# Mechanism of inhibition of protein kinase C by 14-3-3 isoforms

## 14-3-3 isoforms do not have phospholipase $A_2$ activity

Karen ROBINSON,* David JONES,* Yasmina PATEL,* Harry MARTIN,* Joel MADRAZO,*§ Stephen MARTIN,† Steve HOWELL,* Moira ELMORE,‡ Michael J. FINNEN‡ and Alastair AITKEN*||

*Laboratory of Protein Structure and †Laboratory of Physical Biochemistry, National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K., and ‡Yamanouchi Research Institute, Littlemore Hospital, Oxford OX4 4XN, U.K.

The ability of individual members of the 14-3-3 protein family to inhibit protein kinase C (PKC) has been studied by using a synthetic peptide based on the specific 80 kDa substrate for PKC (MARCKS protein) in two different assay systems. Recombinant 14-3-3 and isoforms renatured by a novel method after separation by reverse-phase h.p.l.c. were studied. The detailed effects of diacylglycerol and the phorbol ester phorbol 12-myristate 13-acetate on the inhibition were also investigated. This suggests that one of the sites of interaction of 14-3-3 may be the cysteine-rich (C1) domain in PKC. Since a region in secreted phospholipase $A_2$ ($PLA_2$) shares similarity with this domain, the ability of 14-3-3 to interact with mammalian $PLA_2$ was studied. Cytosolic $PLA_2$ has some similarity to the C2 region of PKC, and the effect of 14-3-3 on this class of $PLA_2$ was also analysed. In contrast with a previous report, no $PLA_2$ activity was found in brain 14-3-3, nor in any of the recombinant proteins tested. These include $\zeta$ 14-3-3 isoform, on which the original observation was made.

## INTRODUCTION

Protein kinase C (PKC) is a family of serine/threonine protein kinases that phosphorylates a wide range of proteins, leading to the regulation of many physiological processes, including secretion, control of neurotransmitter release and membrane conductance. PKC isoenzymes have different distribution, substrate and cofactor specificity, suggesting that different isoforms may control different physiological events (Nishizuka, 1984). The PKC family has a pivotal role in signal transduction (Berridge, 1993), therefore activity must be tightly regulated. One such control mechanism could be via direct inhibition.

A number of protein PKC inhibitors have been studied over the last decade. A 20 kDa heat-stable cytosolic protein was first identified as a PKC inhibitor by Schwantke and Le Peuch (1984). Inhibition of PKC by calmodulin was first shown by Albert et al. (1984). A 17 kDa heat-stable cytosolic protein from bovine brain that inhibited PKC (McDonald and Walsh, 1985) was called PKCI-1 (protein kinase C inhibitor).

A 40 kDa heat-labile membrane-associated monomeric PKC inhibitor has been purified from neutrophils (Balazovich et al., 1992), and annexin V has been identified as an inhibitor of the phosphorylation of some substrates for PKC (Schlaepfer et al., 1992).

The PKC inhibitor isolated in our laboratory (Toker et al., 1990, 1992) called KCIP-1 (kinase C inhibitor protein), is the most potent of the protein PKC inhibitors, having an $IC_{50}$ of 0.85 $\mu$M (for a dimer of $\sim$ 60 kDa). KCIP-1 was purified from sheep brain as a cytosolic heat-labile (at 100 °C) non-competitive inhibitor of PKC, which was independent of cofactor concentration (Toker et al., 1990).

KCIP-1 belongs to a family of proteins known as 14-3-3. The name 14-3-3 was originally given due to their migration position on two-dimensional DEAE-cellulose chromatography and starch-gel electrophoresis (Moore and Perez, 1968). 14-3-3 proteins all have a pI of around 4.5 and mass of 30000 Da on SDS/PAGE, but 60000 Da on size-exclusion chromatography, suggesting a dimeric structure (Toker et al., 1992).

There are seven or eight mammalian brain isoforms of 14-3-3, named $\alpha$–$\eta$ after their respective elution positions on h.p.l.c. (Ichimura et al., 1988; Toker et al., 1992). Five of these have been sequenced (reviewed by Aitken et al., 1992). The 14-3-3 family is highly conserved, and individual isoforms are either identical or contain between one and three conservative substitutions over a wide range of mammalian species (Ichimura et al., 1991). Examples of 14-3-3 proteins have also been found in plants (Hirsch et al., 1992; Brandt et al., 1992), insects (Swanson and Ganguly, 1992) amphibians (Martens et al., 1992), yeast (van Heusden et al., 1992) and the nematode *Caenorhabditis elegans* (EMBL database accession numbers T02212, T00336, T00100 and T01097). In some non-mammalian species, 14-3-3 may play a role in the regulation of gene expression (reviewed by Aitken et al., 1992). More recently, additional evidence for a role in regulation of transcription has come from the group of Ferl (Lu et al., 1992), who have shown that a plant 14-3-3 homologue binds to the G-box promoter element of inducible genes.

In mammals, the first biological function for this family of proteins was studied by Ichimura et al. (1987), who found a 14-3-3 isoform which had similarity to an activator of tyrosine and tryptophan hydroxylases, the rate-limiting step in catecholamine and 5-hydroxytryptamine (serotonin) synthesis in neurons (Yamauchi et al., 1981). However, purified sheep brain 14-3-3 isoforms have been shown not to activate recombinant tyrosine hydroxylase (Sutherland et al., 1993). Recently many other functions have been suggested for this widely distributed family of eukaryotic proteins. To date, no cell type has been identified which does not contain members of the 14-3-3 family. For example, epithelial cells contain a specific isoform called HME1 (Prasad et al., 1992) or stratifin (Leffers et al., 1993). A 14-3-3 protein member(s) called Exo1 stimulates $Ca^{2+}$-dependent

Abbreviations used: DAG, diacylglycerol; KCIP-1, kinase C inhibitor protein-1; MARCKS, myristoylated alanine-rich C-kinase substrate; MARCKS peptide, 21-residue peptide consisting of the phosphorylatable domain of MARCKS; PKC, protein kinase C; $PLA_2$, phospholipase $A_2$; $cPLA_2$, cytosolic $PLA_2$; $sPLA_2$, secreted $PLA_2$; PMA, phorbol 12-myristate 13-acetate.

§ Permanent address: CIGB, Ave. 31, Cubanacan, P.O. Box 6162, Habana, Cuba.

|| To whom correspondence should be addressed.

exocytosis in permeabilized adrenal medulla cells (Morgan and Burgoyne, 1992a; Wu et al., 1992). This stimulation of exocytosis by Exo1 is increased further in the presence of phorbol 12-myristate 13-acetate (PMA). PKC and Exo1 act synergistically (Morgan and Burgoyne, 1992b).

In mammalian pineal gland, serotonin *N*-acetyltransferase demonstrates a 100-fold increase in activity at night, which increases conversion of 5-hydroxytryptamine into melatonin. The $\epsilon$ isoform of 14-3-3 co-purifies with rat pineal *N*-acetyltransferase (Roseboom et al., 1992), thereby suggesting a function for 14-3-3 in regulating diurnal rhythm.

