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Purification to homogeneity, characterization and monoclonal antibodies of phospholipid-sensitive Ca^{2+}-dependent protein kinase from spleen

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A phospholipid-sensitive Ca^{2+}-dependent protein kinase was purified to homogeneity, for the first time, from extracts of pig spleen, employing the steps of DEAE-cellulose, octyl-agarose, Sephacryl S-200 and phosphatidylserine–Affigel 10 affinity chromatographies. The purified enzyme appeared as a single protein band on both analytical (non-denaturing) and sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, having a minimum mol.wt. of 68000 ± 200. The molecular weight of the enzyme was also determined to be 74500 ± 4600 by gel filtration and 80000 based on its sedimentation coefficient (5.52S) and Stokes radius (3.52 ± 0.09 nm), indicating that the enzyme was a monomeric protein. The frictional ratio (f/f_0) of the enzyme was 1.24, indicating it was non-globular in shape. The enzyme had a pI of 5.3, and a pH optimum of 6.5 for its reaction. Amino acid analysis indicated that the enzyme apparently was not similar to myosin light-chain kinase (a calmodulin-sensitive species of Ca^{2+}-dependent protein kinase) or cyclic AMP-dependent and cyclic GMP-dependent protein kinases. The enzyme had an apparent K_m for ATP of $7.5\,\mu$M. Histone H1 and myelin basic protein were effective substrates for the enzyme, with apparent K_m values of 0.3 and $0.2\,\mu$M, and $V_{max.}$ values of 0.06 and $0.09\,\mu$mol/min per mg of enzyme respectively. The enzyme activity was dependent on both phosphatidylserine (apparent $K_a = 6.25\,\mu$g/ml) and Ca^{2+} (apparent $K_a = 160\,\mu$M). Calmodulin was unable to substitute for the phospholipid as a cofactor, nor was it a subunit of the enzyme. Sr^{2+} and Ba^{2+} could partially mimic Ca^{2+} to activate the enzyme in the presence of phosphatidylserine. An endogenous substrate protein (mol.wt. 41000) for the enzyme was found in the total, solubilized fraction of pig spleen. Monoclonal antibodies against the enzyme interacted similarly with the homogeneous and impure enzyme; the antibodies, however, did not bind to cyclic nucleotide-dependent protein kinases.

Ca^{2+} has long been known to play an important role in many biological processes (for review, see Berridge, 1975; Rasmussen & Waisman, 1981). However, the exact mechanisms by which Ca^{2+} exerts its effects on cellular activities have remained obscure. The discovery of calmodulin, a ubiquitous multifunctional Ca^{2+}-binding protein, has shed new light on how Ca^{2+} can interact with and activate various enzymes. By serving as either an enzyme subunit or an obligatory cofactor, calmodulin confers a Ca^{2+}-sensitivity to a wide variety of enzyme systems (for review, see Wang & Waisman, 1979; Cheung, 1980).

Abbreviations used: PMSF, phenylmethanesulphonyl fluoride; SDS, sodium dodecyl sulphate.

Takai *et al.* (1979) have reported the presence in brain of a new species of Ca^{2+}-dependent protein kinase that requires phospholipid (such as phosphatidylserine), rather than calmodulin, as a cofactor. Subsequently, we have reported the widespread occurrence of this phospholipid-sensitive Ca^{2+}-dependent protein kinase in various tissues of a number of animal phyla (Kuo *et al.*, 1980). Endogenous substrate proteins for this enzyme have been detected in several tissues including rat or guinea-pig brain (Wrenn *et al.*, 1980, 1981*a*), heart (Katoh *et al.*, 1981*a*,*b*), pancreas, liver, vas deferens and adrenal (Wrenn *et al.*, 1981*b*), and human platelets (Kawahara *et al.*, 1980), neutrophils and leukaemic cells (D. M. Helfman, W. R. Vogler & J. F. Kuo, unpublished work). Studies on the enzyme

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and its substrate proteins indicate that they are distributed both in the cytosolic and particulate fractions (Katoh & Kuo, 1982). In view of the ubiquitous nature of this protein kinase system and the importance of Ca^{2+} and phospholipid in cellular function, an extensive investigation of phospholipid-sensitive Ca^{2+}-dependent protein kinase seems useful. In the present paper we report the purification to homogeneity, for the first time, of the enzyme from pig spleen extract, some of its properties and initial accounts of studies concerning its monoclonal antibodies.

Experimental

Materials

Component 6 (the unphosphorylated species) of bovine central-nervous-system myelin basic protein (Chou *et al.*, 1976) was a gift from Dr. C.-H. J. Chou and Dr. Robert F. Kibler, Department of Neurology, Emory University School of Medicine. Pharmalyte ampholytes (pH 4.0–6.5), Sephacryl S-200 and staphylococcal protein A were from Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.; Bio-Lyte electrofocusing gel and Affigel 10 were from Bio-Rad Laboratories, Rockville Centre, NY, U.S.A.; DEAE-cellulose (fibrous form), octyl-agarose, Tris, Mes (4-morpholine-ethanesulphonic acid), Pipes (1,4-piperazinediethanesulphonic acid), PMSF, 1,3-dioleoylglycerol, lysine-rich histone (histone H1; type III-S), various phospholipids including phosphatidylserine (bovine brain) and standard proteins for molecular-weight determinations were from Sigma Chemical Co., St. Louis, MO, U.S.A.; ^{32}P and ^{125}I, both carrier-free, were from Amersham Corp., Arlington Heights, IL, U.S.A.; Balb/c mice were from Charles River Breeding Laboratories, Wilmington, MA, U.S.A.; phosphatidylinositol (bovine brain) was from Calbiochem–Behring, La Jolla, CA, U.S.A. All other reagents were of the finest reagent grade available.

