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Properties and distribution of the protein inhibitor (M_r 17000) of protein kinase C

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Ca^{2+}-dependent hydrophobic-interaction chromatography is a powerful tool for the identification and isolation of a variety of Ca^{2+}-binding proteins which expose a hydrophobic site(s) in the presence of Ca^{2+} [Gopalakrishna & Anderson (1982) Biochem. Biophys. Res. Commun. **104**, 830–836; Walsh, Valentine, Ngai, Carruthers & Hollenberg (1984) Biochem. J. **224**, 117–127; McDonald & Walsh (1985) Biochem. J. **232**, 559–567]. Using this approach, we isolated two potent and specific protein inhibitors of protein kinase C, of 17 kDa [McDonald & Walsh (1985) Biochem. J. **232**, 559–567] and 12 kDa [McDonald & Walsh (1986) Biochem. Soc. Trans. **14**, 585–586]. Although these inhibitors were purified by Ca^{2+}-dependent hydrophobic-interaction chromatography and exhibit properties similar to those of calmodulin and related Ca^{2+}-binding proteins, we were unable to demonstrate high-affinity Ca^{2+} binding to these inhibitors, using equilibrium dialysis. Protein kinase C exhibited half-maximal activity at 0.6 μM-Ca^{2+} in the presence of phospholipid and diacylglycerol, and complete inhibition by both inhibitors was observed over the range of Ca^{2+} concentrations examined (10 nM–10 μM). These observations suggest that the inhibitory action of these proteins does not require Ca^{2+}. The inclusion of proteinase inhibitors during isolation of the kinase C inhibitors, as well as two-dimensional peptide mapping and amino acid analysis of the isolated proteins, suggested that the 12 kDa inhibitor is a proteolytic fragment of the 17 kDa protein which is generated during purification. Antibodies raised in rabbits against the bovine brain 17 kDa inhibitor were shown to be specific by Western immunoblotting and the competitive enzyme-linked immunosorbent assay method and were used to study the tissue and species distribution of this protein. The inhibitor was found to be present in several bovine, murine, avian and human tissues, consistent with a role in the regulation of a variety of physiological functions involving the widely distributed protein kinase C.

INTRODUCTION

It is now widely accepted that the Ca^{2+}- and phospholipid-dependent protein kinase (protein kinase C), originally discovered by Inoue *et al.* (1977), plays a major role in coupling extracellular messenger-induced polyphosphoinositide turnover with diverse cellular events (for reviews, see Berridge, 1984; Berridge & Irvine, 1984; Nishizuka, 1984; Kikkawa *et al.*, 1985; Hirasawa & Nishizuka, 1985). Several reports suggest that protein kinase C may be involved in negative-feedback regulation of this extracellular messenger-induced polyphosphoinositide turnover (Orellana *et al.*, 1985; Watson & Lapetina, 1985; Cooper *et al.*, 1985). Such negative-feedback phenomena may occur at the level of the receptor for the extracellular signal by, for example, decreasing the affinity of the receptor for its agonist (Thompoulas *et al.*, 1982; Corvera *et al.*, 1986), and in some cases this has been shown to be closely correlated with receptor phosphorylation (Leeb-Lundberg *et al.*, 1985; May *et al.*, 1985). However, negative-feedback control may also occur distal to the receptor, for example, via inhibition of polyphosphoinositide metabolism (Brock *et al.*, 1985; Naccache *et al.*, 1985; Gispen, 1986).

Previously we (McDonald & Walsh, 1985*a*) reported the isolation of a potent inhibitor of protein kinase C, of 17 kDa, during the large-scale purification of a now well-characterized Ca^{2+}-binding protein of 21 kDa (21 kDa CaBP) from bovine brain (Walsh *et al.*, 1984; McDonald & Walsh, 1985*a*; McDonald *et al.*, 1985, 1987). The 17 kDa inhibitor was found to have an amino acid composition similar to, but distinct from, that of members of the calmodulin family of Ca^{2+}-binding proteins (McDonald & Walsh, 1985*a*), but was by far the most potent inhibitor of protein kinase C when compared with other calciproteins (McDonald & Walsh, 1985*b*). The inhibitory effect of the 17 kDa inhibitor could not be attributed to interaction with substrates or cofactors of protein kinase C (McDonald & Walsh, 1985*b*) or to phosphatase activity (McDonald & Walsh, 1986). In addition, inhibition of kinase C activity was shown to be an intrinsic property of the 17 kDa protein, since inhibition was destroyed by trypsin digestion (McDonald & Walsh, 1986).

We subsequently discovered another heat-stable kinase C inhibitor, of 12 kDa, which co-purified with the 17 kDa inhibitor during Ca^{2+}-dependent hydrophobic-interaction chromatography and could be separated from it by ion-exchange chromatography (McDonald & Walsh, 1986). We report here on the biochemical properties and tissue and species distribution of these two protein kinase C inhibitors, and conclude that the 17 kDa protein is the native inhibitor, whereas the

Abbreviations used: PAGE, polyacrylamide-gel electrophoresis; CaBP, Ca^{2+}-binding protein; ELISA, enzyme-linked immunosorbent assay.

12 kDa protein is an active proteolytic fragment corresponding to the *N*-terminus of the 17 kDa inhibitor.

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP (10–40 Ci/mmol) and $^{45}CaCl_2$ (10–40 mCi/mg of Ca) were purchased from Amersham (Oakville, Ont., Canada). Phenyl-Sepharose CL-4B and DEAE-Sephacel were purchased from Pharmacia (Mississauga, Ont., Canada). Goat anti-(rabbit IgG)–horseradish peroxidase conjugate, goat anti-(rabbit IgG)–alkaline phosphatase conjugate, 4-chloro-1-naphthol, 5-bromo-4-chloroindol-3-yl phosphate, *p*-nitrophenyl phosphate, histone III-S, 1,3-diolein, L-α-phosphatidyl-L-serine, Freund's complete adjuvant, benzamidine and phenylmethanesulphonyl fluoride were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Nitrocellulose membranes, M_r marker proteins and electrophoresis reagents were purchased from Bio-Rad Laboratories (Richmond, CA, U.S.A.), and leupeptin, pepstatin and soya-bean trypsin inhibitor were from U.S. Biochemical Corp. (Cleveland, OH, U.S.A.). General laboratory reagents used were of analytical grade or better and were purchased from Fisher Scientific (Calgary, Alberta, Canada). Bovine brain Ca^{2+}-binding proteins of 27 kDa (Waisman *et al.*, 1983) and 48 kDa and 63 kDa (Waisman *et al.*, 1985) were generously provided by Dr. M. Tokuda and Dr. N. Khanna, calcineurin (β subunit) was from Dr. C. Pallen, and casein kinase I from Dr. T. Singh, all of the Department of Medical Biochemistry, University of Calgary. Human platelets were generously donated by Dr. J. Northup, Department of Pharmacology and Therapeutics, University of Calgary.

