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The major endogenous bovine brain protein kinase C inhibitor is a heat-labile protein

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A crude cytosolic fraction prepared from bovine brain contained protein kinase C, as shown by immunoblotting, but its activity was undetectable, suggesting the presence of interfering factors. Phosphatase, ATPase and protease activities did not account for the absence of detectable protein kinase C activity. The major contributing factor was found to be a heat-labile protein which was separated from the kinase by ion-exchange chromatography. The contribution to the total inhibitory activity of heat-stable proteins was relatively minor, suggesting that they may not function physiologically as protein kinase C inhibitors.

Protein kinase C inhibitor; Bovine brain

1. INTRODUCTION

Protein kinase C (PKC) activity is often underestimated or even undetected in crude tissue extracts such as the cytosolic fraction obtained by high-speed centrifugation of homogenates prepared in the presence of divalent cation chelators (e.g. [1,2]). Fractionation of such extracts by ion-exchange or hydrophobic-interaction chromatography is commonly used to separate the kinase from endogenous factors which mask its activity in the crude extract, thereby allowing its activity to be measured (e.g. [1–3]). Such factors may include protein phosphatases, ATPases, proteases and heat-stable and heat-labile protein inhibitors. The purpose of this study was to evaluate the relative importance of these factors in the inability to detect PKC activity in a crude cytosolic fraction derived from bovine brain. Quantitatively, the most important factor was identified as a heat-labile protein inhibitor. It is proposed that this protein functions to restrict PKC-catalyzed phosphorylations to membrane-, organelle- and cytoskeletal-associated protein substrates by inhibiting cytosolic forms of PKC, which is known to phosphorylate a wide range of proteins in vitro [4].

Abbreviations: EGTA, ethylenebis(oxyethylenenitrilo)tetraacetic acid; PKC, protein kinase C; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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2. MATERIALS AND METHODS

2.1. *Materials*

[γ-^{32}P]ATP (25 Ci/mmol and 4500 Ci/mmol) was purchased from ICN Biomedicals (St. Laurent, Quebec, Canada). A monoclonal antibody recognising both α and β isoenzymes of PKC was purchased from Amersham Corp. (Oakville, Ontario, Canada) and monoclonal antibodies specific for the α, β and γ isoenzymes of PKC were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Calf thymus lysine-rich histone fraction III-S, EDTA, EGTA, Triton X-100 and prestained M_r markers for electrophoresis were purchased from Sigma Chemical Co. (St. Louis, MO). DEAE-Sephacel from Pharmacia (Baie d'Urfe, Quebec, Canada), L-α-phosphatidyl-L-serine (beef brain) and 1,2-diolein from Serdary Research Laboratories (London, Ontario, Canada), and M_r marker proteins and electrophoresis reagents from Bio-Rad Laboratories (Mississauga, Ontario, Canada). General laboratory reagents used were analytical grade or better and were purchased from Fisher Scientific (Calgary, Alberta, Canada).

2.2. *Protein purification*

Rat brain PKC was purified by a modification of the procedure of Wolf et al. [5], using DEAE-Sephacel anion-exchange chromatography and phenyl-Sepharose hydrophobic-interaction chromatography. The isolated protein exhibited a molecular mass of 80 000 Da on SDS-PAGE and was positively identified by Western blotting using a monoclonal antibody to bovine brain PKC which recognises the α and β isoenzymes. The specific activity of the purified kinase using histone III-S as substrate was 324 nmol P_i/mg PKC·min in the presence of Ca^{2+}, phospholipid and diacylglycerol, and 18 nmol P_i/mg PKC·min in the absence of these cofactors.

