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Purification of bovine brain inositol 1,4,5-trisphosphate 3-kinase

Identification of the enzyme by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis

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Inositol 1,4,5-trisphosphate ($Ins P_3$) 3-kinase catalyses the ATP-dependent phosphorylation of $Ins P_3$ to inositol 1,3,4,5-tetrakisphosphate ($Ins P_4$). A method is presented for the rapid purification of $Ins P_3$ 3-kinase from bovine brain by calmodulin (CaM)–Sepharose affinity chromatography. Maximal activation of the purified $Ins P_3$ 3-kinase by Ca^{2+}/CaM was 6–7-fold as compared with the activity measured in the presence of EGTA (1 mM) and 10 μM-$Ins P_3$. At 10 μM-$InsP_3$ and 0.1 mM free Ca^{2+}, half-maximal activation required about 2 nM-CaM. The mechanism of activation by CaM appeared to be an increase in the maximal velocity of the enzyme without a substantial change in the K_m for $Ins P_3$. Further purification was achieved by phosphocellulose chromatography eluted with ATP. Specific activity of the purified enzyme at 37 °C and 10 μM-$Ins P_3$ was 10–20 μmol/min per mg. The apparent M_r of the enzyme, determined by f.p.l.c.-gel filtration, was estimated as about 44000. The purified $Ins P_3$ 3-kinase was subjected to SDS/10%-polyacrylamide-gel electrophoresis. $Ins P_3$ 3-kinase activity was associated with three silver-stained bands, which migrated with apparent M_r values of approx. 52000, 38000 and 35000.

INTRODUCTION

Inositol 1,4,5-trisphosphate ($Ins P_3$) is a second messenger for mobilizing intracellular Ca^{2+} (Berridge & Irvine, 1984). It can be dephosphorylated by soluble and particulate forms of $Ins P_3$ 5-phosphatase, or phosphorylated to produce inositol 1,3,4,5-tetrakisphosphate ($Ins P_4$) by $Ins P_3$ 3-kinase (Irvine *et al.*, 1986; Hansen *et al.*, 1986; Hawkins *et al.*, 1986). These enzymes represent possible targets for the regulation of the $Ins P_3$/$Ins P_4$ signal. $Ins P_3$ 3-kinase has been purified from pig aortic smooth muscle (Yamaguchi *et al.*, 1988) and rat brain (Johanson *et al.*, 1988). However, the yields were very low (i.e. 0.29% for pig aortic smooth muscle and 3.7% for rat brain). The purified enzyme in smooth muscle is Ca^{2+}/CaM-sensitive (Yamaguchi *et al.*, 1988); in contrast, the rat brain $Ins P_3$ 3-kinase is only partially sensitive (Johanson *et al.*, 1988) or insensitive (Morris *et al.*, 1988) to CaM. This could be due to the presence of multiple isoenzymes in different cell types or to limited proteolysis, as for the cyclic nucleotide phosphodiesterase (Beavo, 1988). We report here a rapid method for the purification of the $Ins P_3$ 3-kinase from bovine brain, by using affinity chromatography on CaM–Sepharose. In these experiments, it was crucial to work in the presence of albumin or Triton X-100 to prevent enzyme adsorption on to glass or polystyrene tubes. In addition, purified $Ins P_3$ 3-kinase could be identified as three protein bands on SDS/polyacrylamide gels. This was established by the demonstration of enzyme activity in the gel region corresponding to that of the silver-stained proteins.

MATERIALS AND METHODS

Materials

[2-^{3}H]$Ins P_3$ (sp. radioactivity 3.3 Ci/mmol) was obtained from New England Nuclear Corp.; [5-^{32}P]$Ins P_3$ (sp. radioactivity 1000 Ci/mmol) was from Amersham. 2,3-Bisphosphoglycerate, phenylmethanesulphonyl fluoride, leupeptin and $Ins P_3$ were from Sigma; bovine serum albumin was from Serva. Triton X-100 was from Boehringer. Dowex 1-X8 (formate form, 200–400 mesh) was from Bio-Rad, and DEAE-Sephacel, Blue Sepharose and Superose 12 were from Pharmacia. Phosphocellulose was from Whatman. CaM was purified to homogeneity by the method of Yazawa *et al.* (1980). CaM–Sepharose 4B was prepared by the procedure of Klee & Krinks (1978) from 80 mg of CaM and 15 g of CNBr-activated Sepharose 4B (Pharmacia).