Methods

Purification of phospholipid-sensitive Ca^{2+}-dependent protein kinase from pig spleen extracts. Fresh pig spleens were obtained from a local slaughterhouse and stored at −18°C until the time of enzyme purification. All of the following purification steps were carried out at 4°C.

Step 1 (extraction). Frozen pig spleen (1.4 kg) was homogenized in 3 vol. (w/v) of 20 mM-Tris/HCl (pH 7.5) containing 2 mM-EDTA, 0.3 mM-PMSF and 50 mM-2-mercaptoethanol (extraction solution) for 1 min in a Waring Commercial Blendor and centrifuged at 14000 *g* for 35 min to yield the crude extract. The extract was then filtered through glass wool to remove the fat and the pH was readjusted to 7.5 with 1 M-Tris/HCl (pH 9.0).

Step 2 (DEAE-cellulose chromatography). The extract from the above step was stirred into 2.5 litres of DEAE-cellulose for 2 h and washed four times with 5.0 litres of extraction solution with the use of a large Buchner funnel. The resulting DEAE-cellulose was resuspended in 3 litres of the extraction solution and packed into two columns (5.5 cm × 60 cm), followed by washing each with an additional 2 litres of extraction solution. The enzyme was then eluted using a linear gradient of NaCl dissolved in the extraction solution (0–350 mM; total volume, 4 litres).

Step 3 (octyl-agarose chromatography). The active fractions from the above step were pooled and concentrated to 200 ml, by using an Amicon stirred-cell ultrafiltration system fitted with a YM-10 membrane. The concentrated pool was divided into four aliquots (50 ml each), made to 1 M-$(NH_4)_2SO_4$, and individually loaded on to four octyl-agarose columns (2.5 cm × 7 cm), previously equilibrated with 20 mM-Tris/HCl (pH 7.5) containing 2 mM-EDTA and 50 mM-2-mercaptoethanol (solution A) containing 1 M-$(NH_4)_2SO_4$. Each column was then washed with 250 ml of solution A containing 1 M-$(NH_4)_2SO_4$ followed by 750 ml of solution A containing 0.5 M-$(NH_4)_2SO_4$. Finally, the enzyme was eluted with 750 ml of solution A containing 0.1 M-$(NH_4)_2SO_4$.

Step 4 (Sephacryl S-200 chromatography). The active fractions from the above step were pooled, concentrated to approx. 2 ml, and loaded on to a Sephacryl S-200 column (1.8 cm × 100 cm), previously equilibrated with solution A containing 10% glycerol (to stabilize enzyme activity). The enzyme was eluted with the same solution. The active fractions were pooled, concentrated to about 5 ml, made 30% with respect to glycerol and stored at −80°C. The enzyme was stable for at least several months.

Step 5 (phosphatidylserine–Affigel 10 chromatography). The frozen enzyme solution was first thawed and then made 4 mM with respect to $CaCl_2$ by the addition of the appropriate volume of 100 mM-$CaCl_2$. The enzyme was applied to a column (0.7 cm × 4 cm) of phosphatidylserine–Affigel 10, previously equilibrated with 20 mM-Tris/HCl (pH 7.5) containing 50 mM-2-mercaptoethanol, 4 mM-$CaCl_2$ and 30% glycerol (solution B). The column was washed sequentially with (*a*) 20 ml of solution B, (*b*) 30 ml of solution B containing 1 M-NaCl and (*c*) 20 ml of 20 mM-Tris/HCl (pH 7.5) containing 1 mM-EGTA, 50 mM-2-mercaptoethanol and 30% glycerol. The enzyme was finally eluted from the column with 20 mM-Tris/HCl (pH 7.5) containing 2 mM-EDTA, 10 mM-EGTA, 2 M-NaCl, 50 mM-2-mercaptoethanol and 30% glycerol. Each fraction (2 ml) was then dialysed extensively against 20 mM-Tris/HCl (pH 7.5) containing 2 mM-EDTA,

50mM-2-mercaptoethanol and 30% glycerol (solution C). The first four fractions contained 98% of enzyme activity. Finally, the enzyme was concentrated by application to a small column (0.5cm × 1.5cm) of DEAE-cellulose, previously equilibrated with solution C. The column was then washed with 10ml of solution C and the enzyme eluted with 1ml of solution C containing 150mM-NaCl. The enzyme could be stored at −80°C in glycerol (30%, v/v) or bovine serum albumin (2mg/ml) for several months without significant loss of activity.