The eukaryotic host factor that activates exoenzyme S from *Pseudomonas aeruginosa* has been shown to be a member of the 14-3-3 family (the $\zeta$ isoform; Fu et al., 1993). Exoenzyme S ADP-ribosylates Ras and other GTP-binding proteins. Gross and co-workers have recently sequenced a platelet phospholipase $A_2$ ($PLA_2$) which cleaves the *sn*-2 arachidonoyl group from phosphatidylcholine and have identified it as 14-3-3 $\zeta$ isoform (Loeb and Gross, 1986; Zupan et al., 1992). When those authors over-expressed this protein in *Escherichia coli*, they found that it formed a stable arachidonoyl-enzyme intermediate, which may have a role in cellular trafficking of arachidonic acid. Since arachidonic acid stimulates secretion in many cell types, this function may relate to the secretory function attributed to Exo1 by Morgan and Burgoyne (1992a). However, we have investigated this putative role for 14-3-3 $\zeta$ as a $PLA_2$ and found no activity. Nor did 14-3-3 proteins activate or inhibit $PLA_2$ activity.

In the present study, the mechanism of action of 14-3-3 was investigated; individual isoforms of 14-3-3 were used in order to establish whether all forms could inhibit PKC. In particular, the effects of 14-3-3 $\zeta$ isoform, which had been reported to be an activator of PKC (Isobe et al., 1992), were re-assessed. In keeping with the established nomenclature for mammalian 14-3-3 isoforms, employing Greek letters, we have named the T-cell isoform tau ($\tau$) and the keratinocyte, HME1 or stratifin isoform sigma ($\sigma$).

## MATERIALS AND METHODS

### Materials

All reagents were analytical grade, from BDH, Sigma or Boehringer. Protein assay reagent was obtained from Bio-Rad. H.p.l.c. and f.p.l.c. solvents and water were obtained from Romil.

MARCKS peptide (the 21-residue peptide consisting of the phosphorylatable domain of MARCKS; KKKKRFSFKKSFKLSGFSFKK) was synthesized in-house by Mr. P. Fletcher, using an Applied Biosystems 430 Automated Peptide Synthesizer. Phosphatidylcholine (1-*O*-hexadecyl-2-*O*-arachidonoyl-*sn*-glycero-3-phosphocholine) was obtained from Calbiochem. Phosphatidylserine (L-phosphatidyl-L-serine; dioleoyl, sodium salt) and diacylglycerol (DAG; 1,2-dioleoyl-*sn*-glycerol; $C_{18:1,cis\text{-}9}$) were obtained from Sigma. Coomassie Brilliant Blue R-250 was from Serva. Isogels pH 3–10 were obtained from Flowgen Instruments.

[$\gamma$-$^{32}$P]ATP (222 TBq/mol, 370 MBq/ml) and phosphatidylcholine (L-$\alpha$-1-palmitoyl-2-[1-$^{14}$C]arachidonoyl; 2 GBq/mmol, 1.9 MBq/ml) were obtained from New England Nuclear Research Products. 1-Stearoyl 2-[$^3$H]arachidonoyl phosphatidylcholine and 1-acyl 2-[$^{14}$C]arachidonoyl phosphatidylethanolamine were obtained from Amersham. Enlightening was obtained from NEN Research Products. X-ograph blue X-ray film was obtained from X-graph Ltd. P81 ion-exchange paper was obtained from Whatman.

Human brain 14-3-3 was a gift from Professor R. J. Thompson, University of Southampton. Exo1 was obtained from Professor R. Burgoyne, University of Liverpool; and bovine brain recombinant $\zeta$ 14-3-3 was given by Dr. R. J. Collier and Dr. H. Fu, Harvard Medical School, Boston, MA, U.S.A.

### Purification of 14-3-3

14-3-3 was isolated from sheep brain by a combination of anion-exchange and hydrophobic-interaction chromatography, by the method of Toker et al. (1990). SDS/PAGE was adapted from Laemmli (1970). SDS/polyacrylamide slab gels were run under reducing or non-reducing conditions. Slab gels (20 cm × 20 cm, 3 mm thickness) were used for analytical work, and mini-gels were used to check protein purity at each purification step. All SDS/PAGE was in 12.5% (w/v) acrylamide resolving gel overlaid with 5% (w/v) acrylamide stacking gel.

The concentration of the purified proteins was estimated by amino acid analysis (on a Beckman 121MB or a Beckman 6300 analyser with post-column ninhydrin detection), or by the Bio-Rad protein-concentration assay (according to manufacturers' instructions).

### Reverse-phase separation of 14-3-3 isoforms

Pure 14-3-3 was separated into individual isomers on reverse-phase h.p.l.c., using Aquapore RP300 ($C_8$; 4.6 mm × 100 mm; Toker et al., 1992) and Bakerbond WP-$C_4$ (4.6 mm × 250 mm and 10 mm × 250 mm) columns. The separations employed shallow water/acetonitrile gradients containing trifluoroacetic acid (TFA). The isoforms were eluted between approx. 50 and 56% (v/v) acetonitrile.

### Renaturation of 14-3-3 proteins

14-3-3 isoforms eluted in trifluoroacetic acid/acetonitrile (pH ~ 2) from reverse-phase h.p.l.c. were not restored to their native state when simply neutralized with 50 mM Tris/HCl, pH 7.5. This also resulted in very poor recovery of protein. This was judged by two criteria, c.d. and dimeric structure (see below). The following method was developed to renature 14-3-3 isoforms to a state resembling the native protein.

Fractions of purified $\gamma$, $\zeta$ and $\epsilon$ isoforms (as identified by specific antibodies; Martin et al., 1993) were diluted with 5 vol. of Milli-Q distilled water and concentrated at 4 °C to 400–500 $\mu$l in Centricon tubes (mass cut-off 10000 Da) in a Beckman centrifuge at 4000 *g*. For larger-scale renaturation of isoforms, an Amicon ultrafiltration stir cell (10 ml) was used. The concentrated fractions were diluted again to 10 ml with 20 mM Tris/HCl (pH 7.5)/0.2 M NaCl, re-concentrated to 200–300 $\mu$l and assayed for PKC inhibition. The renaturation of the individual isoforms was verified by c.d. analysis (to check that proteins had refolded to give an $\alpha$-helical content similar to that of native protein) and by gel-exclusion chromatography on a Superose 12 column (to confirm that the protein had regained its dimeric structure).

### Recombinant 14-3-3

Recombinant proteins were expressed in *E. coli* (D. Jones, unpublished work). The $\epsilon$ isoform was expressed as a maltose-binding protein fusion protein, from the vector pMALc2 (New England Biolabs). The rat brain $\epsilon$ cDNA was kindly given by Dr. P. Roseboom, N.I.H. The carrier protein was cleaved with Factor Xa, to leave a glycine residue at the N-terminus of the protein.

The T-cell isoform was expressed as an intact protein from the vector pKK233-2 (Pharmacia). The T-cell 14-3-3 cDNA (human) was kindly given by Dr. P. J. Nielsen (Freiburg, Germany).

Purification strategies were as detailed for the sheep brain isoforms.

### PKC assay

PKC was purified from fresh sheep brain by a modification of the method of Ellis et al. (1987).

