present, the PKC activity under stimulated conditions was determined by addition of sonicated phospholipid vesicles (final concns.: 50 µg of PtdSer/ml, 5 µg of diolein/ml). The other components were as described above.

Ion-exchange chromatography of nuclear extracts

Nuclei (about 5 mg of protein) were extracted with 1.2 % Triton X-100 for 10 min at room temperature. After centrifugation for 15 min at 12000 *g* (4 °C), the supernatant was used for ion-exchange chromatography; the pellet was resuspended in 20 mM-Tris/HCl (pH 7.5)/250 mM-sucrose/5 mM-MgCl₂/1 mM-dithioerythritol (DTE). The supernatant was diluted with the same volume of buffer A (20 mM-Tris/HCl, pH 7.5, 1 mM-EDTA, 1 mM-EGTA, 1 mM-DTE, 1 mM-PMSF, 10 µg of leupeptin/ml) and loaded on a small DEAE-cellulose ion-exchange column (bed vol. 2 ml) equilibrated with buffer A. After washing with 6 ml of buffer A, the column was eluted with a 15 ml gradient from 0 to 1.0 M-NaCl in buffer A, followed by 1 M-NaCl in buffer A; 200 µl fractions were collected. Kinase activities within these fractions were measured with GS peptide as substrate as described above.

Phosphorylation of nuclear-envelope proteins

Membranes were phosphorylated in reaction mixtures containing 20 mM-Hepes, pH 7.4, 1 mM-DTE, 5 mM-magnesium acetate, 0.1 mM-CaCl₂ and additions listed in the Figure legends. Where indicated, endogenous kinases were inactivated by incubation of the membranes before the phosphorylation reaction for 5 min at 65 °C. Purified PKC, added to the samples in some experiments, was prepared from bovine brain as described previously (Krüger *et al.*, 1990). Activators and inhibitors of PKC were added 10 min before the start of the reaction by addition of ATP. After incubation for 1 min at 30 °C, the reaction was stopped by addition of SDS-sample buffer and boiling for 5 min. After SDS/PAGE (Laemmli, 1970), gels were stained with Coomassie Blue, dried and autoradiographed by using Kodak X-OMAT AR films.

Immunoblot analysis

The samples were separated by using SDS/8 % -polyacrylamide gels. They were then electroblotted on to poly(vinylidene difluoride) (PVDF) membranes (Immobilon-P; Millipore). The membranes were incubated in 20 mM-Tris/HCl (pH 7.3)/137 mM-NaCl (TBS), containing 0.1 % Tween-20 (TBS-T) supplemented with 5 % (w/v) non-fat dry milk for 60 min at room temperature. After five washes with TBS-T, affinity-purified isoenzyme-specific antibodies diluted in TBS were added and incubated overnight at 4 °C, followed by five washes with TBS-T. Biotinylated anti-rabbit IgG (Amersham, diluted 1:500 in TBS) was applied to the membrane for 20 min at room temperature and, after five washes with TBS-T, streptavidin-peroxidase (Calbiochem; dilution 1:1000 in TBS) was added for 1 h. Blots were made visible by the ECL system (Amersham) according to the manufacturers' instructions or by staining with diaminobenzidine.

RESULTS

The nuclei prepared as described above appear to be intact and devoid of membrane contamination as judged by phase-contrast microscopy. The N2 fraction contains more than 90 % small (diameter about 7 µm) dense nuclei (reported to be glial nuclei;

Fig. 1. Nuclear membranes support the activity of purified PKC

Endogenous kinases were inactivated by heating for 5 min to 65 °C. PKC activity was determined with peptide GS as substrate. Heated membranes alone show no kinase activity, but support the activity of purified PKC, which can be further stimulated by additional dioloin.

Fig. 2. Phosphorylation of nuclear membranes

(a) Coomassie Blue stain; (b) autoradiography. Lane 1, no Ca²⁺ added, lanes 2–6, 0.1 mM-Ca²⁺; lane 3, 0.1 μM-PMA; lane 4, 1 μM-PMA; lane 5, 1 μM-PMA + 10 μM-Compound 2; lane 6, 1 μM-PMA + 1 μM-Compound 3.

Thompson, 1987). In the N1 fraction larger (diameter about 12 μm) less dense nuclei with one to two nucleoli (identified as neuronal nuclei; Thompson, 1987) are enriched. The other nuclei in this fraction belong to the previously described glial nuclei. Because of better yield, experiments normally were carried out with the N2 fraction.

Specific 5'-nucleotidase activity was always less than 3 % of the specific activity found in plasma membranes, where we found 160–190 nmol/min per mg.