Preparation of phosphatidylserine–Affigel 10. To 25ml of Affigel 10, previously washed with ice-cold isopropyl alcohol, was added 125mg of phosphatidylserine dissolved in 12.5ml of chloroform/methanol (19:1, v/v). This mixture was incubated at 4°C for 36h with gentle agitation in a shaker bath, followed by washing in a Buchner funnel with chloroform/methanol (19:1, v/v). The gel was resuspended in 1M-ethanolamine/HCl (pH8.0) and re-incubated, with gentle agitation, at 4°C for 12h to block any unreacted active ester groups remaining in Affigel 10. Finally, the gel was washed with 20mM-Tris/HCl (pH7.5). For storage, the gel was suspended in 20mM-Tris/HCl (pH7.5) containing 1mM-NaN_3, and the air in the container removed by flushing with N_2 to avoid rancidification of the coupled phospholipid. Determination of the amount of phospholipid coupled to Affigel 10 was as described by Wise *et al.* (1982*a*). The coupling density was determined to be 3mg of phosphatidylserine per ml of packed gel.

Assay for phospholipid-sensitive Ca^{2+}-dependent protein kinase. The assay conditions were essentially the same as those we described previously (Kuo *et al.*, 1980; Wise *et al.*, 1982*a*). Briefly, the standard reaction mixture contained, in a final volume of 0.2ml, 5μmol of Pipes (pH6.5), 2μmol of $MgCl_2$, 5μg of phosphatidylserine, 40μg of lysine-rich histone (histone H1), 0.04μmol of EDTA, with or without 0.1μmol of $CaCl_2$, 1nmol of [γ-^{32}P]ATP [containing (0.8–2.0) × 10^6c.p.m.] and appropriate amounts of enzyme protein. Tris/HCl (5μmol, pH7.5) replaced Pipes where indicated. All reactions were carried out at 30°C and started by the addition of ATP, except in the determination of the K_m for ATP, where reactions were started by addition of enzyme. Enzyme activities were linear as a function of incubation time and enzyme amount in all assays reported herein.

Endogenous substrate protein phosphorylation. Frozen pig spleen (5g) was homogenized in 2vol. of 20mM-Tris/HCl (pH7.5) containing 2mM-EGTA, 0.1% Triton X-100 and 50mM-mercaptoethanol. The homogenate was allowed to stand in ice for 2h, with occasional stirring, and then centrifuged at 105000*g* for 60min. The supernatant (total solubilized protein) was used as the source of protein kinases and their endogenous substrate proteins. The procedures for the phosphorylation of endogenous proteins (100μg of protein/0.2ml of incubation mixture), SDS/polyacrylamide-gel electrophoresis and subsequent autoradiography were as described previously (Wrenn *et al.*, 1980; Katoh *et al.*, 1981*a*,*b*).

Other methods. Immunization of Balb/c mice with homogeneous phospholipid-sensitive Ca^{2+}-dependent protein kinase, cell fusion of splenocytes with P3-NSI/1-Ag4-1 myeloma cells and cloning procedures were performed essentially the same as those described for other antigens (Gefter *et al.*, 1977; Goding, 1980). The antisera and monoclonal antibodies were assayed by the standard solid-phase radioimmunoassay as described by Randolph *et al.* (1977), with ^{125}I-labelled staphylococcal protein A. Cyclic AMP-dependent and cyclic GMP-dependent protein kinases were partially purified from foetal-bovine hearts (Shoji *et al.*, 1977).

Analytical (non-denaturing) and SDS/polyacrylamide-gel electrophoresis of the purified enzyme was carried out as described previously (Helfman & Kuo, 1981; Wise *et al.*, 1982*a*); protein was visually detected with Coomassie Brilliant Blue or by the silver staining method of Oakley *et al.* (1980). Isoelectric focusing was performed by using 2.5% ampholytes (Pharmalyte) with a pH range of 4.0–6.5 (Helfman & Kuo, 1981). Linear-sucrose-density-gradient ultracentrifugation was carried out as described previously (Wise *et al.*, 1982*a*). The sedimentation coefficient and molecular weight were determined by the method of Martin & Ames (1961). The determinations of Stokes radius, molecular weight and fractional ratio were carried out by the method of Siegel & Monty (1966), using Sephacryl S-200. The amino acid composition of the enzyme was determined as described by Benson & Patterson (1965). For this, duplicate enzyme samples (100μg each) were hydrolysed in constant-boiling 6M-HCl for 24 and 72h at 100°C; amino acids were analysed with a Beckman 119 Cl amino acid analyser. Protein was determined by the method of Bradford (1976) with the Bio-Rad protein determination kit and ovalbumin as a standard protein. [γ-^{32}P]ATP was prepared as described by Post & Sen (1967), and radioiodination of staphylococcal protein A was carried out by the method of Randolph *et al.* (1977).

Results

Purification of phospholipid-sensitive Ca^{2+} dependent protein kinase

We have reported previously that spleen and brain have the highest levels of phospholipid-sensitive

Ca^{2+}-dependent protein kinase of any rat tissues tested (Kuo *et al.*, 1980). We also noted that pig spleen had enzyme activity levels comparable with those seen in rat spleen; therefore, this tissue was chosen as the source to purify the enzyme. DEAE-cellulose chromatography of pig spleen extract yielded a single peak of Ca^{2+}-dependent enzyme activity and a large Ca^{2+}-independent protein kinase activity peak (Fig. 1). The concentrated enzyme from the DEAE-cellulose step was quantitatively bound to octyl-agarose in the presence of 1 M-$(NH_4)_2SO_4$. The enzyme was effectively purified (10-fold) and recovered (65%) by eluting it with 0.1 M-$(NH_4)_2SO_4$; about 85–95% of the applied protein remained bound to octyl-agarose. Sephacryl S-200 chromatography yielded a single peak of Ca^{2+}-dependent enzyme activity that was eluted shortly after the major protein peak and a small peak of Ca^{2+}-independent activity (Fig. 2).