2.3. *Extraction of bovine brain PKC*

Procedures were carried out at 4°C or on ice. Bovine brain (0.5 g stored at −70°C) was homogenized using a Brinkmann Polytron for 3 × 15 s in 15 vols. of Buffer A (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 50 mM β-mercaptoethanol) and centrifuged at 100 000 × g for 30 min. The supernatant (≥1) was immediately divided into 0.5-ml aliquots in Eppendorf tubes and stored at −70°C. The pellet was resuspended in 15 vols. of Buffer B (20 mM Tris-HCl, pH 7.5, 5 mM EGTA, 2 mM EDTA, 1 mM phenylsulfonyl fluoride, 50 mM β-mercaptoethanol), stirred for 30 min and centrifuged at 100 000 × g

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for 30 min. The supernatant (S2) was stored as for S1. The pellet was resuspended in 15 vols. of Buffer C (Buffer B + 0.2% Triton X-100 + 10% glycerol), stirred for 30 min and centrifuged at 100 000 × g for 30 min. The supernatant (S3) was stored as for S1 and the pellet was discarded. This protocol for PKC extraction was taken from Leibersperger et al. [6].

2.4. *Enzymatic assays*

PKC activity was routinely measured by the mixed micellar assay [7] under the following conditions: 20 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 0.37 mM phosphatidylserine, 0.093 mM 1,2-diolein, 0.03% (w/v) Triton X-100, 0.2 mg/ml histone III-S, 20 μM [γ-^{32}P]ATP (~ 500 cpm/pmol) and either 0.1 mM free $CaCl_2$ or 1 mM EGTA. Any variations of these reaction conditions are noted in the text. Reactions were initiated by the addition of [γ-^{32}P]ATP and incubated at 30°C. Samples (0.1 ml) of reaction mixtures were withdrawn at selected times and quenched immediately by addition to 0.5 ml of 25% (w/v) trichloroacetic acid, 2% (w/v) sodium pyrophosphate (for quantification of protein-bound [^{32}P]phosphate as previously described [8]) or to 0.1 ml of boiling SDS gel sample buffer (for SDS-PAGE and autoradiography). ATPase activity was measured as described by Ikebe and Hartshorne [9].

2.5. *Other methods*

Protein concentrations were determined by the Coomassie brilliant blue dye-binding assay [10]. SDS-PAGE was carried out using 7.5–20% polyacrylamide gradient slab gels with a 5% acrylamide stacking gel in the presence of 0.1% SDS at 32 mA using the discontinuous buffer system of Laemmli [11]. Western blotting was conducted as previously described [12] and PKC detected following incubation of the nitrocellulose sheets with monoclonal anti-PKC followed by rabbit anti- (mouse IgG) coupled to alkaline phosphatase. The substrate 5-bromo- 4-chloro-3-indolyl phosphate/Nitro blue tetrazolium was used to detect the bound phosphatase [13].