Protein purification

Protein kinase C was purified from bovine brain by the method of Walsh *et al.* (1984) and from human platelets as described by Lim *et al.* (1985). P47 protein (Imaoka *et al.*, 1983) was purified from human platelets as a by-product of the kinase C (Lim *et al.*, 1985). Calmodulin was purified from bovine brain by a modification of the method of Gopalakrishna & Anderson (1982) as described in detail by Walsh *et al.* (1984). The 21 kDa CaBP was purified from bovine brain by a modification of the method of Walsh *et al.* (1984) as described in detail by McDonald *et al.* (1987). The 17 kDa protein inhibitor of kinase C and a novel 12 kDa protein inhibitor of kinase C (see the Results section) were purified from bovine brain by a modification of the method of McDonald & Walsh (1985*a*) as follows. All procedures were carried out at 4 °C. Homogenization of frozen bovine brains (500 g) was carried out as previously described (Walsh *et al.*, 1984) in the presence or absence of proteinase inhibitors (phenylmethanesulphonyl fluoride, 75 mg/l; leupeptin, 1 mg/l; pepstatin, 1 mg/l; soya-bean trypsin inhibitor, 10 mg/l; benzamidine, 10 mM). Centrifugation, precipitation with $(NH_4)_2SO_4$ and Ca^{2+}-dependent hydrophobic-interaction chromatography on phenyl-Sepharose CL-4B were performed as previously described (Walsh *et al.*, 1984). Proteins bound to the phenyl-Sepharose column in a Ca^{2+}-dependent manner were eluted with EGTA. The elution profiles obtained from preparations carried out in the absence or presence of proteinase inhibitors (see above) are shown in Figs. 1 and 2 respectively. Relatively large volumes (200 μl) of selected fractions were freeze-dried before SDS/PAGE analysis in order to detect proteins present in small amounts. Fractions enriched in calmodulin (pool A), the 21 kDa CaBP (pool B) and the 17 kDa and 12 kDa kinase C inhibitors (pool C) were pooled as indicated in Figs. 1 and 2. Pool A was used for the purification of calmodulin (Walsh *et al.*, 1984). Further fractionation of pools B and C to yield purified proteins was identical, and therefore only the protocol as applied to pool C (Fig. 1) will be described in detail here. This pooled material was heat-treated, cooled and centrifuged as previously described (Walsh *et al.*, 1984). The resultant supernatant was dialysed overnight against two changes (10 l each) of 10 mM-NH_4HCO_3 (pH 8.0) and freeze-dried. The residue was dissolved in approx. 20 ml of 20 mM-Tris/HCl (pH 7.5) buffer containing 1 mM-EGTA and 0.5 mM-dithiothreitol and loaded on a column (1 cm × 40 cm) of DEAE-Sephacel previously equilibrated with the same buffer. Unbound proteins were washed through the column with equilibration buffer, and bound proteins were eluted with a linear 0–0.4 M-NaCl gradient generated from 200 ml each of equilibration buffer and equilibration buffer containing 0.4 M-NaCl (Fig. 3). Selected fractions were examined by SDS/PAGE and assayed for their ability to inhibit protein kinase C-catalysed phosphorylation of histone III-S as described below. This final purification step yielded not only the 17 kDa kinase C inhibitor (pool II), but also another kinase C inhibitor of 12 kDa (pool I) and an isoform of the 21 kDa CaBP (pool III). Similar fractionation of the 21 kDa CaBP-enriched pool (pool B, Fig. 1) on DEAE-Sephacel resulted in the purification of the 21 kDa CaBP and both the 17 kDa and 12 kDa kinase C inhibitors. The 12 kDa and 17 kDa inhibitors were obtained in yields of 1.5–2 and 3–4 mg/kg of bovine brain respectively, when isolated in the absence of proteinase inhibitors. However, when a cocktail of proteinase inhibitors (see above) was included in the homogenization buffer, the yield of the 12 kDa inhibitor was decreased to 0.5–1 mg/kg and that of the 17 kDa inhibitor increased to approx. 6 mg/kg of brain.

Immunological procedures

Polyclonal antibodies against purified bovine brain 17 kDa inhibitor of kinase C were raised in two rabbits. The 17 kDa inhibitor (10 μg) in 0.5 ml of phosphate-buffered saline (8 mM-Na_2HPO_4/1.5 mM-KH_2PO_4/0.137 M-NaCl/2.7 mM-KCl, pH 7.2) was mixed with an equal volume of Freund's complete adjuvant and injected intradermally at multiple sites; 1 week later, four 10 μg doses of antigen were injected at multiple sites subcutaneously and intradermally alternately every 3 days. These initial injections were followed at 3-day intervals by a further five booster injections, containing 50 μg, 100 μg, 100 μg, 150 μg and 250 μg, irrespectively, of purified 17 kDa inhibitor. The animals were bled completely 40 days after the first injection of antigen. Anti-(17 kDa inhibitor) was purified as described by Ngai & Walsh (1985). Tissue sample preparation and immunoblotting [with goat anti-(rabbit IgG)–horseradish peroxidase conjugate as secondary antibody] were carried out as previously described (Ngai & Walsh, 1985). In addition, immunoblotting using goat anti-(rabbit IgG)–alkaline phosphatase conjugate as secondary

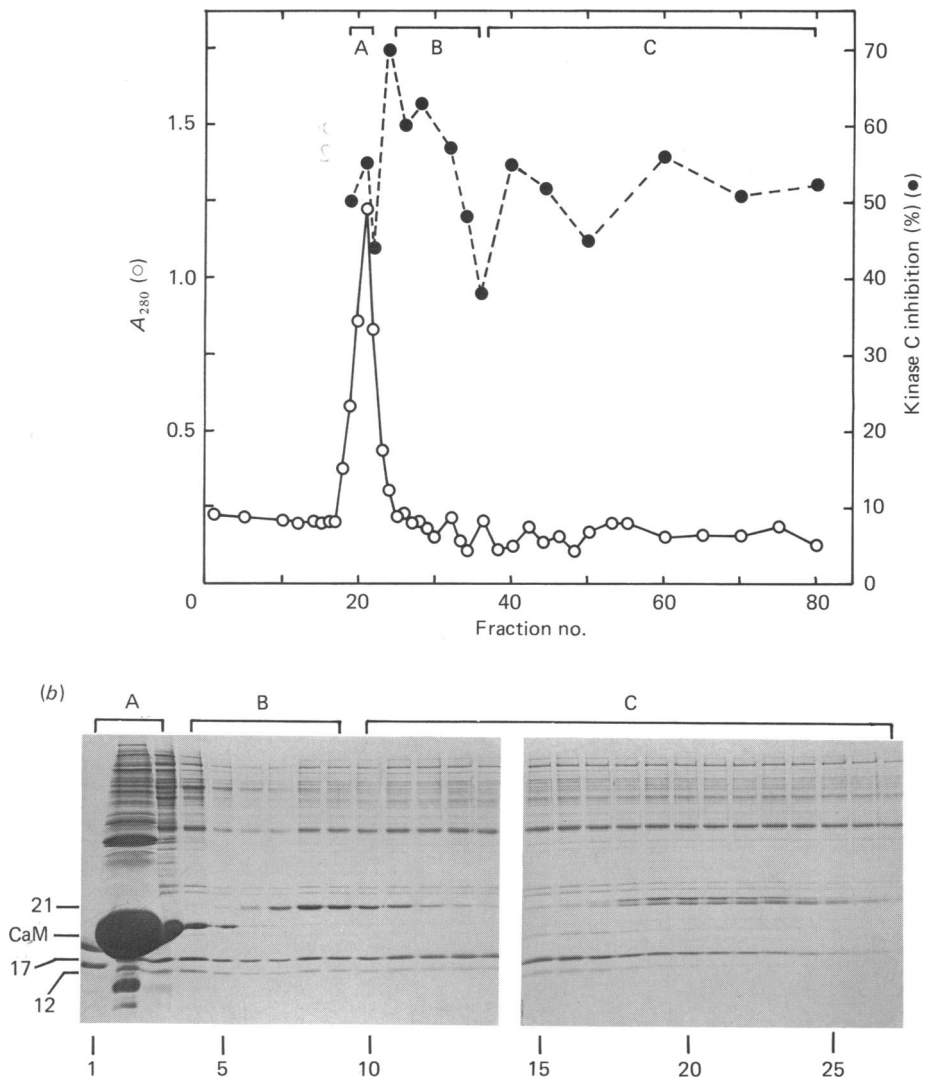

Fig. 1. Ca^{2+}-dependent hydrophobic-interaction chromatography of bovine brain soluble proteins prepared in the absence of proteinase inhibitors