The final purification of the enzyme to homogeneity was accomplished with phosphatidylserine–Affigel 10 affinity chromatography. In typical experiments, about 60–80 mg of the enzyme from the Sephacryl step was applied to 1.5 ml of the affinity gel in the presence of 4 mM-$CaCl_2$ (see the Experimental section for details); about 90% of the protein, or 3% of the activity, applied was not retained by the gel. Washing the gel with solutions containing either 1 M-NaCl or 1 mM-EGTA removed a total of about 5% of the applied protein, or 40% of the applied activity, resulting in enzyme fractions of about 25–40% homogeneous. Final elution with a solution containing 2 M-NaCl and 10 mM-EGTA yielded a homogeneous enzyme preparation, which represented about 2% of the activity applied (Table 1) and was used for all studies reported herein.

Purity of the enzyme

SDS/polyacrylamide-gel electrophoresis of the enzyme from the phosphatidylserine–Affigel 10 step from three separate preparations is shown (Figs. 3*c*–3*f*). The enzyme was essentially homogeneous, as indicated by the presence of a single staining band as visually detected by either Coomassie Brilliant Blue (Figs. 3*c* and 3*d*) or silver stain (Figs. 3*e* and 3*f*). It is noteworthy that a protein-staining band corresponding to calmodulin (mol.wt. 18000) was not present, indicating that calmodulin is not a

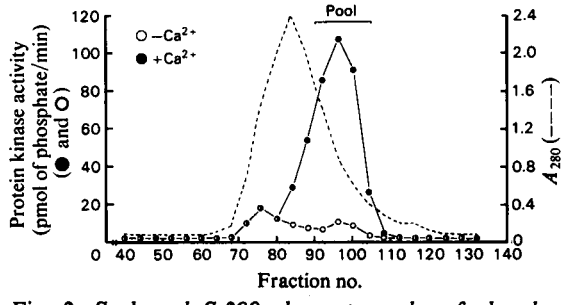

Fig. 2. *Sephacryl S-200 chromatography of phospholipid-sensitive Ca^{2+}-dependent protein kinase from the octyl-agarose step*

The flow rate was 0.1 ml/min and the fraction size was 1.3 ml. A portion of each fraction (0.02 ml) was assayed for protein kinase activity as described in the Experimental section and the legend to Fig. 1.

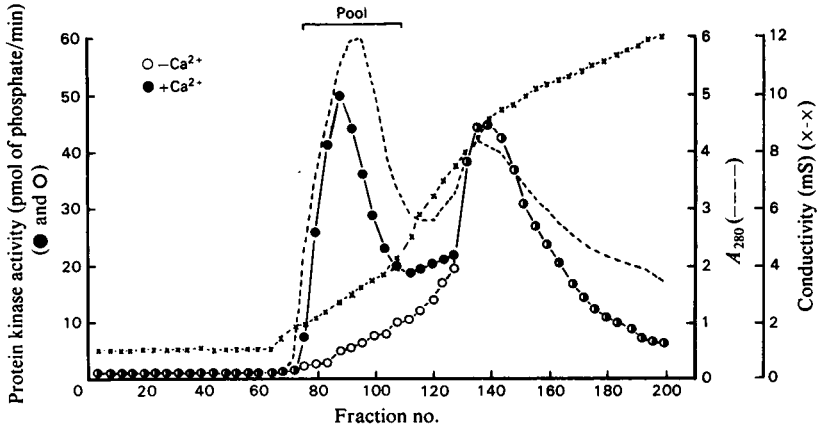

Fig. 1. *DEAE-cellulose chromatography of phospholipid-sensitive Ca^{2+}-dependent protein kinase from pig spleen extract*

The linear gradient of NaCl (dissolved in the extraction buffer) was 0–350 mM. The flow rate was 1.5 ml/min and the fraction size was 18 ml. A portion (0.02 ml) from each fraction was assayed for protein kinase activity in the presence of phosphatidylserine (25 μg/ml), with or without $CaCl_2$ (500 μM), as described in the Experimental section, except that 25 mM-Tris/HCl (pH 7.5) was used.

Table 1. *Summary of the purification of phospholipid-sensitive Ca^{2+}-dependent protein kinase from pig spleen*
The enzyme was assayed as described in the Experimental section. Frozen pig spleen (1.4kg) was used as the starting material. The enzyme activity shown is the net activity stimulated by Ca^{2+} in the presence of phosphatidylserine (25 μg/ml).