The amount of PKC present in the purified nuclei and in nuclear membranes was determined by phorbol-ester-binding assays. We obtained 7.51 ± 2.8 pmol of [³H]phorbol dibutyrate binding/mg of protein (n = 3) for N1 nuclei, 3.88 ± 0.96 pmol/

Fig. 3. PKC activity in isolated nuclei

After treatment with 1.2 % (w/v) Triton X-100 for 10 min at room temperature, PKC activity can be stimulated with PtdSer/diolein (final concns. in the assay: 0.6 % Triton X-100; 8 mol % PtdSer; 2 mol % diolein). Histone H11S (a) and peptide GS (b) were used as substrates. Bars indicate means ± s.d. of triplicate determinations of an experiment representative of three.

mg of protein (n = 3) for N2 nuclei and 4.78 ± 0.76 pmol/mg of protein (n = 4) for nuclear membranes.

Activation of PKC by nuclear membranes

PKC in its soluble form in the cytoplasm is inactive. Activation takes place by translocating it to the plasma membrane. We first addressed the question of whether nuclear membranes too can activate PKC. To study this, kinases present in nuclear membranes were inactivated by short incubation at 65 °C. Then PKC purified from bovine brain was added together with [^γ-³²P]ATP. Inactivated membranes alone showed no kinase activity, but they could activate purified PKC (Fig. 1). However, activation was not maximal: additional dioloin (Fig. 1) or phorbol 12-myristate 13-acetate (PMA) (results not shown) led to a further stimulation of kinase activity.

Properties of nuclear PKC

In order to examine the properties of endogenous nuclear PKC, and to gain a first overview of its substrates, nuclear membranes were incubated with [^γ-³²P]ATP under different conditions. No difference in the phosphorylation pattern occurred whether no Ca²⁺ or 0.1 mM-CaCl₂ was added, or in the presence of 0.1 μM- and 1 μM-PMA (Fig. 2, lanes 1–4). On the other hand, the specific PKC inhibitors Ro 31-7549 (Compound 2) and Ro 31-8220 (Compound 3) totally inhibited the phosphorylation of some proteins (Fig. 2, lanes 5 and 6). The observed lack of stimulation by Ca²⁺ and PMA, together with the action of the inhibitors on the one hand and the phorbol-ester-binding data on the other hand, lead us to assume that the PKC in nuclear membranes may be present in a permanently active, so-called ‘membrane-inserted’, form (Bazzi & Nelsestuen, 1988).

To test whether, after treatment with detergent, phospholipid-stimulatable PKC activity can be demonstrated, as would be

Fig. 4. PKC activity in the presence of streptolysin O, Triton X-100 and inhibitory peptide

Phosphorylation of peptide GS can be inhibited by the inhibitory peptide (final concn. 10 μ M) under activating conditions (PtdSer/diolein; □) as well as under non-activating conditions (▨). Incubation of the nuclei before the phosphorylation assay for 10 min at room temperature with 1000 units of streptolysin O/ml had no significant effect on the PKC activity. Other conditions were as described in the Materials and methods section and the legend to Fig. 3. Bars indicate means $\pm$ S.D. of triplicate determinations of an experiment representative of three.

Fig. 5. Protein kinase activities after ion-exchange chromatography of nuclear extracts

Nuclear extracts were chromatographed on a small DEAE-cellulose anion-exchange chromatography column. The pooled kinase fractions eluted at about 100 mM-NaCl (Peak 1) and at about 200 mM-NaCl (Peak 2) and the non-extractable kinase activity (Pellet) were measured under stimulated (□) and unstimulated (▨) conditions with GS peptide as substrate. The concentration of the inhibitor (Inh.) peptide was 10 μ M. The activities given are the total activities of the respective fraction. The data are mean values of duplicate determinations from one experiment. The experiment was repeated twice with essentially the same result.

expected to be the case for a membrane-inserted PKC (Bazzi & Nelsestuen, 1988), nuclei were treated with Triton X-100 and thereafter the kinase activity was determined with histone HIIIS or peptide GS as substrate. In both cases similar results were obtained: whereas there was no stimulation of kinase activity by phospholipid in nuclei not treated with Triton X-100, such a stimulation could be observed after treatment (Fig. 3).

To exclude the possibility that the accessibility of the substrate could cause any problems, we permeabilized the nuclear membranes with streptolysin O. After permeabilization no differences as compared with the non-permeabilized nuclei could be detected (Fig. 4).