The 100000 *g* supernatant prepared from bovine brain in the absence of proteinase inhibitors was concentrated with $(NH_4)_2SO_4$, dialysed and subjected to Ca^{2+}-dependent hydrophobic-interaction chromatography on a column (1.5 cm × 40 cm) of phenyl-Sepharose CL-4B as described in the Materials and methods section. Only the elution profile after application of the EGTA-containing buffer is shown. The chromatogram was developed at 30 ml/h, and 4 ml fractions were collected. Selected fractions were monitored for protein content (A_{280}, ○) and protein kinase C inhibitory activity (●) (*a*) and examined by 0.1%-SDS/15–20% polyacrylamide-gradient slab-gel electrophoresis (*b*). Platelet kinase C was used in these assays. Similar results were obtained with other preparations by using bovine brain kinase C. Platelet kinase C (80 μl/ml of reaction mixture) was incubated at 30 °C with histone III-S (0.2 mg/ml) in 20 mM-Tris/HCl (pH 7.5)/5 mM-$MgCl_2$/phosphatidylserine (40 μg/ml)/1,3-diolein (0.8 μg/ml)/0.5 mM-$CaCl_2$ in the presence of column fractions (150 μl) in a final reaction volume of 0.25 ml. Reactions were initiated by addition of [γ-^{32}P]ATP (approx. 50000 c.p.m./nmol) to 10 μM. After incubation for 30 min at 30 °C, samples (0.2 ml) of reaction mixtures were withdrawn for quantification of protein-bound [^{32}P]P_i as described by Walsh *et al.* (1983). The activity measured in the absence of column fractions represented 100% activity (0% inhibition). For electrophoresis, 0.2 ml samples of column fractions were freeze-dried and the residues dissolved in 60 μl of 25 mM-Tris/HCl (pH 6.8)/0.5% SDS/0.005% Bromophenol Blue/15% glycerol/0.5% 2-mercaptoethanol and boiled. In (*b*), calmodulin and the 21, 17 and 12 kDa proteins are indicated on the basis of M_r marker proteins electrophoresed simultaneously. Key to gel lanes: 1–27 = fractions 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 70, 75 and 80 respectively. Fractions were pooled, as indicated by the bars, as follows: A = 18–23 (calmodulin); B = 25–36 (21 kDa CaBP); C = 37–80 (17 kDa + 12 kDa proteins). Numbers in the left-hand margin indicate M_r values (× 10^{-3}); CaM = calmodulin.

antibody was performed as follows. After transblotting (Ngai & Walsh, 1985), nitrocellulose membranes were soaked in 100 ml of Tris/saline [20 mM-Tris/HCl (pH 7.5)/0.5 M-NaCl] containing 3% (w/v) bovine serum albumin for 30 min in a glass dish with continuous gentle shaking. The membranes were then soaked in 40 ml of Tris/saline containing 1% (w/v) bovine serum albumin and primary antibody [anti-(17 kDa protein inhibitor), 50 μg/ml] at room temperature overnight with constant gentle agitation. Excess primary antibody was removed

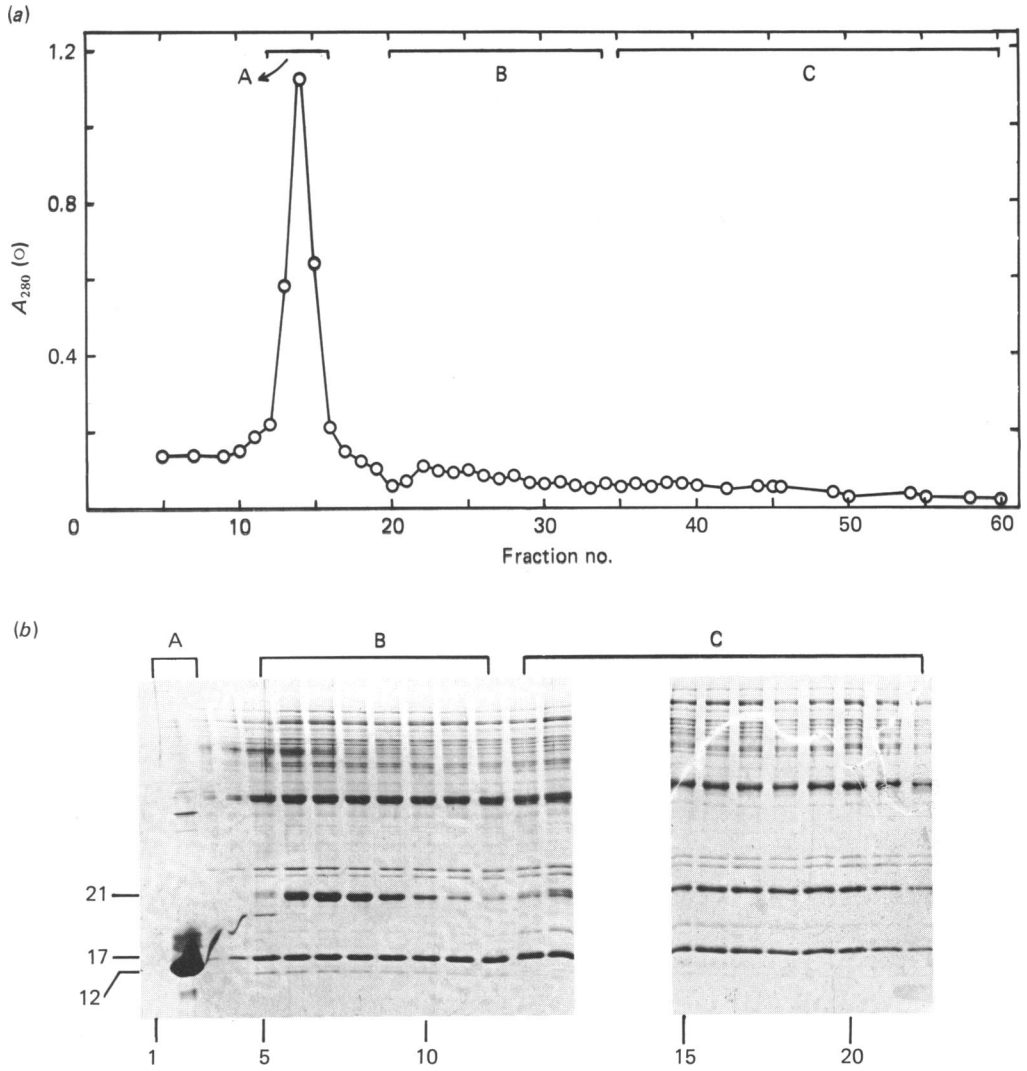

Fig. 2. Ca^{2+}-dependent hydrophobic-interaction chromatography of bovine brain soluble proteins prepared in the presence of proteinase inhibitors

