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(oxyethylenenitri)tetraacetic acid; PKC, protein kinase C; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis

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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 Leibers-
perger 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 
MgCl₂, 0.37 mM phosphatidylserine, 0.063 mM 1,2-diolein, 0.03% 
(w/v) Triton X-100, 0.2 mg/ml histone III-S, 20 µM [γ-³²P]ATP (~ 500 
cpm/µmol) and either 0.1 mM free CaCl₂ or 1 mM EGTA. Any 
variations of these reaction conditions are noted in the text. Reactions 
were initiated by the addition of [γ-³²P]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) trichlo-
roacetic acid, 2% (w/v) sodium pyrophosphate (for quantification of 
protein-bound [³²P]phosphate as previously described [8]) or to 0.1 ml 
of boiling SDS gel sample buffer (for SDS-PAGE and autoradiog-
raphy). 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% SOS at 32 mA using the discontinuous 
gel buffer system of Laemmli [11]. Western blotting was conducted as 
previously described [12] and PKC detected follo\ing incubation of 
the nitrocellulose sheets with monoclonal anti-PKC followed by r
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].

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3. RESULTS

Fig. 1 illustrates the presence, in fractions S1, S2 and 
S3 derived from bovine brain, of PKC (Mr ~ 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 phosphoryla-
tion of histone III-S by this fraction was observed in 
the presence or absence of Ca²⁺, phospholipid and dia-
glycerol (Fig. 2C). On the other hand, S3 did exhibit 
histone kinase activity which was enhanced by Ca²⁺ 
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 Ca²⁺, 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 pro-
tein 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 Ca²⁺/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 
[Ca²⁺] 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 ac-
tivity in the column eluate was examined by assaying 
histone phosphorylation by the peak fraction (no. 69) 
by samples of other column fractions (Fig. 5B). To 
distinguish between heat-labile and heat-stable inhibi-
tory activities, samples of the column fractions were 
either untreated or heated at 100°C for 2 min and cen-
trifuged to remove denatured protein prior to the assay. 
The major inhibitory activity eluted from the column
after PKC and was heat-labile (Fig. 5B). Two peaks of inhibitor 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 I).

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.

![Graph showing phosphorylation of histone III-S](image)

**Fig. 3.** Phosphorylation of histone III-S by S1 and S3. PKC activity in S1 and S3 was assayed in the presence of Ca²⁺, phospholipid and diacylglycerol under conditions described in section 2. S1 and S3 were added to 20% (v/v), i.e. protein concentrations of 0.35 and 0.14 mg/mL respectively. Values represent the means of 7 determinations for S1 and 3 determinations for S3.

**Table 1**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>PKC activity (pmol P)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>530.1 ± 13.2</td>
</tr>
<tr>
<td>Untreated</td>
<td>199.3 ± 4.6</td>
</tr>
<tr>
<td>70°C × 2 min</td>
<td>685.7 ± 7.9</td>
</tr>
<tr>
<td>100°C × 2 min</td>
<td>580.0 ± 8.4</td>
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</table>

*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, incorporated in 5 min) represent the mean ± SD (n=3).
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 Ca²⁺, 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. [³²P]P, 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 (SI). Panel B depicts the corresponding autoradiogram.

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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