Step	Protein (mg)	Specific activity (pmol/min per mg)	Ca^{2+}-stimulation (fold)	Purification (fold)	Recovery (%)
Extract	64800	112	3	1	100
DEAE-cellulose	8100	451	9	4	50
Octyl-agarose	570	4180	14.0	38	33
Sephacryl S-200	150	8690	21.4	78	18
Phosphatidylserine–Affigel 10	0.54	55100	11.5	488	0.4

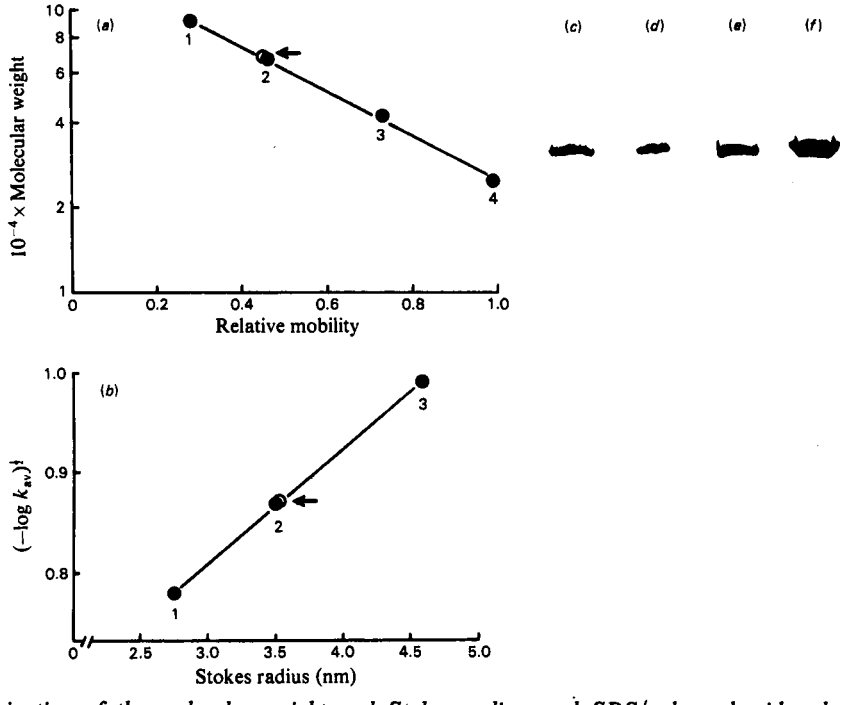

Fig. 3. *Determination of the molecular weight and Stokes radius, and SDS/polyacrylamide-gel electrophoresis of phospholipid-sensitive Ca^{2+}-dependent protein kinase*
SDS/polyacrylamide-gel electrophoresis (*a*, *c–f*) was carried out as described in the Experimental section. For the M_r determination (*a*) the enzyme (10 μg) from the affinity step was electrophoresed on a separating gel containing 7.5% acrylamide, and the marker proteins employed were: 1, phosphorylase *b* (mol.wt. 94000); 2, bovine serum albumin (68000); 3, ovalbumin (45000); 4, α-chymotrypsinogen (25000). For the determination of the Stokes radium (*b*), the enzyme from the octylagarose step (500 μg) was chromatographed on a Sephacryl S-200 column (1.8cm × 100cm) and eluted at a flow rate of 0.1ml/min; 1.3ml fractions were collected. A portion of each fraction (0.02ml) was assayed for protein kinase activity as described in the Experimental section. The following marker proteins were used to calibrate the column: 1, yeast alcohol dehydrogenase (4.5nm); 2, bovine serum albumin (3.55nm); 3, ovalbumin (2.7nm). Three enzyme preparations (*c–f*; 10–12 μg of protein) from the affinity step were electrophoresed in a separating gel containing 10% acrylamide; (*c*) and (*d*) were stained with Coomassie Brilliant Blue and (*e*) and (*f*) with silver. The same enzyme preparation was used in (*c*) and (*e*).

subunit of the enzyme. Analytical (non-denaturing) polyacrylamide-gel electrophoresis of the enzyme from the affinity step also yielded a single protein band corresponding to the enzyme activity (Fig. 4).

Physical properties

Based on its electrophoretic mobility on SDS/polyacrylamide-gel electrophoresis (Fig. 3*a*), the molecular weight of the enzyme was estimated to be

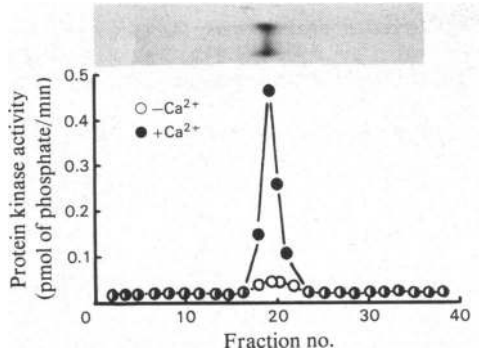

Fig. 4. *Analytical polyacrylamide-gel electrophoresis of phospholipid-sensitive Ca^{2+}-dependent protein kinase from the phosphatidylserine–Affigel 10 step*
The purified enzyme (10 μg) was electrophoresed and a portion (0.03 ml) of each fraction was assayed for protein kinase activity as described in the Experimental section and in the legend to Fig. 1.