The protocol described in Fig. 1 was followed exactly, except that a cocktail of proteinase inhibitors (see the Materials and methods section) was included in the homogenization buffer. Selected fractions eluted from the phenyl-Sepharose CL-4B column with EGTA were examined (*a*) for protein content (A_{280}, ○) and (*b*) by SDS/PAGE. The positions of the 21, 17 and 12 kDa proteins were identified by co-electrophoresis of M_r marker proteins. Key to gel lanes: 1–22 = fractions 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 55 and 60 respectively. Fractions were pooled, as indicated by the bars, as follows: A = 12–16 (calmodulin); B = 20–34 (21 kDa CaBP); C = 36–100 (17 kDa protein). Loading amounts: lanes 1–4 = 50 μl; lanes 5–2 = 200 μl (freeze-dried). Numbers in the left-hand margin indicate M_r values ($\times 10^{-3}$).

by washing with two changes (100 ml each) of Tris/saline containing 0.05% (v/v) Tween-20 and finally with 100 ml of Tris/saline over a 1 h period. Membranes were then treated at room temperature for 2 h with 40 ml of Tris/saline containing 1% (w/v) bovine serum albumin and secondary antibody [goat anti-(rabbit IgG)–alkaline phosphatase conjugate] diluted 1:1000. Excess antibody was removed by washing as described above. Detection was achieved by soaking the membranes in a freshly prepared solution (0.5 mg/ml) of 5-bromo-4-chloro-indol-3-yl phosphate previously dissolved in 0.3–0.5 ml of dimethyl sulphoxide and made up to a total volume of 50 ml with 1 M-Tris/HCl (pH 8.8).

The competitive ELISA method was used to test various antigens for cross-reactivity with anti-(17 kDa inhibitor) by a modification of the method of Engvall (1980). Bovine brain 17 kDa inhibitor was diluted in 15 mM-Na_2CO_3/34 mM-$NaHCO_3$/0.02% NaN_3 (pH 9.6) to a final concentration of 1.0 μg/ml. Samples (0.1 ml) were incubated in microtitre wells for 16 h at 4 °C and washed with phosphate-buffered saline/Tween prior to use. Samples (0.1 ml) of anti-17 kDa inhibitor (0.2 mg/ml) were added to equal volumes of 1-in-3 dilutions of various competing antigens and incubated for 1 h at room temperature. Samples (0.1 ml) of the incubations were then transferred to the previously coated wells and incubated for an additional 2 h at room temperature. After three rinses with phosphate-buffered saline/Tween, 0.1 ml of a 1:1000 dilution of the second antibody [goat anti-(rabbit IgG) conjugated with alkaline phosphatase] was added and incubated for 2 h. The microtitre wells were again rinsed three times with

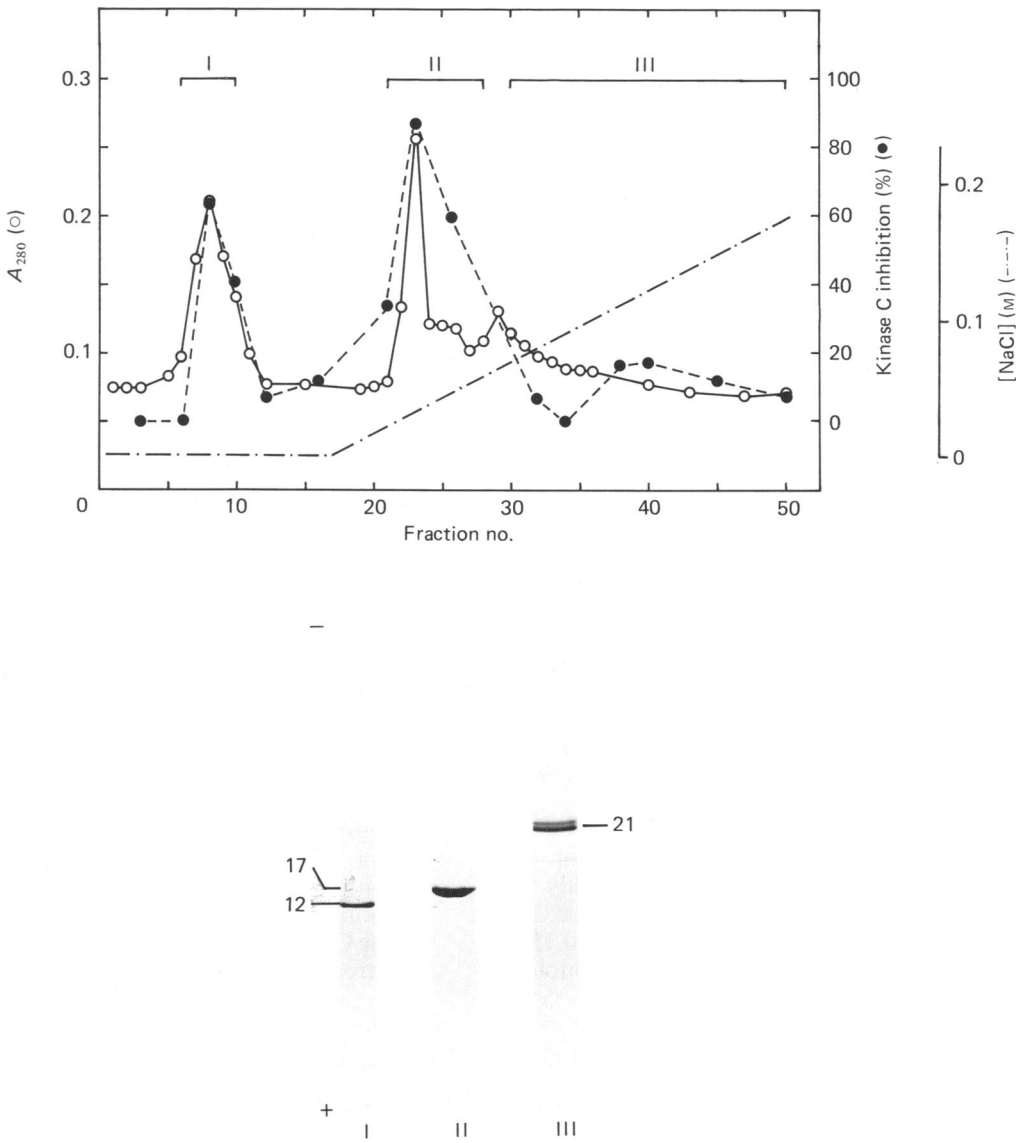

Fig. 3. Ion-exchange chromatography: purification of the 17 kDa and 12 kDa protein inhibitors of protein kinase C

Pool C from the phenyl-Sepharose CL-4B column (Fig. 1), which was enriched in the 17 and 12 kDa proteins, was heat-treated in the presence of Ca^{2+}, dialysed and chromatographed on a column of DEAE-Sephacel (1 cm × 40 cm); flow rate 12 ml/h; fraction size 4 ml. Selected fractions were examined for protein content (A_{280}, ○) and protein kinase C inhibitory activity (●), measured as described in the legend to Fig. 1 by using 100 μl of the indicated column fractions/0.25 ml of reaction mixture. Fractions were pooled on the basis of SDS-PAGE as indicated by the bars: I = 6–10 (12 kDa protein); II = 21–28 (17 kDa protein); III = 30–50 (21 kDa CaBP isoform). The purities of the three protein preparations are shown in the gel insets, with M_r values ($\times 10^{-3}$).

phosphate-buffered saline/Tween, and 0.2 ml of phosphatase substrate (1 mg of *p*-nitrophenyl phosphate in diethanolamine/Mg^{2+} buffer, pH 9.6) was added and incubated for 45 min. The reaction was terminated by addition of 50 μl of 4 M-NaOH, and the intensity of the colour reaction read in an automatic Micro-ELISA reader at 405 nm. Appropriate controls for background were made by omitting either the antigen and/or the primary-antibody step. The highest reading obtained in the absence of competing antigen was taken as 0% inhibition.