Assay of $Ins P_3$ 3-kinase activity

$Ins P_3$ 3-kinase activity was assayed as previously described (Takazawa *et al.*, 1988), with minor modifications. Incubation mixtures (0.1 ml) contained 84 mM-Hepes/NaOH (pH 7.5), 1 mg of bovine serum albumin/ml, 1 mM-ATP, 20 mM-$MgCl_2$, 2.5–12.5 mM-2,3-bisphosphoglycerate, 12 mM-2-mercaptoethanol, 1 mM-EGTA, 1 μM-CaM, diluted enzyme solution, [^{3}H]- or [^{32}P]-$Ins P_3$ (1000 c.p.m./assay), and unlabelled $Ins P_3$ to a final concn. of 0.25–10 μM. $CaCl_2$ was added to adjust the free Ca^{2+} concentration (calculated by using an apparent affinity constant of EGTA for Ca^{2+} of 3.17×10^6 M^{-1} at pH 7.5; Bers, 1982). The reaction was initiated by adding the enzyme, followed by incubation for 10 min at 37 °C. The reaction was terminated by adding 0.9 ml of ice-cold 0.4 M-ammonium formate/0.1 M-formic acid. Samples were immediately applied to 0.5 ml Dowex 1-X8 columns, which were subsequently washed with 2 ml of 0.4 M-ammonium formate/0.1 M-formic acid, followed by 20 ml of 0.7 M-ammonium formate/0.1 M-formic acid. $Ins P_4$ that was formed from $Ins P_3$ by $Ins P_3$ 3-kinase was eluted in 5 ml of 1.2 M-ammonium formate/0.1 M-formic acid. Under these conditions, the product $Ins P_4$ co-eluted with a standard of [^{3}H]$Ins P_4$ (provided by Amersham) on

Abbreviations used: $Ins P_3$, inositol 1,4,5-trisphosphate; $Ins P_4$, inositol 1,3,4,5-tetrakisphosphate; CaM, calmodulin.

anion-exchange h.p.l.c. (Erneux *et al.*, 1987). Enzyme activities were expressed as μmol of InsP_4 produced/min per mg of protein. Dilution of InsP_3 3-kinase to give 5–20% conversion of substrate into product was done in 20 mM-Tris/HCl buffer (pH 7.5) containing 1 mg of bovine serum albumin/ml. We checked that albumin was absolutely required in order to prevent adsorption of purified enzyme to polystyrene or glass tubes. Albumin (1–5 mg) could be replaced by 0.05–1% Triton X-100.

Purification of bovine brain soluble InsP_3 3-kinase

All operations were carried out at 4 °C. Fresh tissue (700 g) was homogenized in a Teflon/glass homogenizer in 1 litre of 20 mM-Tris/HCl (pH 8.3)/0.25 M-sucrose/24 mM-2-mercaptoethanol, containing protease inhibitors (0.4 mM-phenylmethanesulphonyl fluoride, 5 μM-leupeptin). The homogenate was centrifuged at 35000 *g* for 60 min. The supernatant was pooled and kept. The particulate fraction was resuspended in 1 litre of the same buffer, homogenized, and centrifuged for 60 min at 35000 *g*. The supernatant was removed and combined with the supernatant from the first centrifugation. The pH was adjusted to 8.3 by adding cold 1 M-Tris. The total solution was mixed with 450 ml of DEAE-Sephacel for 30 min at 4 °C. The resin was collected in a Buchner funnel, washed with 5 litres of 20 mM-Tris/HCl (pH 8.3)/0.1 mM-EGTA/24 mM-2-mercaptoethanol containing protease inhibitors as above (buffer A), packed in a column and eluted with 1.6 litres of a linear gradient of 0–0.5 M-NaCl in buffer A. The pooled InsP_3 3-kinase peak from the DEAE-Sephacel column was applied to a Blue Sepharose column (2.4 cm × 25 cm). The column was washed with 250 ml of 20 mM-Tris/HCl (pH 7.5)/0.1 mM-EGTA/2 mM-$MgCl_2$/10% glycerol/12 mM-2-mercaptoethanol containing protease inhibitors (buffer B) with 0.75 M-NaCl. The enzyme was eluted with 2.5 M-NaCl in buffer B. The sample was dialysed overnight against 2 × 1 litre of 10 mM-Tris/HCl (pH 7.5)/10 mM-KCl/10% glycerol/12 mM-2-mercaptoethanol containing protease inhibitors (buffer C) and applied to a phosphocellulose column (1.4 cm × 13 cm) which was equibrated in buffer C. The column was washed with buffer C containing 150 mM-potassium phosphate until no protein was eluted. The InsP_3 3-kinase activity was eluted with 500 mM-potassium phosphate in buffer C. The pooled peak fractions (about 13 ml) were adjusted to 0.5 mM-$CaCl_2$ and applied to a CaM–Sepharose column (2.5 cm × 6 cm). The column was washed with 70 ml of 20 mM-Tris/HCl (pH 7.5)/0.2 mM-$CaCl_2$/0.4 M-NaCl/12 mM-2-mercaptoethanol containing protease inhibitors, and subsequently eluted with 50 ml of 20 mM-Tris/HCl (pH 7.5)/2 mM-EGTA/12 mM-2-mercaptoethanol containing protease inhibitors (buffer D). InsP_3 3-kinase activity was eluted in buffer D containing 0.4 M-NaCl and 1% Triton X-100. The pooled enzyme (15 ml) was dialysed overnight against buffer C, stored at −80 °C or used in another purification. A further purification was achieved by the following procedure: after CaM–Sepharose affinity chromatography, InsP_3 3-kinase was applied to a phosphocellulose column (1 ml) equilibrated in buffer C. The column was washed with 150 mM-potassium phosphate and 1% Triton X-100 in buffer C, and InsP_3 3-kinase was eluted with a linear gradient (2 × 10 ml) from 0 to 10 mM-ATP–$MgCl_2$ in buffer C containing 150 mM-potassium phosphate and 0.1% Triton X-100 (36% recovery of activity at 10 μM-InsP_3). The flow rate was 20 ml/h. Protein concentration was determined by the procedure of Peterson (1977).