The PKC assay was based on that of Parker et al. (1984), except for the following: total assay volume was 55 $\mu$l, consisting of 12.5 $\mu$M [$\gamma$-$^{32}$P]ATP (27 kBq/nmol of ATP), $CaCl_2$ (0.1 mM), $MgCl_2$ (13.5 mM), phosphatidylserine (0.04 mg/ml) and MARCKS peptide (36 $\mu$M). This synthetic peptide is based on the phosphorylatable region of the MARCKS protein, a specific substrate for PKC. The sequence of this peptide and sites of phosphorylation are detailed by Amess et al. (1992). The reaction was initiated by the addition of ATP, and after 10 min at 30 °C the reaction was stopped by spotting 40 $\mu$l of the assay volume on to phosphocellulose paper (P81). Unbound [$^{32}$P]ATP was removed by thorough washing in 75 mM $H_3PO_4$. The amount of $^{32}$P incorporated into MARCKS peptide was measured by scintillation counting. A unit of PKC activity is defined as 1 nmol of phosphate incorporated into MARCKS peptide in 1 min at 30 °C, in the presence of saturating concentrations of phosphatidylserine. The assay was carried out in the presence and absence of phosphatidylserine, and expressed as phosphatidylserine-dependent kinase activity, in order to correct for PKM (the proteolysed cofactor-independent form of PKC).

### PKC inhibitor assay

The PKC assay (described above) was modified to analyse the inhibitor proteins as follows. To a standard concentration of PKC (with known activity), the required concentration of inhibitor was added (total assay volume 55 $\mu$l), and inhibitory activity was expressed as a percentage of phosphatidylserine-dependent kinase activity.

### Dispersed phospholipid vesicles

These were prepared as follows: 5 $\mu$l of phosphatidylserine (20 mg/ml in chloroform/methanol, 1:2, v/v) was dried under nitrogen; 227 $\mu$l of 20 mM Tris/HCl, pH 7.5 was added, the mixture was vortex-mixed, and sonicated on ice at 2 $\mu$m amplitude, for 3 × 30 s bursts, with 30 s rest intervals, by using a MSE Soniprep 150 (fitted with a 3 mm probe tip).

### Mixed-micelle vesicles

These were prepared by drying together, under a stream of nitrogen, 8 $\mu$l of DAG (10 mg/ml in chloroform/methanol, 1:2, v/v) and 15 $\mu$l of phosphatidylserine (20 mg/ml in chloroform/methanol, 1:2, v/v). Then 100 $\mu$l of 1% (v/v) Triton X-100 in 20 mM Hepes, pH 7, was added to the dried lipid. This solution was vortex-mixed, followed by a 5 min incubation at 30 °C.

### PMA/DAG assay

The required concentration of DAG (final assay concn. of 6–600 $\mu$M) or PMA (final assay concn. of 10–1000 nM) was dried down with 5 $\mu$l of phosphatidylserine (20 mg/ml) under nitrogen, and prepared as described for phosphatidylserine vesicles. The assay was then carried out as above.

### Titration curve

The pH optimum for dissociation of the dimeric 14-3-3 isoforms was determined on an Isogel (a pre-poured agarose gel containing carrier ampholytes; Flowgen). Before loading the sample, the carrier ampholytes were pre-focused (1 W for 10 min). This established a pH gradient which increases left to right as indicated by the arrow (Figure 2). The pre-focused gel was rotated through 90° and the sample of native 14-3-3 isoforms was loaded in a strip equidistant between anode and cathode. The gel was again placed in the electrophoresis apparatus and re-run at 1 W for 10 min, followed by 1000 V and 25 W for 40 min. This analysis can also indicate changes in oligomeric state of a protein due to exposure of charged groups previously masked.

### Circular dichroism

Far-u.v. c.d. spectra were recorded from 260 to 185 nm in a Jasco J-600 spectropolarimeter operated with an instrument time constant of 0.5 s. The spectra were recorded at 22 °C for protein solutions at concentrations in the range 0.1–1.0 mg/ml in 0.1, 0.2 or 1 mm fused silica cuvettes. The spectra shown here represent the average of at least four scans. All data are reported in terms of molar c.d. absorption coefficient, $\Delta\epsilon$, based on an assumed mean residue weight of 111 Da. The residue molar ellipticity, $[\theta]_{mrw}$, may be obtained from the relationship $[\theta]_{mrw} = \Delta\epsilon \times 3300$.

The analysis of secondary-structure content from far-u.v. c.d. spectra was performed by the method of Hennessey and Johnson (1981).

### $PLA_2$ assay

(*a*) Assay by mass spectrometry

Phosphatidylcholine (52 $\mu$M) was incubated in the presence of 0.1 mM $CaCl_2$ and 20 mM Tris/HCl, pH 7.5, with cobra venom $PLA_2$ (7 units), pig pancreatic $PLA_2$ (7 units, both from Sigma), 8.3 $\mu$M KCIP-1 (mixed 14-3-3 isoforms) or 8.3 nM $\zeta$ 14-3-3 in a total assay volume of 55 $\mu$l. A range of concentrations of mixed brain 14-3-3 isoforms was also tested for ability to inhibit this group of $PLA_2$. The assays were initiated by addition of phosphatidylcholine. Samples were incubated at 30 °C for 60 min. Lipids were extracted with chloroform, and added to diethanolamine matrix before running on a VG 70-250 SE fast-atom-bombardment mass spectrometer at an operating voltage of 8 kV using a 30 kV $Cs^+$ ion gun.

(*b*) Assay by h.p.l.c.

Sheep brain 14-3-3 isoforms or recombinant $\tau$ 14-3-3 were assayed in the presence or absence of equimolar amounts of two forms of $PLA_2$, $cPLA_2$ (cytoplasmic, from dimethyl sulphoxide-differentiated HL-60 cells; Clark et al., 1990) and $sPLA_2$ (secreted from interleukin-1-stimulated human aortic smooth-muscle cells; Mizushima et al., 1989). The $cPLA_2$, from a 100000 ***g*** supernatant of HL60 cells lysed in the presence of a protease inhibitor cocktail, was purified by ion-exchange chromatography on Q-Sepharose, followed by size-exclusion chromatography on Superdex 200. In the $cPLA_2$ assay a 14-3-3/$PLA_2$ mixture was incubated at 37 °C for 1 h in the presence of 25 nCi of 1-stearoyl 2-[$^3$H]arachidonoyl phosphatidylcholine vesicles and 1 mM $Ca^{2+}$. The $sPLA_2$ secreted into the culture medium of the human aortic smooth-muscle cells was assayed without further purification. In the $sPLA_2$ assay a 14-3-3/$PLA_2$ mixture was incubated at 37 °C for 1 h in the presence of 10 nCi of 1-acyl 2-[$^{14}$C]arachidonoyl phosphatidylethanolamine micelles at 0.05% Triton X-100 and 10 mM $Ca^{2+}$. Assays were stopped and extracted with Dole's reagent (acidified propan-2-ol/heptane; Dole, 1956). The extract was applied to a 1 ml Bond-elut silica column (Varian), which bound the unhydrolysed phospholipid; unbound non-esterified fatty acid was collected and analysed by scintillation counting. In addition, trichloroacetic acid precipitant from the non-lipid fraction was analysed for the presence of radioactivity, to check whether 14-3-3 formed a covalent bond with the radiolabelled fatty acid (as suggested by Zupan et al., 1992).

### Investigation of a putative $PLA_2$–arachidonoyl complex

The possibility that a covalent arachidonoyl complex is formed was investigated by a method proposed by Zupan et al. (1992) in four preparations of 14-3-3 proteins. These were: (1) purified 14-3-3 from sheep brain; (2) purified recombinant $\epsilon$ isoform; (3) *E. coli* membranes from a strain expressing $\epsilon$ 14-3-3; (4) sheep brain 14-3-3 membrane fraction.