Protein determinations

Protein concentrations were determined by the Coomassie Blue dye-binding assay (Spector, 1978), with dye reagent and γ-globulin standard purchased from Pierce Chemical Co. (Rockford, IL, U.S.A.), or by spectrophotometric measurements using the following absorption coefficients: calmodulin, $A^{1\%}_{277} = 1.9$ (Klee, 1977); 21 kDa CaBP, $A^{1\%}_{277} = 9.1$ (McDonald & Walsh, 1985*a*); 17 kDa inhibitor, $A^{1\%}_{281} = 16.2$ (see the Results section).

Electrophoresis

Electrophoresis was performed in 7.5–20% or 15–20% polyacrylamide-gradient slab gels (1.5 mm thick), with a 5%-acrylamide stacking gel, in the presence of 0.1% (w/v) SDS at 36 mA, in the discontinuous buffer system of Laemmli (1970). Gels were stained in 45% (v/v) ethanol/10% (v/v) acetic acid containing 0.14% (w/v)

Coomassie Brilliant Blue R-250 and diffusion-destained in 10% acetic acid. Subunit M_r values were determined by comparison of electrophoretic mobilities in denaturing gels with those of M_r-marker proteins by the method of Lambin (1978).

Amino acid analysis

Triplicate samples of bovine brain 12 kDa protein inhibitor were hydrolysed *in vacuo* at 110 °C in 6 M-HCl containing 0.1% (w/v) phenol and 0.02% (v/v) 2-mercaptoethanol for 24, 48 and 72 h before amino-acid-composition analysis with a Beckman model 6300 amino acid analyser. Tryptophan was determined after methanesulphonic acid hydrolysis as described by Simpson *et al.* (1976), and cysteine after performic acid oxidation as described by Hirs (1967). *N*-Terminal sequence analysis was performed in a Beckman 890c sequencer equipped with a Sequemat autoconverter. The resultant amino acid phenylthiohydantoin derivatives were identified by h.p.l.c. in a Beckman Ultrasphere-ODS reverse-phase C_{18} column.

Enzyme assays

Protein kinase C activity was assayed essentially by the method of Kikkawa *et al.* (1982) as described in detail by Walsh *et al.* (1984). Details of enzymic assay reaction conditions are provided in the text. Casein kinase I activity was assayed by the method of Singh *et al.* (1982).

Ca^{2+} binding

Ca^{2+} binding to the 17 kDa protein inhibitor was determined by equilibrium dialysis as previously described (McDonald & Walsh, 1985*a*) at 0.1 mM free Ca^{2+} in the presence of 20 mM-Mops (pH 7.1)/0.15 M-KCl/3.0 mM-$MgCl_2$. The validity of the methodology was verified with calmodulin from bovine brain, which was shown to bind 3.2 mol of Ca^{2+}/mol under identical conditions.

RESULTS

Purification of protein kinase C inhibitors

We have shown previously that several proteins in a crude extract of bovine brain bind to phenyl-Sepharose CL-4B in a Ca^{2+}-dependent manner (Walsh *et al.*, 1984). Fig. 1 shows the proteins eluted from such a column upon chelation of Ca^{2+} with EGTA. Much larger amounts (0.2 ml) of column fractions were analysed by SDS/PAGE than previously (compare Fig. 1*b* here with Fig. 1*b* in Walsh *et al.*, 1984), in order to see clearly the minor protein components in the EGTA eluate. Apart from calmodulin (the major eluted protein), the 21 kDa CaBP (McDonald & Walsh, 1985*a*,*b*) and 17 kDa and 12 kDa proteins were detected. In addition, a 21 kDa/23 kDa doublet was eluted from the column later than the 21 kDa CaBP. This appears to be an isoform of the 21 kDa CaBP, since both bands of the doublet cross-react with anti-21 kDa CaBP and the isolated doublet exhibits an amino acid composition and two-dimensional peptide map which are indistinguishable from those of the 21 kDa CaBP (J. R. McDonald, U. Gröschel-Stewart & M. P. Walsh, unpublished work). In the present work we have focused on the 17 kDa and 12 kDa proteins, which have been described as inhibitors of protein kinase C (McDonald & Walsh, 1985*a*,*b*, 1986). Fig 1(*a*) also demonstrates the inhibition of kinase C activity by EGTA-eluted column fractions. The 17 kDa and 12 kDa protein inhibitors of kinase C were purified to electrophoretic homogeneity by heat treatment in the presence of Ca^{2+} and ion-exchange

Table 1. Comparison of amino acid compositions of the 17 kDa and 12 kDa protein inhibitors of kinase C

	Composition (mol of residue/mol)				
Protein kinase C inhibitor...	12 kDa				17 kDa*
Amino acid	24 h	48 h	72 h	Average	
Lysine	7.8	7.9	7.8	7.8	12.9
Histidine	1.9	1.9	2.4	2.1	4.3
Arginine	5.2	5.6	–	5.4	5.3
Aspartic acid	11.0	11.2	10.4	10.9	15.7
Threonine	6.9	6.6	5.8	7.6†	6.2
Serine	7.9	6.0	4.7	9.4†	10.2
Glutamic acid	13.6	13.8	13.6	13.7	21.0
Proline	5.6	5.4	6.6	5.8	7.8
Glycine	11.9	12.3	13.7	12.6	21.3
Alanine	14.2	14.5	–	14.4	13.8
Cysteine‡	1.0	0.8	0.9	0.9	2.2
Valine	7.0	8.1	7.7	7.6	8.8
Methionine	1.7	1.5	1.5	1.6	1.8
Isoleucine	4.1	4.8	4.5	4.5	8.6
Leucine	8.5	8.8	8.1	8.5	11.8
Tyrosine	2.4	2.5	2.6	2.5	3.7
Phenylalanine	3.2	3.4	3.6	3.4	3.9
Tryptophan§	1.1	–	0.9	1.0	1.0

* From McDonald & Walsh (1985*a*).
† Extrapolated to zero time of hydrolysis.
‡ Determined by performic acid oxidation (Hirs, 1967).
§ Determined by methanesulphonic acid hydrolysis (Simpson *et al.*, 1976).

chromatography, as described in the Materials and methods section. The elution profile from the ion-exchange column is shown in Fig. 3, which shows the electrophoretic purity of the products and the association of kinase C inhibitory activity with both the 17 kDa and 12 kDa proteins.

The inhibitory effects of the isolated 17 kDa and 12 kDa proteins (each at a concentration of 50 μg/ml) on bovine brain protein kinase C were compared with histone III-S as substrate. Mean inhibition ($\pm$S.D.) of $75\pm3\%$ ($n = 6$) was observed with the 17 kDa inhibitor and $85\pm2\%$ ($n = 5$) with the 12 kDa inhibitor. In addition to inhibiting histone phosphorylation, both 17 kDa and 12 kDa inhibitors also inhibited protein kinase C-catalysed phosphorylation of platelet P47 protein, which has been shown to be a substrate of kinase C in intact platelets stimulated by phorbol esters (Sano *et al.*, 1983) (results not shown).