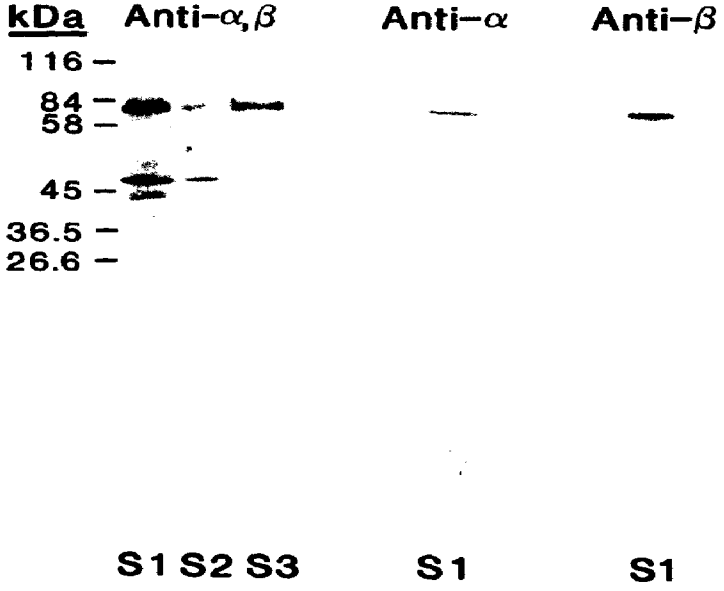

Fig. 1. Detection of PKC by Western blotting of bovine brain extracts. S1, S2 and S3 were subjected to SDS-PAGE and immunoblotting as described in section 2. The positions of prestained M_r markers are indicated at the left. PKC was detected using monoclonal antibodies recognising both the α and β isoenzymes (Anti-$\alpha\beta$) or just the α (Anti-α) or β (Anti-β) isoenzymes.

3. RESULTS

Fig. 1 illustrates the presence, in fractions S1, S2 and S3 derived from bovine brain, of PKC (M_r ~ 80 000) detected with a monoclonal antibody recognising the α and β isoenzymes of PKC. Monoclonal antibodies specific for α or β isoenzymes were demonstrated both to be present in the S1 fraction (Fig. 1). The γ isoenzyme was not detected in S1 using a monoclonal γ-specific antibody at the same protein loading level. Whereas PKC was predominant in S1 (Fig. 1), no phosphorylation of histone III-S by this fraction was observed in the presence or absence of Ca^{2+}, phospholipid and diacylglycerol (Fig. 2C). On the other hand, S3 did exhibit histone kinase activity which was enhanced by Ca^{2+} and the lipid cofactors (Fig. 2C). Therefore, S1 contains a factor(s) which masks PKC activity. A quantitative comparison of histone phosphorylation by S1 and S3 in the presence of Ca^{2+}, phospholipid and diacylglycerol is shown in Fig. 3. PKC activity was not detected in S1 using either the mixed micellar assay (Figs. 2 and 3) or the liposomal assay described by Kikkawa et al. [14] (data not shown).

Sahyoun et al. [15] have identified in rat liver a protein phosphatase which dephosphorylates lysine-rich histone phosphorylated by PKC. However, S1 was found not to contain significant amounts of histone phosphatase (Fig. 4). This experiment, however, does not rule out the presence of Ca^{2+}/calmodulin-dependent (type 2B) phosphatase. This was achieved by treatment of histone III-S (phosphorylated with rat brain PKC and dialyzed to remove ATP) with S1 or buffer: no significant dephosphorylation occurred during a 5-min reaction. Direct measurement of ATPase activity under standard PKC assay conditions in the presence of S1 revealed that < 2.5% of the ATP was hydrolyzed during a 10-min reaction. Furthermore, PKC activity was not detected in S1 assayed at 5-times the standard free [Ca^{2+}] or 5-times the standard [lipid] (data not shown). In addition, there was no evidence of proteolysis of PKC during the reactions depicted in Fig. 2, as shown by Western blotting of the reaction mixture at the end of the reaction (data not shown).

These results suggested that the inability to detect PKC activity in S1 is due to endogenous inhibitory activity. This activity was found to be non-dialyzable and was destroyed by trypsin treatment. Fractionation of S1 by ion-exchange chromatography unmasked the PKC activity (Fig. 5A). The presence of inhibitory activity in the column eluate was examined by assaying inhibition of PKC activity of the peak fraction (no. 60) by samples of other column fractions (Fig. 5B). To distinguish between heat-labile and heat-stable inhibitory activities, samples of the column fractions were either untreated or heated at 100°C for 2 min and centrifuged to remove denatured protein prior to the assay. The major inhibitory activity eluted from the column