Fig. 6. Immunoblot analysis of PKC isoenzymes

Proteins were separated on SDS/8% acrylamide gels, blotted on PVDF membranes and probed for the PKC isoenzymes as described in the Materials and methods section. (a) Nuclear membranes: the antibodies against isoenzymes α and γ gave clear signals, whereas the antibody against isoenzyme β showed almost no reactivity. In each case a doublet of bands occurred. In contrast, in cytosolic (b) and plasma-membrane (c) fractions all three isoenzymes were present, each as a single band. The arrowhead indicates 80 kDa. The upper bands of the doublets are at approx. 82 kDa.

To ensure that we are dealing with phosphorylation by PKC, we used an inhibitory peptide, which has the sequence of the pseudosubstrate region of PKC and is a specific and competitive inhibitor of the phosphorylation of peptide GS by PKC (House & Kemp, 1987). The inhibitory peptide inhibited both the unstimulated and the phospholipid-stimulated phosphorylation of peptide GS (Fig. 4). About 20% of the maximal kinase activity remained, which probably is due to the activity of other kinases able to phosphorylate peptide GS (see below). Similar results were obtained when nuclear membranes instead of nuclei were employed for the assays (results not shown).

A further confirmation that the observed kinase activity was, at least for the most part, PKC activity was obtained from experiments involving ion-exchange chromatography. Nuclei were extracted with 1.2% Triton X-100 and the extract was chromatographed on a small DEAE-cellulose anion-exchange column as described in the Materials and methods section. Whereas no significant kinase activity was detected in the flow-through and wash fractions, two peaks of activity were eluted within the NaCl gradient: a major peak at approx. 100 mM-NaCl, and a second minor peak at approx. 200 mM-NaCl. The peak fractions were pooled and dialysed against the starting buffer. The kinase activity was measured together with the kinase activity which could not be extracted (pellet fraction) (Fig. 5). The kinase activity within the first peak comprised about 73% of the total activity and showed strong dependence on phospholipid: without phospholipid the kinase activity was near background level. The activities of the second peak and of the pellet fraction comprised 8.8 and 18% of the total activity respectively. Whereas the kinase activity within the first peak could be inhibited by the inhibitory peptide by 87%, the activity of the second peak (less than 10% of the total activity) could be only partially inhibited, and the activity of the pellet fraction was hardly affected at all.

PKC isoenzymes in nuclear membranes

Finally, we investigated which isoenzymes are present in the membranes of brain nuclei. For this purpose nuclear membrane proteins were separated by SDS/PAGE, blotted on a PVDF membrane and probed for PKC isoenzymes with isoenzyme-specific antibodies. We obtained clear signals with the antibodies against isoenzymes α and γ , in each case comprising a doublet of bands, whereas the antibody against isoenzyme β showed almost no reactivity (Fig. 6a). We therefore conclude that the α and γ isoenzymes are present in nuclear membranes. As a control, in cytosolic and plasma-membrane fractions we found all three isoenzymes (Figs. 6b and 6c).

DISCUSSION

In this paper we report that PKC, which we first identified by phorbol ester binding, is present in brain nuclei. Contamination

of the nuclear preparation by plasma membrane as the source of the PKC detected was very unlikely, because: (1) the specific activity of 5'-nucleotidase, a marker enzyme specific for plasma membrane, was very low in nuclei and nuclear membranes, always being below 3% of that found for isolated plasma membranes; (2) we found that the PKC content in isolated plasma membranes from brain (14–20 pmol of [³H]PDBu binding/mg of protein) is in any case too low to account for the observed nuclear PKC; (3) the proportions of the α , β and γ isoenzymes were different for nuclei, cytosol and plasma membranes.

Since little is known about the composition and properties of nuclear membranes, we asked whether they can activate PKC. We could show that exogenous PKC is activated by nuclear membranes and that this activity can be further stimulated by diolein. On the other hand, endogenous PKC activity could not be stimulated by phorbol ester or diolein, so that we had to show by other means that at least part of the kinase activity measured is due to PKC, although present in a special 'membrane-inserted' form (see below).

First, we used PKC inhibitors [Ro 31-7549 (Compound 2) and Ro 31-8220 (Compound 3)], which were described to have improved selectivity for PKC over staurosporine and K252a. The substances used inhibit PMA-induced phosphorylation of a known PKC substrate in a cellular system with IC₅₀ values of 4.4 μ M and 0.7 μ M respectively (Davis *et al.*, 1989). Under our conditions PKC in any case should be the kinase which is primarily affected. Indeed, these inhibitors lead to a general decrease in phosphorylation, as evidenced by the autoradiograph, but some bands disappeared completely or were very strongly depressed. These bands therefore most likely represent substrates phosphorylated by endogenous PKC. Apparently, these inhibitors act at the catalytic domain and are thus able to inhibit also the membrane-inserted PKC.