Comparison of 17 kDa and 12 kDa kinase C inhibitors

If proteinase inhibitors were included during homogenization of the tissue, the amount of 17 kDa inhibitor eluted from the hydrophobic column with EGTA was increased, while the amount of 12 kDa inhibitor was decreased (cf. Figs. 1 and 2). This was reflected in the final relative yields of purified 17 kDa and 12 kDa inhibitors obtained in the absence and presence of proteinase inhibitors (see the Materials and methods section). These observations suggested that the 12 kDa inhibitor may be a proteolytic fragment of the 17 kDa inhibitor generated during isolation. This conclusion was substantiated by two-dimensional tryptic-peptide mapping of isolated 12 kDa and 17 kDa inhibitors, which revealed ten common peptides (results not shown). In addition, the amino acid composition of the 12 kDa inhibitor is included, within experimental error, in that of the 17 kDa protein (Table 1). Both proteins were found, by *N*-terminal sequence analysis, to be *N*-terminally blocked, suggesting that the 12 kDa protein corresponds to the *N*-terminus of the 17 kDa inhibitor.

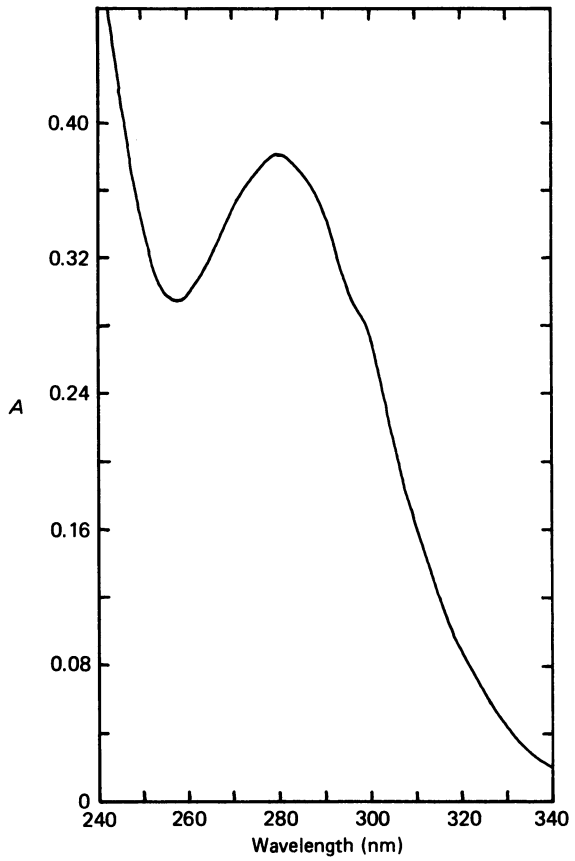

Fig. 4. U.v.-absorption spectrum of the 17 kDa protein kinase C inhibitor

The 17 kDa protein (1.0 ml) was dialysed overnight against two changes (5 l each) of 20 mM-Tris/HCl (pH 7.5), and the protein concentration was determined to be 0.163 mg/ml. The u.v.-absorption spectrum was recorded in a Beckman DU-8B spectrophotometer.

U.v.-absorption spectrum of the 17 kDa inhibitor

The u.v.-absorption spectrum of the 17 kDa inhibitor is shown in Fig. 4. An absorption maximum was observed at 281 nm, with a shoulder at 290–295 nm, indicative of the presence of tryptophan. This is consistent with the results of amino acid analysis, which revealed the presence of 1 mol of tryptophan/mol of 17 kDa protein (McDonald & Walsh, 1985*a*). An absorption coefficient, $A^{1\%}_{281} = 16.2$, of the 17 kDa inhibitor was calculated from the u.v.-absorption spectrum and the protein concentration.

Ca^{2+}-binding properties of the 17 kDa inhibitor

The 17 kDa kinase C inhibitor shares a number of properties with calmodulin and other members of the calmodulin family of Ca^{2+}-binding proteins: a high content of acidic amino acid residues (Table 1), consistent with its pI (5.5–5.6), Ca^{2+}-dependent interaction with a hydrophobic matrix (phenyl-Sepharose CL-4B) and heat-stability in the presence of Ca^{2+}. These observations suggested that the 17 kDa inhibitor may be a Ca^{2+}-binding protein (McDonald & Walsh, 1985*a*). However, we have been unable to demonstrate the binding of $^{45}Ca^{2+}$ to the 17 kDa inhibitor by equilibrium dialysis (see the Materials and methods section). We have shown previously that the 17 kDa inhibitor, in contrast with calmodulin and some related Ca^{2+}-binding proteins, does not undergo a Ca^{2+}-dependent electrophoretic-mobility shift, nor does it bind $^{45}Ca^{2+}$ after SDS/PAGE and transblotting on to nitrocellulose membranes (McDonald & Walsh, 1985*a*). The 17 kDa inhibitor may therefore interact with phenyl-Sepharose in an indirect manner. To examine this possibility further, the entire EGTA eluate from phenyl-Sepharose CL-4B column chromatography (identical with that in Fig. 1) was pooled, dialysed against two changes (10 l each) of 20 mM-Tris/HCl (pH 7.5)/0.1 mM-$CaCl_2$/1 mM-dithiothreitol and re-applied to a new phenyl-Sepharose CL-4B column previously equilibrated with the same buffer. The 17 kDa inhibitor bound to this column and was eluted with EGTA. The 12 kDa inhibitor, on the other hand, did not bind to the column in the presence of Ca^{2+}. Furthermore, the isolated 17 kDa inhibitor bound to phenyl-Sepharose CL-4B in a Ca^{2+}-dependent manner. We can therefore rule out the possibility that Ca^{2+}-dependent binding of the 17 kDa inhibitor to the hydrophobic resin requires a factor such as phospholipid which is lost on dialysis, but rather it is direct binding.

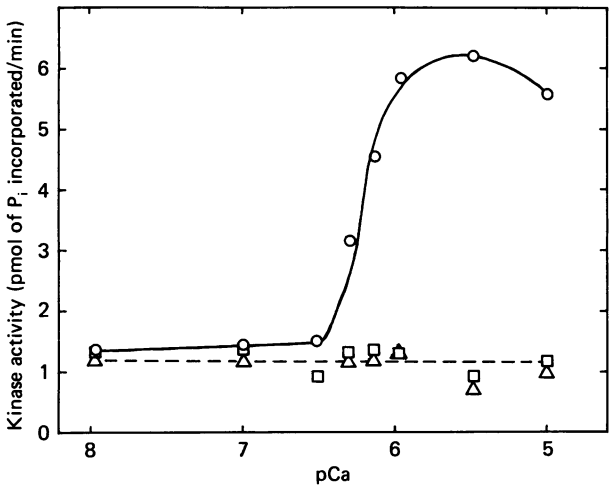

Fig. 5. Effects of 17 kDa and 12 kDa protein kinase C inhibitors at different Ca^{2+} concentrations

All proteins were dialysed against three changes (10 vol. each) of 20 mM-Mops (pH 7.1)/0.1 mM-EGTA/3.0 mM-$MgCl_2$/0.5 mM-dithiothreitol over a 24 h period. Protein kinase C activity was assayed at various concentrations of free Ca^{2+} (added from a 10 mM-$CaCl_2$ stock solution) determined by assuming a pK_d (Ca^{2+}) for EGTA of 10.955 (Fabiato, 1981) under the following conditions: human platelet protein kinase C (16 μl/ml of reaction mixture; 9.8 μg of protein/ml) was incubated at 30 °C with histone III-S (0.2 mg/ml) in 20 mM-Tris/HCl (pH 7.5)/5 mM-$MgCl_2$/phosphatidylserine (40 μg/ml)/1,3-diolein (0.8 μg/ml) in the absence of inhibitor (○) or presence of 17 kDa (□) or 12 kDa (△) proteins (10 μg/ml). Reactions were initiated by addition of [γ-^{32}P]ATP (approx. 50000 c.p.m./nmol) to a final concentration of 10 μM. The reaction volume was 2.0 ml. Samples (0.2 ml) of reaction mixtures were withdrawn at 3, 6, 9, 12, 15, 18, 21 and 24 min for quantification of protein-bound [^{32}P]P_i as described by Walsh *et al.* (1983).