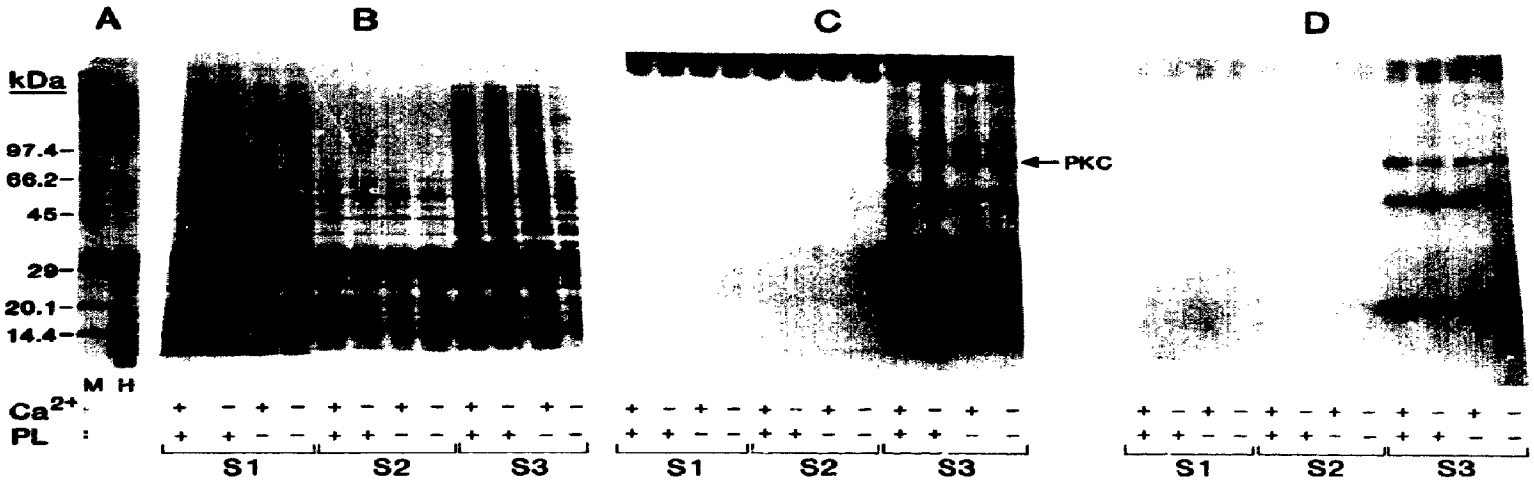

Fig. 2. PKC activity is only detected in fraction S3. S1, S2 and S3 were incubated with (panels B and C) or without (panel D) histone III-S under PKC assay conditions (see section 2) in the presence or absence of Ca^{2+} and phospholipid/diacylglycerol (PL) as noted. At the end of the 5-min incubation, samples of reaction mixtures were subjected to SDS-PAGE and autoradiography. Panel A shows the Coomassie blue staining pattern of M_r markers (M) and the histone III-S substrate (H). Panel B shows the stained gel of the S1, S2 and S3 reaction mixtures containing the histone substrate. Panel C shows the autoradiogram of panel B, and panel D the autoradiogram of control reactions from which the histone was omitted. Autophosphorylation of PKC is apparent in panels C and D.

after PKC and was heat-labile (Fig. 5B). Two peaks of inhibitory activity were detected following heat-treatment of column fractions: one in the flow-through fractions and one in fractions 64–68, the trailing edge of the PKC peak (Fig. 5), consistent with previous observations [16]. The heat-labile inhibitor was also found to be labile to heating at 70°C for 5 min (Table 1).

4. DISCUSSION

It has often been observed that PKC activity is underestimated in assays of crude tissue extracts due to the presence of endogenous interfering factors. We have investigated the nature of these factors in bovine brain and report that the major component involved is a heat-labile protein inhibitor. This inhibitor, present in a cytosolic fraction obtained by high-speed centrifugation of a brain homogenate prepared in the presence of EDTA to solubilize membrane-associated (but not integral) PKC, was separated from PKC by ion-exchange chromatography. Recombination of inhibitor-containing fractions with PKC resulted in inhibition of PKC activity. The inhibitor was shown to be non-dialyzable and sensitive to proteolysis.