The preparations were incubated with [$^{14}$C]phosphatidylcholine (50 $\mu$M; sp. radioactivity 120000 d.p.m./nmol), in the presence of 10 mM $CaCl_2$, for 3 min at 37 °C. Reaction was terminated by addition of an equal volume of double-concentration solubilizing buffer, before SDS/PAGE. Coomassie-Blue-stained gels were immersed in 150 ml of fluophor (Enlightening, 1:5 dilution). Gels were dried and exposed to X-ray film, at −70 °C.

### Phospholipid binding

Phospholipid binding was carried out by a method adapted from Boustead et al. (1988). For this, 1.25 $\mu$mol of phosphatidylserine, or a mixture of phosphatidylserine and phosphatidylcholine, was dried into an Eppendorf tube under nitrogen, on ice. Then 1 ml of buffer A (2 mM $CaCl_2$, 100 mM KCl, 1 mM EGTA, 2 mM $MgCl_2$, 20 mM Hepes, pH 7.4) was added to the dried lipid. This solution was sonicated on ice at 2 $\mu$m amplitude for 3 × 30 s bursts with 30 s rest intervals.

A mixture of 150 $\mu$l of phospholipid vesicles (187.5 nmol) and 10 $\mu$g of protein (14-3-3 or annexin V) was made up to 500 $\mu$l with buffer A. This mixture was incubated at room temperature for 15 min, followed by centrifugation at 12000 ***g*** for 10 min. The supernatant (S1) was removed and reserved. The pellet was washed with a further 500 $\mu$l of buffer A and centrifuged at 12000 ***g*** for 10 min; supernatant (S2) was removed. Then 300 $\mu$l of acetone was added to the pellet, and incubated at −20 °C for 30 min. Samples were centrifuged at 12000 ***g*** for 10 min, and supernatant (S3) and pellet were separated and reserved. Pellet and S1 fractions were analysed for the presence of protein by SDS/PAGE.

### $Ca^{2+}$ binding in 14-3-3 protein

This was tested by four methods.

#### (*a*) Gel filtration

A 500 $\mu$g sample of 14-3-3 protein was incubated in a volume of 0.5 ml with 0.5 $\mu$g of $^{45}CaCl_2$ (10 $\mu$Ci) at 37 °C for 2 h and then run on a 30 ml Superose 12 column; 0.5 ml fractions were collected and counted for radiolabel.

#### (*b*) $^{45}$Ca overlay on nitrocellulose

For this, 5 $\mu$g of 14-3-3 was run on a SDS/PAGE gel and on a 10% (w/v) non-denaturing PAGE gel. The 14-3-3 proteins were electroblotted on to nitrocellulose, which was then probed with 0.3 mCi of $^{45}$Ca for 30 min at room temperature by the method of Garrigos et al. (1991). The nitrocellulose X-ray film was exposed for 7 days.

#### (*c*) Equilibrium dialysis

A 600 $\mu$g portion of 14-3-3 in a volume of 0.5 ml was dialysed against 1 mM EDTA for 16 h to remove any bound $Ca^{2+}$. The EDTA was then removed by dialysis against several changes of distilled water for 36 h. The 14-3-3 was then dialysed against 10 ml of 20 mM Tris/HCl, pH 7.4, containing 12.5 ng of $^{45}CaCl_2$ (0.25 $\mu$Ci) for 22 h at 4 °C, after which the concentration of radiolabel inside and outside the dialysis tubing was compared. Controls of serum and homogenized sheep brain were also analysed.

#### (*d*) Electrophoretic migration in the presence and absence of $Ca^{2+}$

14-3-3 protein was electrophoresed on SDS/PAGE in the presence of either 1 mM $Ca^{2+}$ or 0.1 mM EDTA.

## RESULTS

### Physical properties of 14-3-3 proteins

C.d. studies indicated that sheep brain 14-3-3 isoform mixture was predominantly $\alpha$-helical (63.7%), with 5.3% $\beta$-sheet (Figure 1). This Figure also shows that both recombinant and reverse-phase-purified 14-3-3 which had been renatured (as described in the Materials and methods section) have secondary structure identical with that of native 14-3-3. Recombinant and renatured 14-3-3 were dimeric, according to Superose 12 gel-exclusion chromatography, thus confirming a correct tertiary and quaternary structure (results not shown). Denatured 14-3-3 (boiled or after purification on reverse-phase h.p.l.c. at low pH) produced a c.d. profile which showed a lower ratio of $\alpha$-helix to random coil. Therefore, without careful renaturation, the protein exhibits some changes in secondary structure, which may be indicative of some refolding which could also affect quaternary (dimeric) structure.

In the titration curve shown in Figure 2, an abrupt change in migration of the Coomassie-Blue-staining material is seen at the position marked A. This corresponds to the pI of 14-3-3 isoforms. However, a change in the rate of migration is also seen at point B, which may be due to the dissociation of 14-3-3 to monomers. Dissociation would expose additional charged groups and alter the rate of movement of the different bands of Coomassie-Blue-staining material (native 14-3-3 dimers). This change in migration is observed over a range of pH between 3.7 and 4 (point B), and the order of isoform-band migration also alters. The titration curve therefore strongly suggests that the dissociation of dimers occurs within this pH range. This result is in accord with the elution from reverse-phase h.p.l.c. of 14-3-3 isoforms as individual monomeric species in an acidic buffer (Toker et al., 1992) and could explain the inability in the present study to recover 14-3-3 from gel-exclusion chromatography at pH values below pH 4.5.

Superose 12 gel size-exclusion chromatography was used to examine the stability of 14-3-3 across the pH range suggested by the titration-curve analysis. 14-3-3 was dimeric at pH 7.5, 5.5 and 5 (results not shown). Below this, at pH 4.5, 4 and 3, 14-3-3 was not eluted, presumably due to low solubility at or below the pI.

### Inhibitory activity of 14-3-3 isoforms

Two assay techniques are commonly employed to determine PKC activity, the mixed-micelle assay and the dispersion assay. These differ in that the mixed-micelle assay contains DAG in addition to phosphatidylserine, and the lipid vesicles in the mixed-micelle assay are larger than those of the dispersion assay. Details are given in the Materials and methods section. Sheep brain 14-3-3 isoforms inhibit PKC in both types of assay, showing that inhibition is independent of the physical properties