SDS/polyacrylamide-gel electrophoresis

All steps were performed at 4 °C. Enzyme preparation was made to 62 mM-Tris/HCl, pH 6.8, 3% SDS, 5% 2-mercaptoethanol and 10% glycerol, and immediately run (without boiling) on a SDS/10%-polyacrylamide mini slab gel (Laemmli, 1970). After electrophoresis at 200 V (60 min), the gel was cut into two parts: one part was silver-stained (Merrill & Goldman, 1984), and the other was cut into 1.5 mm sections. Each slice was homogenized in 0.5 ml of 84 mM-Hepes/NaOH, pH 7.5, containing 1 mg of bovine serum albumin/ml, 1 mM-EGTA, 12 mM-2-mercaptoethanol and 25% sucrose. After incubation for 12 h at 4 °C, each fraction was centrifuged (4000 *g*, 30 min) and the supernatant was assayed for activity in the presence of 1% Triton X-100 for 30 min incubation at 37 °C. Recovery of enzyme activities were about 25–60% of the material loaded on the gel in several separate experiments. We verified that Triton X-100 (1%) had no effect on basal or CaM-activated InsP_3 3-kinase activity.

RESULTS AND DISCUSSION

Purification by CaM–Sepharose

Crude enzyme preparations indicated that the bovine brain InsP_3 3-kinase could be activated by Ca^{2+}/CaM (Ryu *et al.*, 1987; Takazawa *et al.*, 1988). We decided to use this interaction to purify the kinase and then characterize its stimulation by Ca^{2+}/CaM. InsP_3 3-kinase could be purified from bovine brain by DEAE-Sephacel chromatography, followed by Blue Sepharose and phosphocellulose chromatography. The next stage of purification inolves adsorption to CaM–Sepharose in the presence of $CaCl_2$, followed by elution with EGTA. When eluted in the presence of EGTA alone, recoveries of InsP_3 3-kinase activity were particularly low, less than 8% of the total activity applied to the CaM–Sepharose column. The inclusion of bovine serum albumin (1 mg/ml) or Triton X-100 (1%) in the EGTA-containing buffer substantially increased enzyme recovery (up to 60% with albumin; results not shown). This is presumably due to a strong association of the enzyme to the glass column. We have observed the same 'loss of activity' by enzyme dilution in buffer in the absence of albumin or detergent. Optimal recovery of activity at the CaM–Sepharose step was achieved in the presence of EGTA (2 mM), NaCl (0.4 M) and Triton X-100 (1%) (Fig. 1). The affinity step typically gave a recovery of enzyme activity between 42 and 53%. In the experiment shown in Fig. 1, recovery of activity at 10 μM-InsP_3 was only 5% when eluted in the presence of EGTA alone. Most of the activity (53%) was eluted in buffer containing EGTA and detergent. When added to the Ca^{2+}-containing buffer, Triton X-100 (1%) was not sufficient to elute the enzyme. Moreover, the 1% Triton X-100 did not prevent Ca^{2+}/CaM stimulation of InsP_3 3-kinase activity (results not shown). These data indicate that the high-affinity interaction between immobilized CaM and InsP_3 3-kinase is Ca^{2+}-dependent, and that the interaction is not influenced by high concentration of Triton X-100. Similar results have been reported for the CaM-sensitive cyclic nucleotide phosphodiesterase (Kincaid & Vaughan, 1988). A typical purification, starting with two