As an additional hint that the proteins the phosphorylation of which could be best inhibited with Compound 2 and Compound 3 are PKC substrates, we take the observation that these proteins are strongly hyperphosphorylated when purified PKC was added (R. Beckmann, K. Buchner & F. Hucho, unpublished work).

In assays *in vitro* for PKC activity associated with nuclear membranes, we used peptide GS as substrate. This peptide, derived from glycogen synthase, was modified, resulting in higher specificity for phosphorylation by PKC (House *et al.*, 1987). In combination with a pseudosubstrate-peptide PKC inhibitor which is a specific and competitive inhibitor of the phosphorylation of peptide GS by PKC (House & Kemp, 1987), this substrate peptide can be used to measure PKC activity specifically even in permeabilized cells (Alexander *et al.*, 1990). We can demonstrate a kinase activity associated with nuclear membranes which phosphorylates peptide GS and can be inhibited by a pseudosubstrate-peptide PKC inhibitor. Furthermore, this kinase activity can be stimulated by phosphatidylserine/diolein, but only after detergent treatment. Taken together, the observations strongly indicate that a special form of PKC activity is associated with brain nuclei.

Additional strong evidence that the kinase activity that we measured is indeed for the most part due to PKC activity came from experiments involving ion-exchange chromatography. Most of the kinase activity detected (73% of the total activity) phosphorylating peptide GS was eluted at 100 mM-NaCl, as expected for PKC. Furthermore, this kinase activity shows strong phospholipid-dependence and can be strongly inhibited (87% inhibition) by the pseudosubstrate-peptide inhibitor. The kinase activity under non-stimulating conditions is much lower than in the other experiments (see Fig. 4). This is very probably due to the removal of, e.g., endogenous lipids by the

chromatographic procedure. In the former experiments the PKC is present together with these components. As discussed above, it is probably present in a 'membrane-inserted' form and thus shows considerable activity, which, however, can be stimulated only after detergent treatment.

The kinase activity of the second peak (8.8% of the total activity) could be partially inhibited by the inhibitor peptide. As it is known that PKC can be eluted from DEAE-cellulose columns in two peaks (a major and a minor one) (Kikkawa *et al.*, 1983), the activity in the second peak could be at least partially due to PKC activity. However, because of the low amount of activity, the identity of the kinase(s) underlying this activity could not be further clarified.

The kinase activity in the pellet fraction could not be stimulated with phospholipid, although Triton X-100 was present in the assay. Furthermore, this activity showed only little inhibition by the inhibitory peptide, so that we conclude that the kinase showing this activity is not PKC, but another kinase (or kinases) able to phosphorylate peptide GS. This kinase most probably is mainly responsible for the residual kinase activity found after inhibition with the inhibitory peptide in the experiments depicted in Fig. 4.

In summary, the experiments using ion-exchange chromatography showed that under our conditions, besides PKC (peak 1), no other significant kinase activity could be detected in nuclei which can be effectively inhibited by the pseudosubstrate-peptide inhibitor.

We found PKC activity associated with nuclear membranes; we cannot exclude that PKC is also present in the nuclear matrix. However, reports using immunocytochemistry indicate a localization at the nuclear envelope or at the periphery of the nucleus. This holds for studies on brain (Girard *et al.*, 1985), as well as studies on cultured cells such as 3T3 fibroblasts or HL 60 cells. In most of these cases, however, PKC was found in nuclei only after stimulation of the cells with phorbol ester or other mitogens (Cambier *et al.*, 1987; Chen *et al.*, 1987; Thomas *et al.*, 1988; Leach *et al.*, 1989; Fields *et al.*, 1990; Hocevar & Fields, 1991). Whether PKC is located at the outer or inner membrane is not known. The nuclear substrates which were demonstrated to be phosphorylated after phorbol ester stimulation are lamin B (Fields *et al.*, 1988; Hornbeck *et al.*, 1988) and DNA topoisomerase II (Sahyoun *et al.*, 1986; Rottmann *et al.*, 1987), suggesting that the PKC is facing the interior of the nucleus. How this can be accomplished after translocation from the cytosol remains to be clarified, as up to now nothing is known about transport of PKC. The enzyme does not have a typical nuclear location signal, necessary for transport through nuclear-pore complexes.