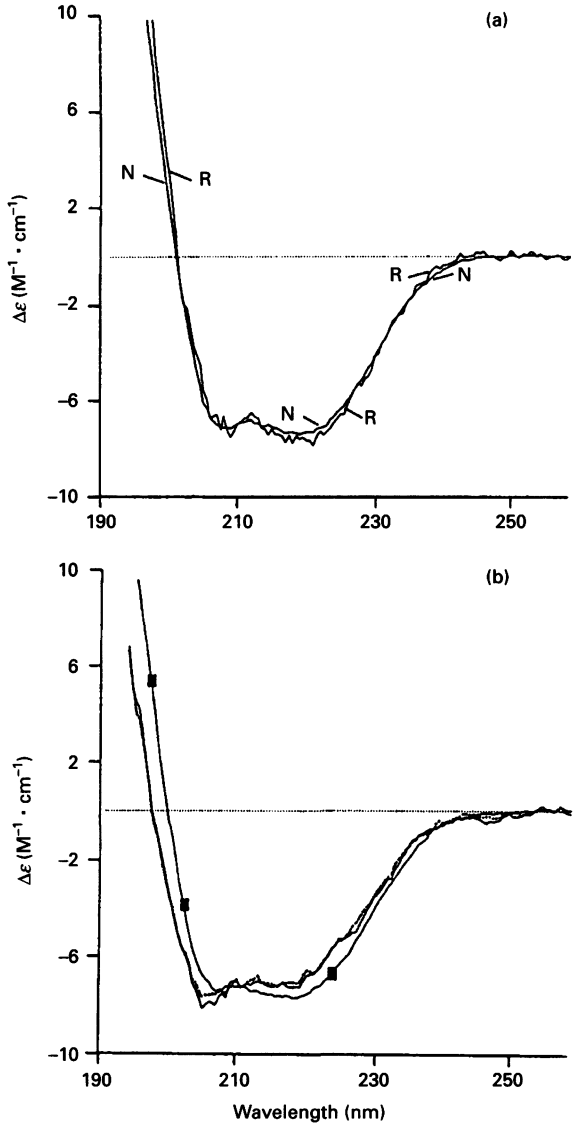


**Figure 1 Circular dichroism of 14-3-3**

(**a**) C.d. spectra of recombinant $\tau$ 14-3-3 isoform (R) and native mixed brain 14-3-3 isoforms (N). The similarities in the spectra indicate identical secondary structure. (**b**) Spectra of renatured $\gamma$, $\epsilon$ and $\zeta$ 14-3-3 (■). These are identical with that of native 14-3-3. The average spectra for these h.p.l.c.-separated (but not renatured) 14-3-3 isoforms (——) and heat-denatured 14-3-3 isoform mixture (· · · · · ·) are superimposable, but are different from the native 14-3-3 spectrum, indicating loss of $\alpha$-helix. The analysis of native secondary structure was carried out by the Hennessey and Johnson (1981) procedure, which indicated 63.7% $\alpha$-helix, 5.3% $\beta$-sheet, 13.3% turn and 17.7% random coil. This fits the secondary-structure prediction detailed by Toker et al. (1992).

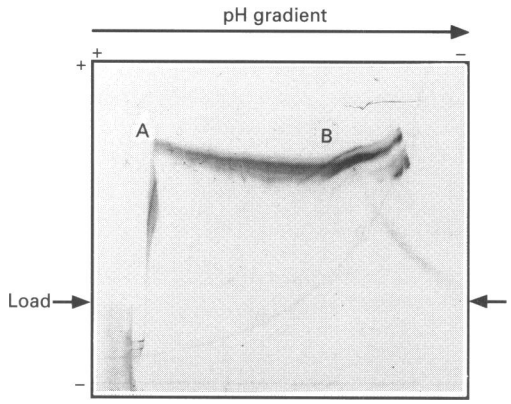


**Figure 2 Titration curve of human 14-3-3**

Two-dimensional pH separation of a pure native preparation of human 14-3-3. The arrow represents increase in pH gradient from left to right. A 40 $\mu$l portion of human 14-3-3 (133 $\mu$g) was loaded in a strip across the pH gradient, at an equal distance from anode and cathode. pH values were obtained from a blank gel which was run as described in the Materials and methods section, cut into 1 $cm^2$ squares, and the pH of each square was determined in the presence of 1 ml of 10 mM KCl (after 1 h equilibration time). The two areas where shift in migration occurs are labelled A and B.

**Table 1 $IC_{50}$ of 14-3-3 isoforms**

All assays were carried out by the dispersion assay with 1 unit of PKC/ml and MARCKS peptide as substrate except for * [value obtained by Toker et al. (1990) with histone IIIS as substrate]. All values in this study are means of triplicate assays at a minimum of three concentrations of 14-3-3, ±S.E.M. except where indicated: † mean of three triplicate assay curves; ‡ ±range of triplicate assays.

| Protein | $IC_{50}$ ($\mu$M) |
|---|---|
| 14-3-3 brain isoforms | 0.8±0.1 |
| 14-3-3 brain isoforms (histone substrate)* | 0.85 |
| 14-3-3 brain isoforms (mixed micelle assay) | 4.5±0.3 |
| Recombinant $\zeta$ 14-3-3 | 0.9±0.1‡ |
| Renatured $\zeta$ 14-3-3 | 1.1±0.2 |
| Recombinant $\tau$ 14-3-3 | 0.32†±0.01 |
| Exo1 | 0.8±0.1‡ |

of the lipid vesicle and is not an artefact of one particular assay system. However, the $IC_{50}$ of KCIP-1 in the mixed-micelle assay is 4.5 $\mu$M, rather than 0.85 $\mu$M in the dispersion assay (Table 1). This difference may be in part due to the requirement for DAG in the mixed-micelle assay, which, as shown below, will overcome 14-3-3 inhibition of PKC at high concentration. Assays described below were performed by using the dispersion assay.

Table 1 shows that Exo1 inhibits PKC with an $IC_{50}$ similar to that of the sheep brain 14-3-3 isoform mixture. The recombinant $\zeta$ isoform inhibited PKC to a similar extent as the sheep brain 14-3-3 isoform mixture, whereas the recombinant $\tau$ isoform inhibited PKC with an $IC_{50}$ of 0.32 $\mu$M (Figure 3). The $\epsilon$ recombinant isoform, with the fusion protein enzymically removed, inhibited PKC with an $IC_{50}$ of ~ 0.4 $\mu$M, but the maltose-binding carrier protein/$\epsilon$ 14-3-3 fusion (i.e. with the carrier protein not cleaved) had much decreased activity (~ 10% inhibition at 0.7 $\mu$M).

Table 1 also shows that renatured and recombinant $\zeta$ isoforms inhibited PKC to a similar extent. Renatured $\gamma$ and $\epsilon$ isoforms were both more potent inhibitors than purified isoform mixture and $\zeta$ isoform. Two separate triplicate assays of renatured $\gamma$ isoform indicated an $IC_{50}$ of ~ 0.6 $\mu$M. $\epsilon$ 14-3-3 assayed in triplicate at 0.32 $\mu$M gave 50% inhibition of PKC.

It was previously reported that phorbol ester can partially overcome 14-3-3 inhibition of PKC (Toker et al., 1990). Since phorbol esters interact at the same site on PKC, DAG may also interfere with 14-3-3 inhibition of PKC. Figures 4(a) and 4(b) indicate that phorbol ester (PMA) has a higher potency than diacylglycerol, overcoming inhibition at a concentration of

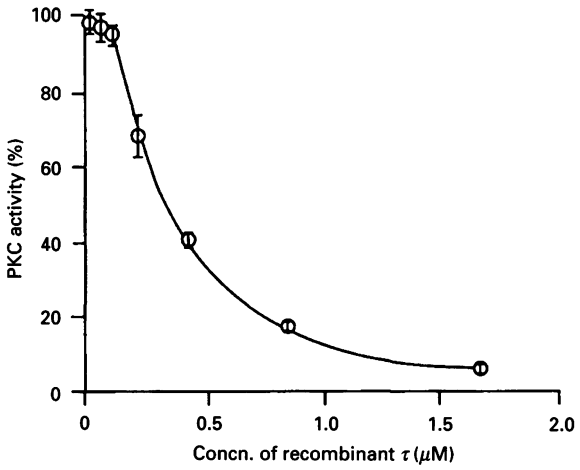


**Figure 3 Inhibition curve for 14-3-3 τ isoform**

Various concentrations of recombinant τ isoform were incubated with PKC in the dispersion assay. Height of bars represent double the S.E.M. at each concentration point. An $IC_{50}$ of 0.32 μM was calculated from the mean of three separate experiments, with S.E.M. ± 0.01.