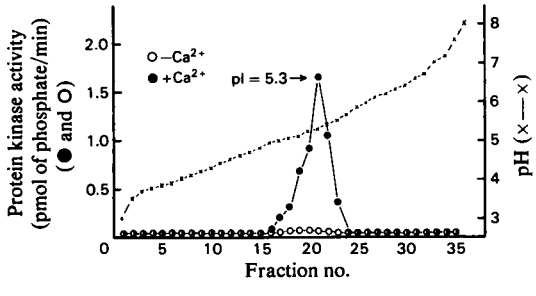

Fig. 5. *Isoelectric focusing of phospholipid-sensitive Ca^{2+}-dependent protein kinase.*
The enzyme (40 μg) from the phosphatidylserine–Affigel 10 step was electrofocused for 18 h as described in the Experimental section. Portions (0.04 ml) of each fraction were assayed for protein kinase activity.

68 500 ± 250 (five determinations; mean ± S.E.M.). Using Sephacryl S-200 chromatography the Stokes radius of 3.52 ± 0.085 nm (Fig. 3*b*) and a mol.wt. of 74 253 ± 4593 (five determinations) was obtained for the enzyme. The enzyme focused as a single symmetrical peak on isoelectric focusing with a pI of 5.3 (Fig. 5). The sedimentation coefficient was determined to be 5.5 S by linear-sucrose-density-gradient ultracentrifugation. The molecular weight of the enzyme based on its Stokes radius and sedimentation coefficient was calculated to be 80 000. The frictional ratio (f/f_0) was calculated to be 1.24, indicating that the enzyme was a non-globular protein. The properties of phospholipid-sensitive

Table 2. *Summary of the physical properties of phospholipid-sensitive Ca^{2+}-dependent protein kinase from pig spleen, and comparison with those of the enzyme from bovine heart*

Parameter	Spleen enzyme*	Heart enzyme†
Molecular weight	74 525 ± 4593‡	113 600 ± 7400‡
	68 500 ± 250§	83 500 ± 500§
	80 000‖	99 500‖
	76 000¶	
Sedimentation coefficient (S)	5.52	5.6
Stokes radius (nm)	3.52 ± 0.085	4.29 ± 0.16
Frictional ratio (f/f_0)	1.24	1.40
Isoelectric point	5.30	5.2; 5.5; 5.8

* The present study.
† Taken from Wise *et al.* (1982*a*).
‡ Determined by gel filtration.
§ Determined by SDS/polyacrylamide-gel electrophoresis.
‖ Calculated from sedimentation coefficient and Stokes radius.
¶ Calculated from amino acid analysis.

Ca^{2+}-dependent protein kinase purified from pig spleen (results from the present study) and those of the enzyme from bovine heart reported previously (Wise *et al.*, 1982*a*) are compared (Table 2).

Amino acid composition

The amino acid composition of the purified phospholipid-sensitive Ca^{2+}-dependent protein kinase is presented (Table 3). It is compared with the composition reported by others for cyclic AMP- and cyclic GMP-dependent protein kinases (Lincoln & Corbin, 1977) and myosin light-chain kinase (Adelstein & Klee, 1981).

Catalytic properties

The pH optimum of the enzyme activity was between 6.5 and 7.0. The stimulation by Ca^{2+} at pH 6.5 was higher than that seen at pH 7.5, although the maximal activity attainable was comparable under the two conditions (the enzyme was routinely assayed at pH 6.5 or 7.5).

The enzyme had an apparent K_m for ATP of 7.5 μM when assayed using histone H1 as substrate, with a $V_{max.}$ of 0.064 μmol/min per mg of enzyme. Myelin basic protein was the most effective phosphate acceptor tested, histone H1 being less effective and histones H2B and H4 much less effective. The K_m and $V_{max.}$ for myelin basic protein were 0.2 μM and 0.092 μmol/min per mg of enzyme respectively, compared with those values of K_m and $V_{max.}$ for histone H1 of 0.3 μM and 0.058 μmol/min per mg of protein respectively. We noted that phosphorylation

Table 3. *Comparison of the amino acid composition of phospholipid-sensitive Ca^{2+}-dependent protein kinase (PL-Ca-PK) with those shown by others for cyclic AMP- and cyclic GMP-dependent protein kinases (A-PK and G-PK) and for myosin light-chain kinase (MLCK)*

	Content (residues/mol)			
Amino acid	PL-Ca-PK (pig spleen)	A-PK* (bovine heart)	G-PK* (bovine lung)	MLCK† (turkey gizzard)
Aspartic acid	8.56	11.28	11.23	9.80
Threonine	5.35	4.27	5.07	6.12
Serine	5.95	5.66	6.85	7.71
Glutamic acid	10.30	13.39	12.88	13.86
Proline	6.06	4.85	4.66	5.18
Glycine	10.22	6.93	8.22	5.73
Alanine	7.75	6.81	6.99	7.60
Valine	8.14	6.00	5.07	7.38
Half-cystine	0.27	1.50	1.37	2.40
Methionine	1.52	2.08	2.47	1.94
Isoleucine	4.39	4.16	3.84	4.61
Leucine	10.49	8.55	8.08	5.97
Tyrosine	2.95	2.89	2.47	2.21
Phenylalanine	5.95	5.43	3.97	2.87
Lysine	4.63	7.85	8.08	10.28
Histidine	1.98	1.96	1.23	1.16
Arginine	5.65	5.20	6.30	3.76
Total	662	1687	1481	1120
Molecular weight	76000	186000	165000	125000

* Calculated from the data of Lincoln & Corbin (1977).
† Taken from Adelstein & Klee (1981).

of an endogenous protein (mol.wt. 41000) in spleen was stimulated by Ca^{2+} plus phosphatidylserine, whereas phosphorylation of another protein (mol.wt. 48000) was stimulated only by phosphatidylserine (Fig. 6). No substrates for protein kinases activated by Ca^{2+} plus calmodulin, cyclic AMP or cyclic GMP were detected under the experimental conditions employed.