Effect of [Ca^{2+}] on inhibition of kinase C by the 17 kDa and 12 kDa proteins

The effects of the 17 kDa and 12 kDa inhibitors on protein kinase C activity were examined as a function of Ca^{2+} concentration. In Fig. 5, the activity of protein kinase C is presented as a function of pCa in the absence of inhibitor and in the presence of either the 17 kDa or 12 kDa inhibitor. Half-maximal activity of protein kinase C in the absence of inhibitor occurred at 0.6 μM-Ca^{2+}. Maximal inhibition by both inhibitors was observed at all concentrations of Ca^{2+} studied. These experiments were conducted in the presence of a 5-fold lower concentration of enzyme and inhibitor than usual in order to facilitate the accurate calculation of initial reaction velocities. The maximal kinase C activity observed at 3.3 μM-Ca^{2+} was 322 pmol of P_i incorporated/min per mg of protein, with histone III-S as substrate. This compares favourably with the average rate observed under normal assay conditions (374 pmol of P_i/min per mg).

Specificity of anti-(17 kDa inhibitor)

Polyclonal antibodies raised in rabbits against the bovine brain 17 kDa inhibitor were found to be highly

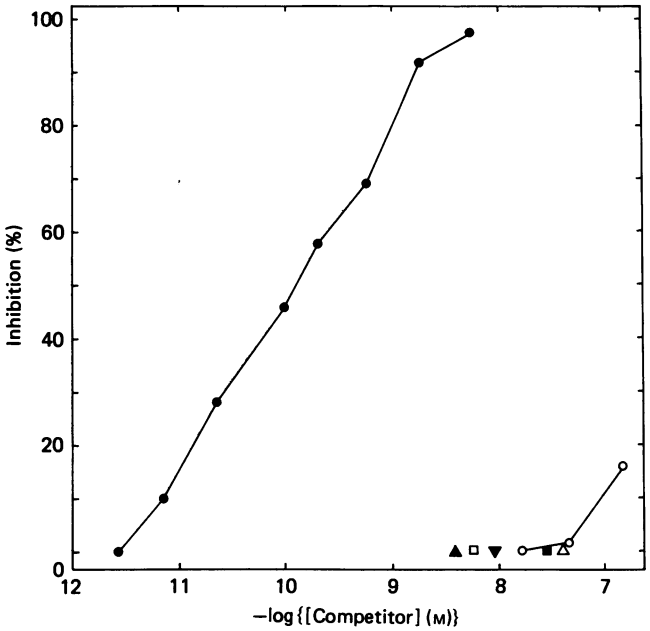

Fig. 6. Comparison of cross-reactivities of various antigens with anti-(17 kDa protein inhibitor of kinase C) by the competitive ELISA method

Various antigens were tested for cross-reactivity with anti-(bovine brain 17 kDa inhibitor) as described in the Materials and methods section. Symbols for competing antigens: ●, bovine brain 17 kDa inhibitor of kinase C; ○, calmodulin; ■, calcineurin β-subunit; □, 27 kDa CaBP; ▲, 48 kDa CaBP; △, 63 kDa CaBP; ▼, bovine brain 12 kDa inhibitor of kinase C.

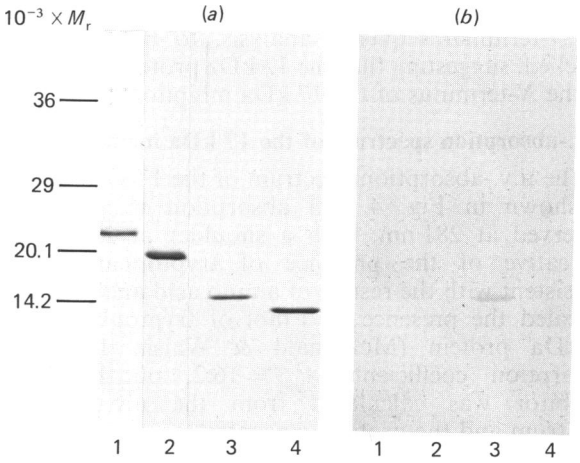

Fig. 7. Specificity of anti-(17 kDa inhibitor)

The purified proteins indicated (5–10 μg of each) were subjected to SDS/PAGE and either stained with Coomassie Blue (*a*) or transblotted on to a nitrocellulose membrane and treated with primary antibody [anti-(17 kDa inhibitor)] and secondary antibody [anti-(rabbit IgG)–horseradish peroxidase conjugate] before detection of the secondary antibody as described in the Materials and methods section (*b*). Key to lanes: 1, 21 kDa CaBP; 2, calmodulin; 3, 17 kDa inhibitor of kinase C; 4, 12 kDa inhibitor of kinase C. Numbers at the left indicate M_r ($\times 10^{-3}$) of marker proteins electrophoresed simultaneously.

specific, as determined by the competitive ELISA method (Fig. 6). The antibodies did not cross-react with any of several bovine brain Ca^{2+}-binding proteins, even at concentrations several orders of magnitude higher than that at which the 17 kDa inhibitor produced maximal inhibition of antibody binding. Very slight cross-reactivity was observed at the highest concentration of calmodulin examined, which may be due to trace contamination of the 17 kDa inhibitor used for antibody production, or trace contamination of the calmodulin preparation with 17 kDa inhibitor. Western immunoblotting experiments confirmed the specificity of the anti-(17 kDa inhibitor) (Fig. 7). The weakly cross-reactive band, of 27 kDa, in lane 3 (Fig. 7) was not observed when immunoblotting was done with goat anti-(rabbit IgG)–alkaline phosphatase conjugate. Furthermore, immunoblotting of a total homogenate of bovine brain revealed a single cross-reactive protein band, of 17 kDa (results not shown). Identical results were obtained whether goat anti-(rabbit IgG)–horseradish peroxidase conjugate or goat anti-(rabbit IgG)–alkaline phosphatase conjugate was used as secondary antibody. Anti-(17 kDa inhibitor) had no effect on the capacity of the 17 kDa inhibitor to inhibit kinase C activity, even when included at a weight ratio to inhibitor of 50:1.

Tissue and species distribution of the 17 kDa inhibitor

Several bovine tissues were screened for the presence of the 17 kDa protein inhibitor by immunoblotting of total homogenates as described in the Materials and methods section. In all tissues examined, except bovine trachea, a single cross-reactive band of 17 kDa was observed. The intensity of the cross-reactive band varied from one tissue to another, as indicated in Table 2 by the number of plus signs. Control immunoblots treated identically, except that either the primary antibody was omitted or it was replaced by non-immune globulin, or the secondary antibody was omitted, remained completely clear, thereby establishing the specificity of the results. The efficiency of protein transfer to nitrocellulose membranes was routinely verified by Amido Black staining. The data in Table 2 indicate that the content of 17 kDa inhibitor is generally highest in secretory tissues (kidney, liver and brain) and striated muscles, and lowest in smooth muscles (aorta, stomach, intestine and trachea). We have also demonstrated the presence of the 17 kDa inhibitor in rat liver, rat kidney, chicken gizzard and human platelets, suggesting a broad tissue and species distribution.