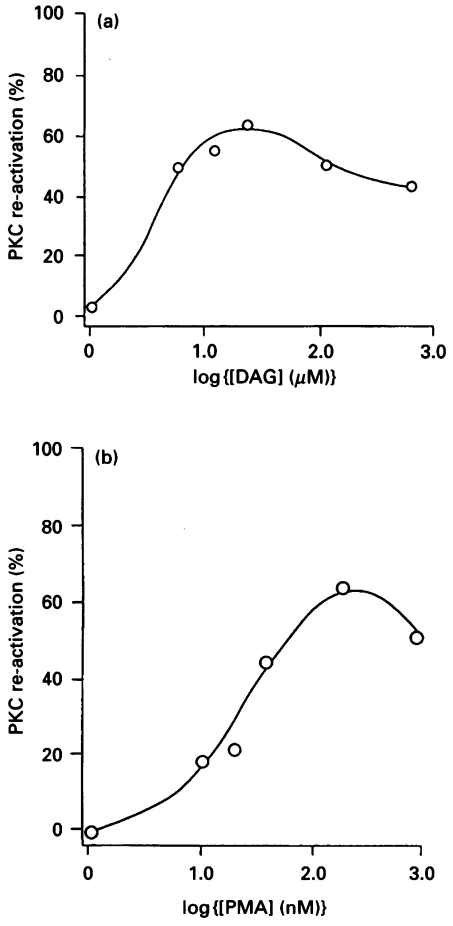


**Figure 4 Effects of DAG and phorbol ester on 14-3-3 inhibition of PKC**

The dispersion assay was carried out in the presence of (**a**) DAG (final assay concn. 6–600 μM) or (**b**) PMA (final assay concn. 10–1000 nM).

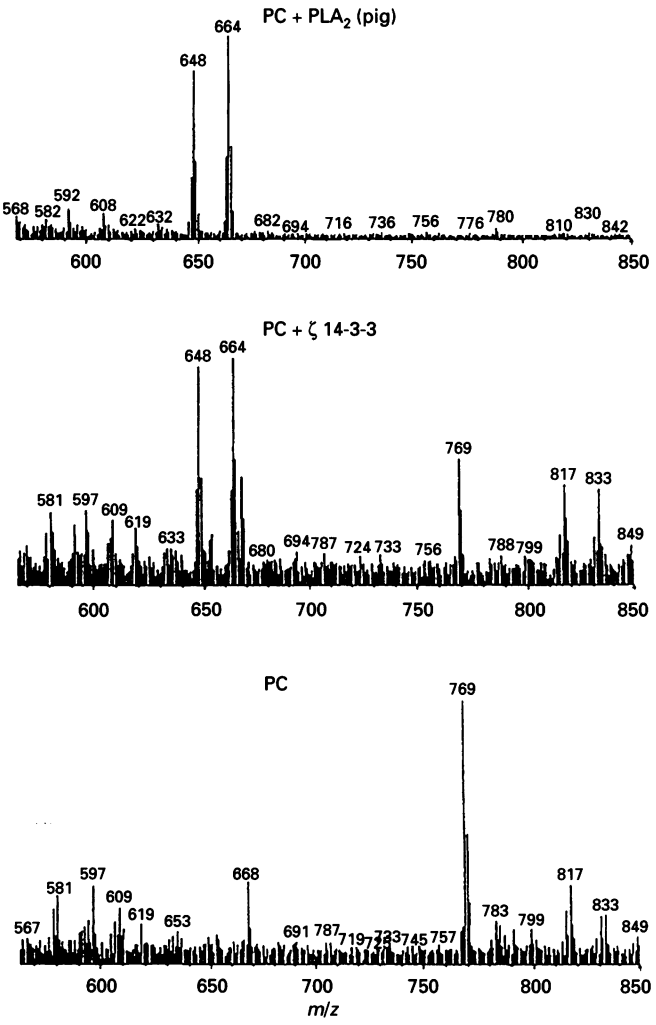


**Figure 5 $PLA_2$ mass-spectrometric assay**

Pig pancreatic $PLA_2$ (top) or ζ 14-3-3 isoform (middle) were preincubated with 52 μM phosphatidylcholine (PC) in the presence of 0.1 mM $CaCl_2$ and 20 mM Tris/HCl, pH 7.5, for 60 min at 30 °C. Lipids were extracted with chloroform and analysed by fast-atom-bombardment mass spectrometry. A control of PC is also shown (bottom). The quasimolecular ion $[M+H]^+$ of PC is evident at *m/z* 769, except in the sample after incubation in the presence of $PLA_2$, where the PC has been hydrolysed. Peaks at *m/z* 817, 833 and 849 are due to PC epoxides. Peaks at *m/z* 648 and 664 are impurities extracted from polyethylene tubes.

200 nM rather than 40 μM. However, both DAG and PMA overcame inhibition by a maximum of approx. 60% in the presence of phosphatidylserine.

## Do 14-3-3s have $PLA_2$ activity?

It was recently suggested by Gross and co-workers that 14-3-3 has $PLA_2$ activity (Zupan et al., 1992). They state that, whereas 14-3-3 purified from platelets releases radiolabelled arachidonic acid from phosphatidylcholine, the recombinant ζ isoform forms a covalent acyl-enzyme complex with the arachidonoyl hydrolysis product, which can only be detected as a 30 kDa band by SDS/PAGE followed by autoradiography. However, this experiment was carried out by incubating the membrane fraction of *E. coli* expressing the recombinant ζ 14-3-3 isoform with radiolabelled phosphatidylcholine. Therefore a separate $PLA_2$, present in the *E. coli* membrane fraction, could have mediated the

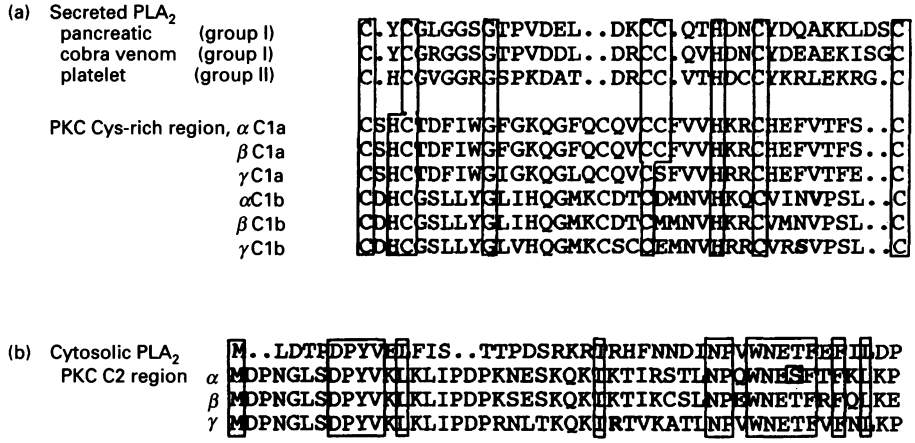


**Figure 6 Sequence similarities between $PLA_2$s and PKC**

Alignments are shown of mammalian PKC $\alpha$, $\beta$, $\gamma$ with: (a) $sPLA_2$ (group I and II); the pancreatic and platelet enzymes are human sequences; (b) human $cPLA_2$. Boxes show areas of identity; **bold** letters indicate amino acids that are not totally conserved in a particular isoform of PKC for all known mammalian species.