A surprising result of the experiments presented here was that the PKC in brain nuclei is present in a permanently active membrane-inserted form. This form is Ca²⁺-independent, and binds phorbol ester without being stimulated. Only after removal from the membrane with detergent does the enzyme behave 'normally' again, i.e. it can be stimulated by phospholipid (Bazzi & Nelsestuen, 1988). The term 'membrane-inserted PKC' was coined by these authors to indicate the very tight association with phospholipid vesicles, which could not be reversed by EDTA, but only by treatment with detergent. Whether PKC is indeed converted into an integral membrane protein, or whether post-translational modifications play a role, remains unclear. However, as the occurrence of the membrane-inserted PKC was first observed in investigations employing both purified PKC and phospholipid vesicles, conversion into this form seems to be due to protein-lipid interactions and not to interaction with other proteins such as the very recently described 'receptors for activated C-kinase (RACKs)' (Mochly-Rosen *et al.*, 1991).

The membrane-inserted PKC which we found in nuclei is permanently active, but is not maximally activated, as demonstrated by the possibility to stimulate the enzyme further after detergent treatment. The observation that without detergent endogenous PKC cannot be stimulated is at first sight contradictory of the experiments including exogenous PKC, where stimulation with diolein could be detected. However, the PKC added to nuclear membranes could not be expected to be converted into the membrane-inserted form entirely or to a large extent, so that at least a large part of the added enzyme remains in a stimutable form.

Whether the PKC found in liver nuclei (Masmoudi *et al.*, 1989; Rogue *et al.*, 1990) is also at least in part membrane-inserted and permanently active is not clear, because in these studies detergent extracts of nuclei were used to assay PKC activity. The lack of PMA stimulation in our system which led us to the assumption of a membrane-inserted PKC is in contradiction to the observation with liver nuclei, where nuclei were stimulated with PMA, washed and then assayed for PKC activity (Buckley *et al.*, 1988). This led to a very rapid and dramatic (200-fold) increase in phospholipid-stimulated PKC activity. Others, however, could not confirm this enormous effect (Rogue *et al.*, 1990). Those authors found after PMA stimulation of isolated nuclei only an 8–10-fold increase in phospholipid-stimulated PKC in detergent extracts of the nuclei. This could be interpreted as a translocation of PKC from an unknown intranuclear pool to the nuclear membrane upon PMA stimulation. Why this pool could not be activated by phospholipid in nuclear extracts of control nuclei is unknown. The differences from our work may be due to different assay conditions and/or to tissue differences. One important difference is that we found the α and γ subspecies associated with nuclear membranes, whereas the liver nuclear PKC was reported to be the β II subspecies (Rogue *et al.*, 1990).

The lack of the β isoform of PKC in brain nuclei is in accordance with immunohistochemical work on brain (Hosoda *et al.*, 1989) and is a further indication of isotype-specific subcellular distribution of PKC. The β subspecies is the most abundant PKC form in cerebral cortex (Shearman *et al.*, 1987).

A recent report claimed that the ζ isoform is associated with rabbit brain nuclei (Hagiwara *et al.*, 1990). However, those authors found immunoreactivity with a protein of 80 kDa, whereas the calculated molecular mass of the ζ subspecies is 64 kDa. This size was also found after SDS/PAGE of this subspecies expressed in COS cells (Ono *et al.*, 1989). What the reason for this discrepancy is, and if there is perhaps a ζ subspecies of 80 kDa present in bovine brain nuclei cross-reacting with our antibodies against the α and γ subspecies, remains to be determined.

One interesting observation in our immunoblot analysis was the occurrence of a doublet of bands for each isoform (Fig. 6). These doublets may perhaps be due to different phosphorylation states. Patel & Stabel (1989) observed a doublet of bands after SDS/PAGE for the γ -isoform expressed in a baculovirus system and showed that, after treatment with potato acid phosphatase, both bands shifted to a single band of lower apparent molecular mass. Recently it was shown that there is a correlation between the extent of phosphorylation and the activity of PKC (Mitchell *et al.*, 1989). We therefore speculate that the observed doublets of PKC immunoreactive bands at least in part reflect the active state of the membrane-inserted enzyme.

The origin of the permanently active PKC in nuclear membranes is not clear up to now. As activation of PKC by elevated Ca^{2+} levels causes part of the enzyme to become stably associated with membranes (Wolf *et al.*, 1985), probably owing to conversion into the membrane-inserted form, repeated activation *in vivo* may lead to cumulative membrane insertion of

the enzyme. It was suggested that the membrane-inserted PKC may represent in this way a kind of 'cellular memory of previous activation' (Burgoyne, 1989). The regulation of the permanently active PKC present in nuclear membranes, perhaps by the action of a calpain-like proteinase, and the role of the components of the nuclear envelope remain to be elucidated.

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