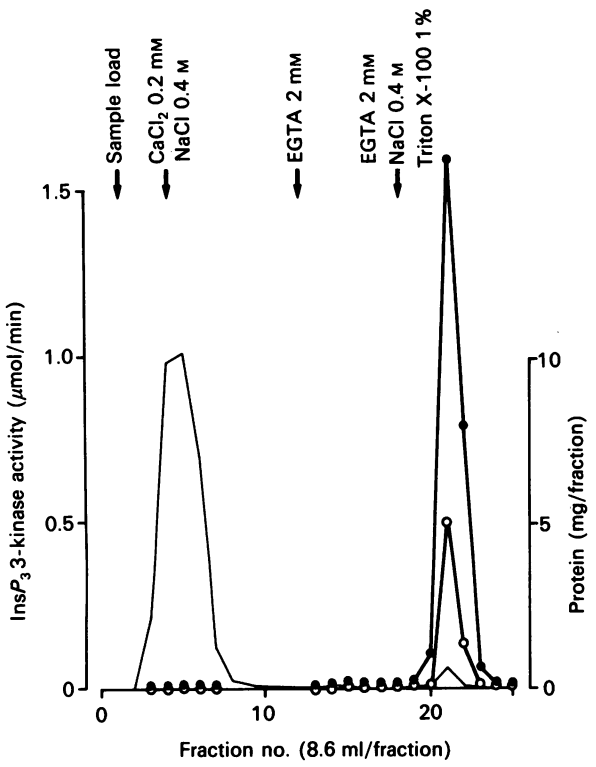

Fig. 1. Separation of $InsP_3$ 3-kinase by CaM–Sepharose affinity chromatography

$InsP_3$ 3-kinase (12 mg in 25 ml) partially purified by DEAE-Sephacel, Blue Sepharose and phosphocellulose (I) chromatography was applied to CaM–Sepharose as described in the Materials and methods section. The column was eluted as indicated. $InsP_3$ 3-kinase was assayed at 10 μM-$InsP_3$ in the presence of both 1 μM-CaM and 100 μM free Ca^{2+} (●) or 1 mM-EGTA (○; free Ca^{2+} below 1 nM). Protein content is indicated as a continuous line. This profile is representative of experiments with three different preparations.

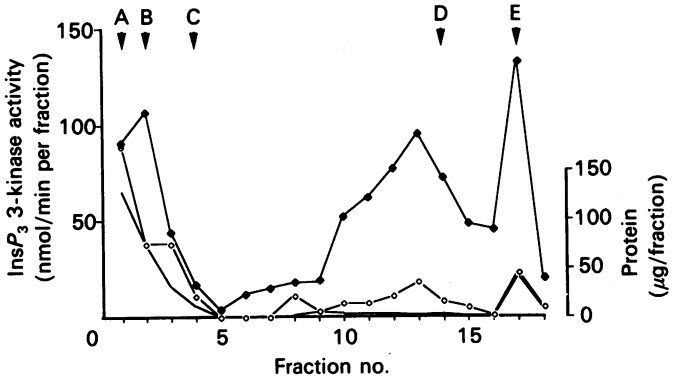

Fig. 2. Phosphocellulose (II) chromatrography of $InsP_3$ 3-kinase

The pooled enzyme after CaM–Sepharose (0.22 mg in 13 ml) was loaded on a 1 ml phosphocellulose column (A). The column was washed with 150 mM-potassium phosphate and 1% Triton X-100 (B). The enzyme was eluted with a linear gradient (2 × 10 ml) of 0–10 mM-ATP–$MgCl_2$ in the presence of 150 mM-potassium phosphate (from C to D). The column was washed with 10 mM-ATP–$MgCl_2$ (D), and then with 500 mM-potassium phosphate (E). The volume of fraction 1 was 13 ml, those of fractions 2 and 3 were 6 ml, and those of later fractions were 2 ml. $InsP_3$ 3-kinase activity was assayed under the same conditions as in Fig. 1.