**Table 2 $PLA_2$ activity in the presence of 14-3-3**

$cPLA_2$ (cytoplasmic from dimethyl sulphoxide-differentiated HL-60 cells) and $sPLA_2$ (secreted from interleukin-1-stimulated human aortic smooth-muscle cells) were incubated in the absence and presence of 14-3-3 mixed isoforms or $\tau$ 14-3-3 as detailed in the Materials and methods section.

| Sample | $PLA_2$ activity (d.p.m.) |
|---|---|
| $cPLA_2$ | 906 ± 41 |
| $cPLA_2$ + 14-3-3 | 1003 ± 42 |
| $cPLA_2$ + $\tau$ 14-3-3 | 958 ± 162 |
| $sPLA_2$ | 355 ± 26 |
| $sPLA_2$ + 14-3-3 | 394 ± 9 |
| $sPLA_2$ + $\tau$ 14-3-3 | 347 ± 50 |

acylation of 14-3-3. To re-examine this possibility, two preparations (mixed 14-3-3 isoforms purified from sheep brain and purified recombinant $\epsilon$ isoform) were incubated with radiolabelled phosphatidylcholine, subjected to SDS/PAGE and autoradiographed. The membrane fraction of *E. coli* expressing the recombinant isoform was also incubated with radiolabelled phosphatidylcholine. Finally, to determine if 14-3-3 merely acts as an arachidonoyl acceptor for $PLA_2$, $sPLA_2$ and radiolabelled phosphatidylcholine were incubated with purified 14-3-3 mixture or purified recombinant $\epsilon$ 14-3-3 isoform. All autoradiographs from the above experiments were blank (results not shown).

Both 14-3-3 mixture and recombinant $\zeta$ isoform were assayed for $PLA_2$ activity on phosphatidylcholine, and the release of arachidonic acid was monitored by fast-atom-bombardment mass spectrometry as described in the Materials and methods section. No peak at the mass of phosphatidylcholine was observed when incubated with $PLA_2$, but the phosphatidylcholine peak remained when preincubated with 14-3-3 (Figure 5). Purified 14-3-3 and purified recombinant $\tau$ isoform were assayed for $PLA_2$ activity by using conditions developed for $cPLA_2$ and $sPLA_2$. No $PLA_2$ activity was detected (results not shown). Since both $PLA_2$ and $sPLA_2$ have similarity to distinct regions on the regulatory domain of PKC (Figure 6), effects of 14-3-3 on $PLA_2$ activity were also investigated; results are shown in Table 2. In addition, pancreatic $PLA_2$ was not inhibited by 14-3-3 in the mass-spectrometry assay (results not shown).

### Does 14-3-3 bind phospholipid?

Annexins are $Ca^{2+}$- and phospholipid-binding proteins, and some annexins inhibit $PLA_2$ (Russo-Marie, 1992). Since 14-3-3 has an amino acid sequence similar to a motif in the C-terminus of annexins (Aitken et al., 1990), the possibility that 14-3-3 may bind phospholipids was examined. 14-3-3 did not bind phosphatidylserine vesicles or phosphatidylserine/phosphatidylcholine phospholipid vesicles in a $Ca^{2+}$-dependent or -independent manner (results not shown), in contrast with the positive controls (annexin V isoforms). Table 2 also indicates that, in contrast with annexins, 14-3-3 does not inhibit $PLA_2$.

### Does 14-3-3 bind $Ca^{2+}$?

Incubation of 14-3-3 with $^{45}Ca^{2+}$ followed by gel filtration on Superose 12 showed that 0.5 mg of protein contained only 400 c.p.m. of $^{45}Ca^{2+}$, 0.01% of the total radioactivity. In the $^{45}Ca^{2+}$ overlay assay, 14-3-3 was blotted on to nitrocellulose from native gels and from SDS/PAGE gels (Garrigos et al., 1991). No $Ca^{2+}$ binding was detectable in either sample, whereas a sample of sheep brain homogenate, run in parallel, contained several $Ca^{2+}$-binding proteins. Furthermore, no $Ca^{2+}$ binding could be shown by equilibrium dialysis of 600 $\mu$g of 14-3-3 protein against 12.5 ng of $^{45}CaCl_2$, whereas controls using dilute serum or whole brain homogenate resulted in an 8-fold increase in $^{45}Ca^{2+}$ concentration inside the dialysis sac. Finally, there was no visible effect on the mobility of 14-3-3 proteins in SDS/PAGE in the presence of either 1 mM $Ca^{2+}$ or 0.1 mM EGTA. All these results show that 14-3-3 does not bind $Ca^{2+}$.

## DISCUSSION

Individual 14-3-3 isoforms have distinct tissue distributions (reviewed by Aitken et al., 1992). This may reflect differences in isoform function; therefore it is important to establish, as we have done for all isoforms investigated in this study, whether all 14-3-3 family members inhibit PKC. This analysis is particularly

problematical, since they may form heterodimers (Toker et al., 1992) and a suitable method for separation of native 14-3-3 isoforms has not yet been found. The reverse-phase h.p.l.c. method has low recovery and short column life, and activity is not easily restored. However, by using the renaturation procedure developed for this study, biological activity of 14-3-3 isoforms separated by h.p.l.c. can be recovered. It has been reported that $\zeta$ 14-3-3 isoform renatured from reverse-phase h.p.l.c. by neutralizing the eluted peak with Tris buffer was an activator of PKC (Isobe et al., 1992). However, maximal activation was less than 2-fold under their assay conditions, which included DAG. The renatured isoforms analysed in the present study were the three most abundant brain isoforms, $\gamma$, $\epsilon$ and $\zeta$. We have also analysed the activity of 14-3-3 recombinant isoforms and shown that these retain the PKC-inhibitory activity of the wild-type proteins. The recombinant and renatured $\zeta$ isoform in the present study both inhibited PKC to a similar extent ($IC_{50}$ 0.9 and 1.1 $\mu$M respectively; Table 1). In addition, recombinant $\epsilon$ 14-3-3 inhibited PKC with an $IC_{50}$ of $\sim$ 0.4 $\mu$M, whereas the value for renatured $\epsilon$ 14-3-3 was 0.32 $\mu$M. $\zeta$ 14-3-3 together with $\delta$ 14-3-3 (which we have shown to be in all probability identical in primary structure; Martin et al., 1993) comprise $\sim$ 30% of total brain 14-3-3. The finding that $\zeta$ 14-3-3 is a slightly less potent PKC inhibitor than are mixed brain isoforms ($IC_{50}$ 0.85 $\mu$M) is consistent with the greater potency of the other major isoforms ($\gamma$ and $\epsilon$, $\sim$ 26% and 21% of total protein, $IC_{50}$ 0.6 and 0.32–0.4 $\mu$M respectively).