Table I

Inhibitory activity is heat-sensitive

Inhibitor[a]	PKC activity (pmol P_i)
None	530.1 ± 13.2
Untreated	199.3 ± 4.6
70°C × 2 min	685.7 ± 7.9
100°C × 2 min	580.0 ± 8.4

[a]Inhibitor refers to pooled fractions 70–76 from the ion-exchange column. PKC activity of fraction no. 60 (10 μl/250 μl assay volume) was measured in the absence of inhibitor and in the presence of 50 μl of untreated inhibitor or inhibitor heat-treated as indicated. Values (pmol P_i incorporated in 5 min) represent the mean ± SD (n=3).

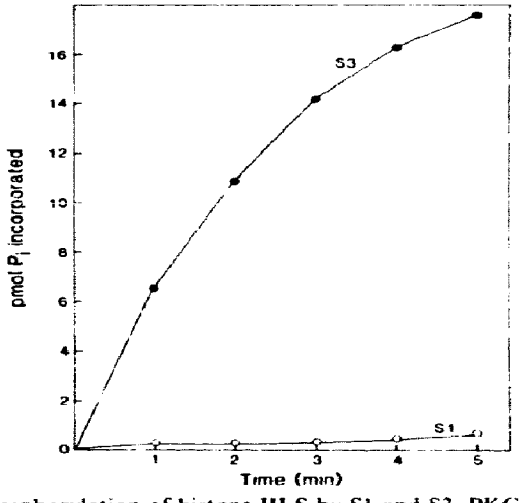

Fig. 3. Phosphorylation of histone III-S by S1 and S3. PKC activity in S1 and S3 was assayed in the presence of Ca^{2+}, phospholipid and diacylglycerol under conditions described in section 2. S1 and S3 were added to 20% (v/v), i.e. protein concentrations of 0.25 and 0.14 mg/ml, respectively. Values represent the means of 7 determinations for S1 and 3 determinations for S3.

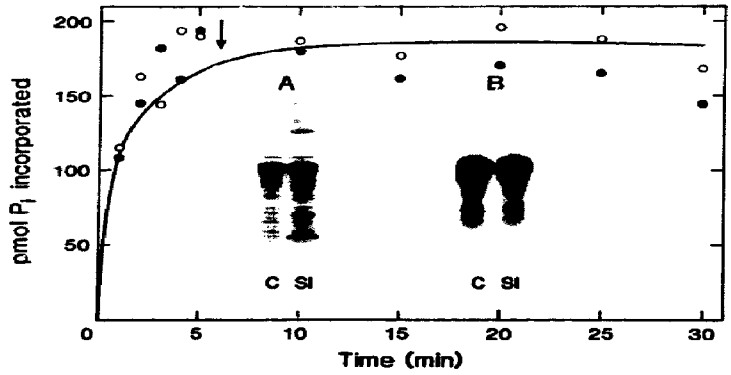

Fig. 4. S1 does not contain significant histone phosphatase activity. Histone III-S (0.24 mg/ml) was phosphorylated in duplicate by purified rat brain PKC (1.4 μg/ml) under standard conditions in the presence of Ca^{2+}, phospholipid and diacylglycerol as described in section 2. After 6 min incubation (indicated by the arrow), EGTA (1 mM) was added to both reaction mixtures to inactivate the kinase and S1 was added to one reaction mixture (closed circles) and buffer to the other (open circles). These additions diluted the histone to 0.2 mg/ml to ensure that reaction conditions were then identical to those of Figs. 2 and 3. [^{32}P]P_i incorporation was quantified at the times indicated. At the end of the reaction, samples of reaction mixtures were subjected to SDS-PAGE and autoradiography. Panel A shows the Coomassie blue staining pattern of the control (buffer) reaction mixture (C) and the S1-containing reaction mixture (S1). Panel B depicts the corresponding autoradiogram.