Table 2. Distribution of 17 kDa inhibitor of kinase C in bovine tissues

Various bovine tissues (1.0 g) were homogenized in 6 vol. of 0.3 M-KCl/50 mM-imidazole/HCl (pH 6.9)/1 mM-EGTA/0.5 mM-$MgCl_2$/0.25 mM-phenylmethanesulphonyl fluoride with the aid of a Brinkman Polytron equipped with a PT10ST probe generator. A sample (0.5 ml) of each homogenate was added to 0.5 ml of SDS-gel sample buffer [50 mM-Tris/HCl (pH 6.8)/1% SDS/0.01% Bromophenol Blue/30% (v/v) glycerol/1% 2-mercaptoethanol] and boiled before SDS/ PAGE and immunoblotting using anti-(17 kDa protein). The relative intensities of cross-reactivity of 17 kDa proteins in the various tissue homogenates are indicated by plus signs; n.d., not detected.

Tissue	Cross-reactivity
Aorta	++
Brain	++++
Cardiac muscle	++++
Intestine	+
Kidney	+++++
Liver	+++
Lung	++
Oesophagus	++++
Skeletal muscle	++++
Stomach	++
Trachea	n.d.

DISCUSSION

Ca^{2+}-dependent hydrophobic-interaction chromatography has been widely used in the isolation of a variety of Ca^{2+}-binding proteins which expose a hydrophobic site(s) in the presence of bound Ca^{2+}. For example, this approach has been used to purify calmodulin (Gopalakrishna & Anderson, 1982), calelectrins (Südhof *et al.*, 1984), protein kinase C (Walsh *et al.*, 1984) and a novel 21 kDa CaBP of unknown function (Walsh *et al.*, 1984; McDonald & Walsh, 1985*a*; McDonald *et al.*, 1985). When we originally applied this technique to the study of bovine brain Ca^{2+}-binding proteins, we observed several other proteins which bound to phenyl-Sepharose CL-4B in a Ca^{2+}-dependent manner (Walsh *et al.*, 1984). We subsequently purified two of these proteins (12 kDa and 17 kDa) and found them to be potent inhibitors of protein kinase C (McDonald & Walsh, 1985*a*,*b*, 1986). We demonstrate here that the 12 kDa protein is a proteolytic fragment derived from the 17 kDa protein and corresponds to its *N*-terminal region. The inclusion of a cocktail of proteinase inhibitors during tissue homogenization allows the isolation of the 17 kDa kinase C inhibitor in improved yields of approx. 6 mg/kg of bovine brain.

Two factors suggested that the 17 kDa and 12 kDa inhibitors may be Ca^{2+}-binding proteins: firstly, they interact with phenyl-Sepharose CL-4B in a Ca^{2+}-dependent manner, and, secondly, they are low-M_r, acidic, heat-stable proteins similar to calmodulin and related Ca^{2+}-binding proteins. Our early observations that, unlike calmodulin and the 21 kDa CaBP, the 17 kDa inhibitor did not exhibit a Ca^{2+}-dependent electrophoretic-mobility shift or bind $^{45}Ca^{2+}$ after SDS/PAGE and transblotting on to a nitrocellulose membrane could be due to a weaker affinity of the 17 kDa inhibitor for Ca^{2+} or its failure to renature to any extent after electrophoresis and/or transblotting. We addressed this question directly by measuring $^{45}Ca^{2+}$ binding to the isolated 17 kDa inhibitor by equilibrium dialysis. Under conditions in which we could determine binding of 3.2 mol of Ca^{2+}/mol of calmodulin, no binding of Ca^{2+} to the 17 kDa inhibitor protein was detected.

We reasoned that, if inhibition of kinase C activity by the 17 kDa and 12 kDa proteins was Ca^{2+}-dependent, this may be apparent by studying the Ca^{2+}-dependence of kinase C activity in the presence and absence of inhibitors. If inhibition required Ca^{2+} and the inhibitors had lower affinities for Ca^{2+} than did kinase C, as

predicted from our findings discussed above, then one would expect to observe kinase C activity to increase in the range of 0.1–1 μM-Ca^{2+} in the presence or absence of inhibitor and subsequently to decrease at higher Ca^{2+} concentrations only in the presence of inhibitor as the inhibitor bound Ca^{2+} and became active. However, our data (Fig. 5) show that, whereas half-maximal activation of protein kinase C is observed at 0.6 μM-Ca^{2+} in the absence of inhibitors, in the presence of 17 kDa or 12 kDa inhibitors complete inhibition is observed at all concentrations of Ca^{2+}. This result could be explained if the affinity of 17 kDa and 12 kDa proteins for Ca^{2+} was equal to or greater than that of kinase C. Alternatively, the inhibitory effect of the 17 kDa and 12 kDa proteins is Ca^{2+}-independent. If the kinase C inhibitors are indeed unregulated, they resemble the well-studied heat-stable protein inhibitor of cyclic AMP-dependent protein kinase (Walsh *et al.*, 1971). These inhibitors may be regulated *in vivo* in some other way that remains to be elucidated.

It is well known that protein kinase C is widely distributed among tissues and species (Kuo *et al.*, 1980; Minakuchi *et al.*, 1981). If the 17 kDa inhibitor does indeed play a role physiologically in the regulation of kinase C activity, one would expect it also to be broadly distributed. Using specific polyclonal anti-(bovine brain 17 kDa protein) in immunoblotting experiments, we have demonstrated the presence of this inhibitor in a variety of bovine tissues and rat liver and kidney, chicken gizzard and human platelets.

The 17 kDa kinase C inhibitor has been shown to inhibit kinase C prepared from bovine brain (McDonald & Walsh, 1985*a*,*b*), human platelets (the present work) and bovine aorta (K. Dell & D. L. Severson, personal communication), with either histone III-S or human platelet P47 protein as substrate. The inhibitor appears to be specific for protein kinase C; it has no effect on myosin light-chain kinase (McDonald & Walsh, 1985*a*), cyclic AMP-dependent protein kinase catalytic subunit (McDonald & Walsh, 1986), casein kinase I (results not shown) or calmodulin-dependent cyclic nucleotide phosphodiesterase (McDonald & Walsh, 1985*a*). The 17 kDa protein inhibitor may therefore be more useful than the less specific isoquinolinesulphonamides (Hidaka *et al.*, 1984) and the less potent synthetic myelin-basic-protein peptide analogues (Su *et al.*, 1986) as tools in probing specific events *in vitro* and *in vivo* regulated by protein kinase C.

In conclusion, the high specificity of action and widespread tissue and phylogenetic distribution of the 17 kDa inhibitor is consistent with the hypothesis that this protein functions in the regulation of kinase C activity. Activation of the 17 kDa inhibitor, by a mechanism not yet defined, may equip the cell with an effective mechanism for the inactivation of protein kinase C, thereby: (i) abolishing the physiological responses associated with kinase C activation, e.g. neurotransmitter release (see Nishizuka, 1984); (ii) abolishing the postulated negative-feedback control of extracellular messenger-induced polyphosphoinositide turnover (see the Introduction); and (iii) avoiding the necessity of removing the second messengers, Ca^{2+} and diacylglycerol, in order to inactivate kinase C. This would allow other cellular biochemical pathways which are dependent on Ca^{2+} (e.g. activation of calmodulin-dependent kinases) and diacylglycerol (e.g. prostaglandin synthesis) to proceed.

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