Phosphatidylserine was the most effective phospholipid cofactor tested, having an apparent K_a of 6μg/ml (Fig. 7). Cardiolipin and phosphatidic acid, although having similar K_a values, could only support the enzyme activity up to about 30% of that seen with phosphatidylserine. Other phospholipids, such as phosphatidylethanolamine, phosphatidylcholine and phosphatidylinositol (from both soya bean and bovine brain), were essentially ineffective. Calmodulin, previously shown to be unable to serve as a cofactor for less pure enzyme preparations (Takai *et al.*, 1979; Kuo *et al.*, 1980; Wise *et al.*, 1982*a*), could not substitute for phosphatidylserine. Previous studies by Kaibuchi *et al.* (1981), using the partially purified brain enzyme,

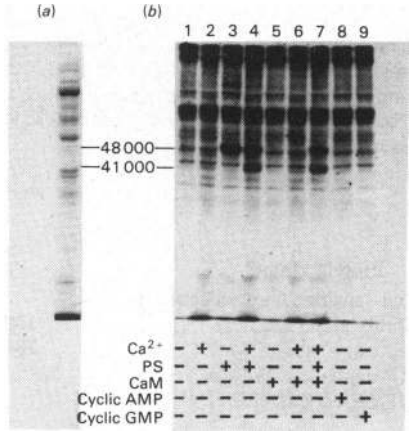

Fig. 6. *Effects of Ca^{2+}, phosphatidylserine, calmodulin and cyclic nucleotides on phosphorylation of endogenous proteins in the total solubilized fraction of pig spleen*
Additions (to 0.2ml of incubation mixture), as indicated, consisted of $CaCl_2$ (0.1μmol), phosphatidylserine (PS, 5μg), calmodulin (CaM, 1μg), cyclic AMP (0.2nmol) and cyclic GMP (0.2nmol); EGTA (0.04μmol) was present in all incubations. The separating gel was 10% acrylamide containing 0.1% SDS. The protein applied was 20μg/lane. Other experimental procedures were as described in the Experimental section and by Wrenn *et al.* (1980). (*a*) Proteins stained with Coomassie Brilliant Blue; (*b*) autoradiogram.

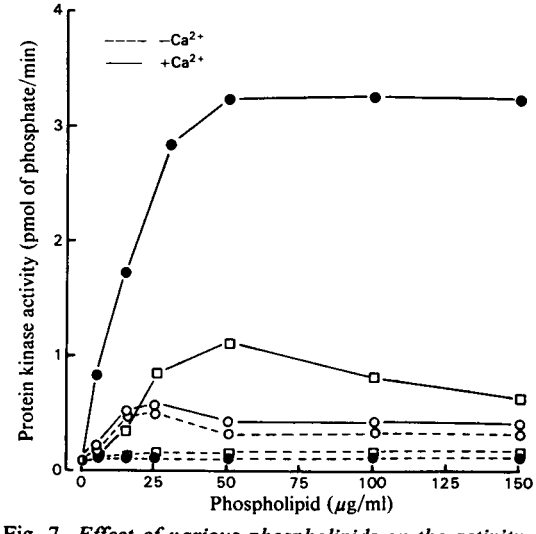

Fig. 7. *Effect of various phospholipids on the activity of phospholipid-sensitive Ca^{2+}-dependent protein kinase*
The enzyme (0.06μg) was assayed as described in the Experimental section except in the presence of various phospholipids at the indicated concentrations: ●, phosphatidylserine; ○, cardiolipin; □, phosphatidic acid.

Table 4. *Comparison of immunoreactivity of antisera and monoclonal antibodies raised against phospholipid-sensitive Ca^{2+}-dependent protein kinase with various protein kinases*

Binding of antibodies to various enzymes were carried out by the solid-phase radioimmunoassay (Randolph *et al.*, 1977), with 100 μl of antisera (after diluting 100-fold) or 100 μl of culture medium of hybridomas after the cells were removed. The values shown are means ± S.E.M. from three or four determinations and have been corrected for the background activity seen in the absence of antibodies, which was about 5–10% of the values seen in their presence.

	Binding of ^{125}I-protein A (c.p.m.)			
		Clone		
Protein kinase	Antisera	17.581.83	17.51.18	17.405.14
Phospholipid-sensitive Ca^{2+}-dependent				
From affinity step (2 μg)	1355 ± 102	1072 ± 35	1038 ± 32	471 ± 25
From Sephacryl step (10 μg)	1815 ± 66	1913 ± 97	1954 ± 75	1499 ± 44
Cyclic AMP-dependent (15 μg)		146 ± 7	184 ± 26	64 ± 8
Cyclic GMP-dependent (50 μg)		21 ± 10	14 ± 9	32 ± 5

indicate that the ability of phosphatidylserine to serve as cofactor is enhanced by phosphatidylethanolamine, diminished by phosphatidylcholine and sphingomyelin and unaffected by phosphatidylinositol. In the present studies, however, we noted that the homogeneous or partially purified spleen enzyme (for example, from the Sephacryl step) was inhibited to various degrees by these phospholipids. Diacylglycerols (such as 1,2- and 1,3-dioleoylglycerol) have been shown to increase the affinity of less pure brain (Kishimoto *et al.*, 1980; Kuo *et al.*, 1980) and nearly homogeneous heart enzyme (Wise *et al.*, 1982*a*) for phosphatidylserine and Ca^{2+}. We noted here that 1,3-dioleoylglycerol (5 and 15 μg/ml) had only a slight effect on the pure spleen enzyme in decreasing its K_a for phosphatidylserine from 6 to about 3 μg/ml, and its K_a for Ca^{2+} from 160 to about 140 μM.