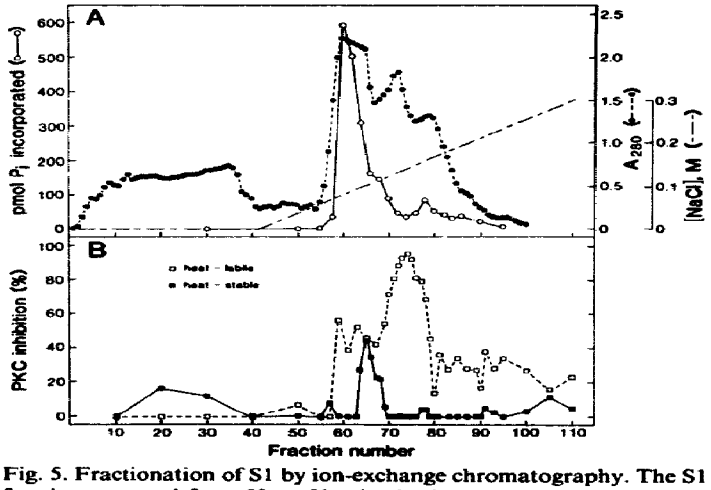

Fig. 5. Fractionation of S1 by ion-exchange chromatography. The S1 fraction prepared from 50 g of bovine brain was applied to a column (1 × 40 cm) of DEAE-Sephacel previously equilibrated with Buffer A at a flow rate of 20 ml/h collecting 4-ml fractions. After washing the column with Buffer A, bound proteins were eluted with a [NaCl] gradient (— - —). The protein elution profile is shown by measurements of A_{280} (●- - -●), and PKC activity (○—○) of selected fractions was measured in the presence of Ca^{2+}, phospholipid and diacylglycerol under standard conditions using 20 μl of each fraction in a total reaction volume of 250 μl, 10 μM [γ-^{32}P]ATP and a 5-min incubation period (panel A). Selected fractions were also assayed for heat-labile (□- - -□) and heat-stable (■—■) PKC inhibitory activity (panel B). Heat-labile activity was determined by measuring PKC activity of fraction no. 60 (10 μl) in the presence of samples (50 μl) of selected fractions and subtracting the activity in the absence of fraction no. 60. An ATP concentration of 10 μM and a 5-min incubation period were used with a total reaction volume of 250 μl. Heat-stable activity was measured similarly following heat-treatment of samples of the selected fractions at 100°C for 2 min and centrifugation. Control PKC activity (0% inhibition) for panel B was 144.5 ± 9.3 pmol P_i incorporated in 5 min (n=15).

While heat-stable PKC activity was detected in bovine brain following ion-exchange chromatography (this study) or hydrophobic-interaction chromatography [3], it is apparent that the heat-labile inhibitory activity described here is quantitatively much more important. This raises the possibility that PKC inhibition by heat-stable proteins may not be a physiological function. Several inhibitors of PKC, some of which are heat-stable, have been reported [16–23]. Four of these have been purified: calmodulin [17,21], a sheep brain inhibitor existing as multiple isoforms of 29–33 kDa [23], and 17 kDa and 12 kDa inhibitors from bovine brain [16]. However, we recently found that the PKC inhibitory activity of the 12 kDa protein is associated with a trace contaminating protein, not the 12 kDa protein itself, which was identified as the immunophilin, FK506-binding protein (Pearson, J.D. et al., submitted).

It is unlikely that the heat-labile inhibitor reported here is the same as the sheep brain inhibitor described by Toker et al. [23] since the former is labile to heating at 70°C for 5 min whereas the latter is insensitive to such treatment.

In conclusion, we have demonstrated that the major factor responsible for the inability to detect PKC activity in a bovine brain cytosolic fraction is a heat-labile protein inhibitor. This protein may function physiologically to prevent the phosphorylation of proteins by active cytosolic forms of PKC and thereby restrict PKC-catalyzed phosphorylations to membrane-, organelle- and cytoskeletal-associated protein substrates.

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