In order to eliminate problems with the PKC assay due to batch-to-batch variations in the substrate histone and cofactor phosphatidylserine, the former was replaced by a synthetic peptide (MARCKS peptide) and the latter with synthetic phosphatidylserine. The $IC_{50}$ of sheep brain 14-3-3 isoform mixture remained the same with the new substrate, indicating that 14-3-3 is directly associating with PKC and is not substrate-specific inhibition. The latter was a possibility if the basic protein, histone, interacted with the acidic 14-3-3. The importance of subtracting PKM (cofactor-independent activity) from the true level of PKC in the purified kinase preparation to be assayed cannot be over-emphasized.

14-3-3 has now been shown to inhibit PKC in two assay systems in the presence of two different types of lipid vesicle, indicating that inhibition is independent of the physical properties of the membrane. In addition, 14-3-3 has been shown not to bind $Ca^{2+}$ or phospholipid in a $Ca^{2+}$-dependent manner, thus supporting previous observations that inhibition is independent of the concentration of these cofactors (Toker et al., 1990). Our present results with brain 14-3-3 isoform mixture do not exclude the possibility of phospholipid binding of particular isoform(s). Recent studies have indicated a low level of $Ca^{2+}$-independent binding of $\gamma$ and $\epsilon$ 14-3-3 isoforms to phospholipid vesicles (D. Roth, A. Morgan, H. Martin, D. Jones, G. J. M. Martens and R. D. Burgoyne, unpublished work).

In the present paper, Exo1 has been shown to inhibit PKC in the dispersion assay, in contrast with the results of Morgan and Burgoyne (1992b), who found no effect on the activity of PKC. PKC and Exo1 act in synergy to increase $Ca^{2+}$-dependent exocytosis in permeabilized adrenal chromaffin cells (Morgan and Burgoyne, 1992a). In this study we have clearly shown that 14-3-3 is not a $PLA_2$ (which could have mobilized arachidonic acid to stimulate secretion). Our results therefore preclude this mechanism for synergy of Exo1 and PKC in $Ca^{2+}$-dependent secretion.

Inhibition of PKC by 14-3-3 was overcome to a maximum of 60% with DAG at 40 $\mu$M or phorbol ester at 0.2 $\mu$M (Figure 4). In previous studies, with a concentration range of DAG routinely used in laboratories (i.e. 1–5 $\mu$M), this effect was not observed (Toker et al., 1990). However, the present results confirmed the previous observation (at a single concentration) that phorbol ester could partially overcome PKC inhibition. Although it is possible that binding of DAG or phorbol ester produces a conformation of PKC which is less susceptible to inhibition, it is more likely that the interaction site of 14-3-3 on PKC is at or near the DAG/phorbol ester binding site, i.e. the cysteine-rich (C1) region. This region has been reported by Maraganore (1987) to have sequence similarity to a cysteine-rich potential phospholipid-binding site in $PLA_2$. This prompted a reassessment of the possible role of 14-3-3 in regulating $PLA_2$.

Zupan et al. (1992) produced evidence that the recombinant $\zeta$ isoform has $PLA_2$ activity. However, in the present study brain 14-3-3 mixture (which includes $\zeta$) as well as recombinant $\epsilon$, $\tau$ and $\zeta$ isoforms have been shown by two assay methods not to act as $PLA_2$ enzymes. Morgan et al. (1993) have also shown that Exo1 [which are clearly member(s) of the 14-3-3 family] have no $PLA_2$ activity.

Secreted types of $PLA_2$ are found in various snake venoms, which contain both groups I and II enzymes. Pancreatic $PLA_2$ is group I and $sPLA_2$ from platelets is group II. These enzymes contain a region with similarity to the cysteine-rich regions (C1a and C1b) of PKC (Figure 6). $cPLA_2$, which may be regulated by effectors of signal-transduction mechanisms, is a totally distinct intracellular protein without any apparent homology to the above secreted forms of $PLA_2$. However, it does appear to have some similarity to the C2 region of PKC (Clark et al., 1991) (see Figure 6). We therefore tested the effects of 14-3-3 on members of both these enzyme families. The results in Table 2 confirm that 14-3-3 does not affect $cPLA_2$ and group II $PLA_2$ activity. 14-3-3 was also shown to have no effect on pancreatic $PLA_2$ (group I; results not shown).

The 14-3-3 family has a motif which shows similarity with the pseudosubstrate site on PKC (Parker, 1989). This motif may bind the active site of PKC, thus blocking access of substrate, but a second interaction site must be present to account for the non-competitive inhibition (Toker et al., 1990). This site may be the sequence in 14-3-3 which shows similarity to the C-terminus of the annexin family of $Ca^{2+}$–phospholipid- and membrane-binding proteins (Aitken et al., 1990). Annexin V is a potent inhibitor of PKC (Schlaepfer et al., 1992). Mochly-Rosen et al. (1991) have investigated 'Receptors for Activated C Kinase' (RACKs) and the mechanism by which PKC is translocated to the plasma membrane when activated by $Ca^{2+}$. They have shown that the C-terminus of annexins (and the similar sequence in KCIP-1) can prevent PKC association with RACKs proteins. Since KCIP-1/14-3-3 does not inhibit PKM (Toker et al., 1990), the second interaction site may involve the regulatory domain of PKC. This is supported by the DAG/phorbol ester re-activation results, which suggest involvement of the cysteine-rich C1 region of PKC (Maraganore, 1987). The consequences for the function of 14-3-3 as a regulator of PKC activity may be as follows: 14-3-3 may function in the cytosol to maintain activity of cytosolic PKC at a low level. Stimulation of PtdIns$P_2$ hydrolysis produces the two second messengers, Ins$P_3$ and DAG. The former causes an increase in $Ca^{2+}$ levels, which may assist translocation of PKC to the plasma membrane. If translocation is not prevented by 14-3-3, the highly elevated local concentration of DAG may overcome the 14-3-3 inhibition. The recombinant $\epsilon$ isoform, expressed with a fusion protein attached to the N-terminus, was a poor PKC inhibitor. An additional site of PKC–KCIP-1 interaction may therefore be near the N-terminus of 14-3-3.

One of the recombinant 14-3-3 isoforms investigated in this study is the T-cell ($\tau$) 14-3-3 isoform. This is the first 14-3-3 that is not a major brain isoform (Nielsen, 1991) to be investigated as

a PKC inhibitor. PKC $\gamma$ isoenzyme has been reported to be brain-specific (Nishizuka, 1984). It is therefore of particular interest that we have shown (D. Jones, K. Robinson and A. Aitken, unpublished work) that the $\tau$ isoform is an inhibitor of $\gamma$ PKC. In contrast with recombinant and renatured $\zeta$ 14-3-3, the $\tau$ 14-3-3 isoform has an $IC_{50}$ value (0.32 $\mu$M) which is less than half that of the brain isoform mixture.

Future experiments will reveal whether 14-3-3 inhibits $Ca^{2+}$-independent PKCs or $Ca^{2+}$-independent PKC $\zeta$ isoform, which also lacks one of the cysteine-rich repeat regions (Hug and Sarre, 1993). If inhibition is associated with the DAG-binding motif on PKC, perhaps the latter will not be inhibited.

The secondary-structure studies which have revealed a high $\alpha$-helical content for this family of dimeric proteins are being extended to three-dimensional X-ray crystallography, which will indicate more clearly the sites of interaction with PKC and mechanism of inhibition.

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