When assayed in the presence of Ca^{2+} and phosphatidylserine, Mg^{2+}, Mn^{2+} and Co^{2+} were found to be effective at supporting enzyme activity, having optimal concentrations of 10, 1 and 0.5 mM respectively. Mn^{2+} and Co^{2+}, however, were only able to support the enzyme activity to the extent of 73 and 50% respectively of that seen with Mg^{2+}. In other studies, only Sr^{2+} and Ba^{2+}, at concentrations higher than 1 mM, were found to be able to partially substitute for Ca^{2+} in activating the enzyme.

Immunoreactivity of antisera and monoclonal antibodies

We have isolated 10 hybridomas that secrete antibodies directed against pure phospholipid-sensitive Ca^{2+}-dependent protein kinase. Initial accounts of the antibody studies are presented (Table 4). Both antisera from mice and monoclonal antibodies elaborated by three randomly selected hybridomas (17.581.83, 17.51.18 and 17.405.14) were found to interact with the homogeneous and impure enzyme from the affinity and Sephacryl steps respectively (Table 4). These antibodies, however, were much less reactive toward cyclic AMP-dependent and cyclic GMP-dependent protein kinases, indicating an immunological specificity for the Ca^{2+}-dependent enzyme.

Discussion

The present paper describes the purification to homogeneity of phospholipid-sensitive Ca^{2+}-dependent protein kinase from pig spleen extracts. The crucial final step was affinity chromatography on phosphatidylserine–Affigel 10, in which the phospholipid is coupled to the gel via its amine group of the serine moiety. Previously, we purified (80–95% homogeneous) the enzyme from bovine heart extracts by using phosphatidylserine–Affigel 102, in which the phospholipid is coupled to the gel via its carboxy group of the serine moiety (Wise *et al.*, 1982*a*). Although a total of about 50% of the Ca^{2+}-dependent enzyme activity applied to phosphatidylserine–Affigel 10 could be recovered from it with the use of various elution conditions, only the combination of EGTA and NaCl selectively eluted the activity to yield a homogeneous preparation. The yield of the pure enzyme was very low and amounted to about 2% of the applied enzyme, or 0.4% of the original activity present in extracts. Several reasons that could account for the low yield have been discussed (Wise *et al.*, 1982*a*). An improvement of the yield either by modifying the affinity step or incorporating other procedures is necessary for certain studies requiring a larger amount of the enzyme. A better understanding of the mechanism(s) by which phospholipid confers a Ca^{2+}-sensitivity to the enzyme, in particular the exact manner of interactions among the individual components in the enzyme–Ca^{2+}–phosphatidyl-

serine ternary complex, would definitely help develop more effective and specific elution conditions of the enzyme from the phospholipid affinity gel. In other studies in our laboratory, using $^{45}CaCl_2$ binding to the enzyme, we have found that the Ca^{2+}–phospholipid–enzyme ternary complex appears to be quite stable (R. C. Schatzman & J. F. Kuo, unpublished work). This would explain, in part, the difficulty in eluting the enzyme from the affinity column. An approach to the purification of the enzyme in high yield may be antibody affinity chromatography utilizing the monoclonal antibodies we have produced.

The molecular and catalytic properties of the heart enzyme have been reported previously (Wise *et al.*, 1982*a*,*b*). It appears that the enzymes from the two sources were slightly different in that the spleen enzyme (*a*) had a lower molecular weight, Stokes radius and frictional ratio, (*b*) had a higher K_a for Ca^{2+}, (*c*) was relatively unaffected by dioleoylglycerol in its affinity for Ca^{2+} and phosphatidylserine and (*d*) had a lower specific activity in terms of phosphorylating histone or myelin basic protein. In addition, the spleen enzyme, compared with the heart or brain enzyme, was relatively resistant to inhibition by chlorprothixene and haloperidol (Schatzman *et al.*, 1981) and did not exhibit a stimulatory phospholipid co-operativity as reported by Kaibuchi *et al.* (1981) for the brain enzyme. The spleen enzyme, also quantitatively bound to phosphatidylserine–Affigel 102, was not eluted from it with EGTA and NaCl, a condition used for eluting the heart (Wise *et al.*, 1982*a*) or brain enzyme (R. S. Turner & J. F. Kuo, unpublished work) from this gel, or used for eluting the spleen enzyme from phosphatidylserine–Affigel 10 (the present work). Aside from those mentioned above, the spleen enzyme was indistinguishable from the heart or brain enzyme, particularly with respect to the specificity for phospholipid cofactor, metal ion activator and substrate proteins.

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