bovine brains, required about 3 days and gave a final yield of 15% with a 7000-fold enrichment (Table 1). After CaM–Sepharose, specific activity at 10 μM-$InsP_3$ was 4.0–6.0 μmol/min per mg of protein. Further purification could be achieved by phosphocellulose chromatography with elution by ATP. In the experiment shown in Fig. 2, specific activity of pooled fractions (fractions 10–16) eluted in the 0–10 mM-ATP gradient was 16 μmol/min per mg (Table 1). This represented an approx. 28000-fold increase in specific activity as compared with the crude soluble fraction. These values were greater than previously reported in pig aortic smooth muscle and brain tissue (Ryu *et al.*, 1987; Johanson *et al.*, 1988; Yamaguchi *et al.*, 1988).

Enzymic and physical characterization of the CaM–Sepharose-purified enzyme

The apparent M_r of the $InsP_3$ 3-kinase was estimated by f.p.l.c.-gel fitration on Superose 12. In the presence of Triton X-100, recovery of enzyme activity was over 70% and the apparent M_r of the kinase was 44000 (Fig. 3). When gel filtration was performed in the absence of detergent, recoveries of activity were lower and the apparent M_r was 35000 (results not shown). These M_r values are lower than previously reported for the purified

Table 1. Purification of bovine brain $InsP_3$ 3-kinase

$InsP_3$ 3-kinase activity was assayed at 10 μM-$InsP_3$ in the presence of 0.1 mM free Ca^{2+} and 1 μM-CaM or 1 mM-EGTA for basal activity. Total and specific activities are given in the presence of Ca^{2+}/CaM. Results are from one representative preparation out of four.

Step	Protein mg	Total activity (μmol/min)	Specific activity (μmol/min per mg)	Purification (fold)	Yield (%)	CaM stimulation (fold)
Crude soluble	12 700	7.40	0.000583	1	100	3.5
DEAE-Sephacel	3290	6.37	0.00193	3.3	86	4.5
Blue Sepharose	40	3.45	0.0861	148	47	4.8
Phosphocellulose I	7.9	2.69	0.343	588	36	5.8
CaM–Sepharose	0.26	1.11	4.27	7320	15	6.8
Phosphocellulose II	0.020	0.325	16.2	27 800	4.4	7.3

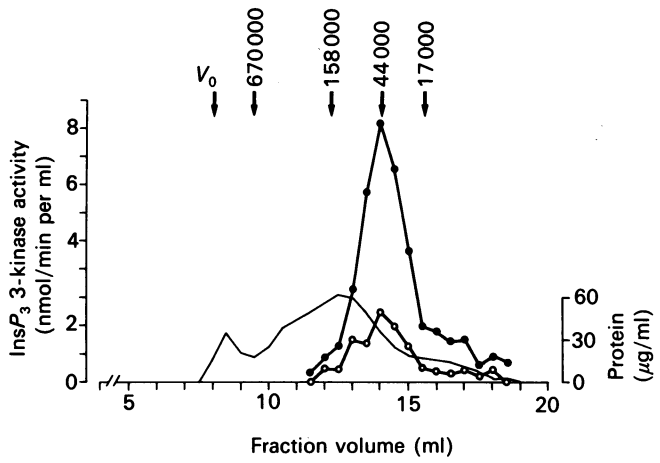

Fig. 3. Separation of $InsP_3$ 3-kinase by f.p.l.c.-gel filtration on Superose 12

$InsP_3$ 3-kinase (0.3 mg in 0.2 ml) partially purified as described in the legend of Fig. 1 was applied to an f.p.l.c. Superose 12 column equilibrated at room temperature in 20 mM-Tris/HCl (pH 7.5)/0.2 mM-$CaCl_2$/10% glycerol/0.5% Triton X-100/0.2 M-NaCl/12 mM-2-mercaptoethanol containing protease inhibitors. The flow rate was 0.5 ml/min, and 0.5 ml fractions were collected. The column was calibrated with M_r standards, including thyroglobulin (670000), γ-globulin (158000), ovalbumin (44000) and myoglobin (17000). $InsP_3$ 3-kinase was assayed as described in the legend of Fig. 1. The data are representative of experiments with three different preparations.

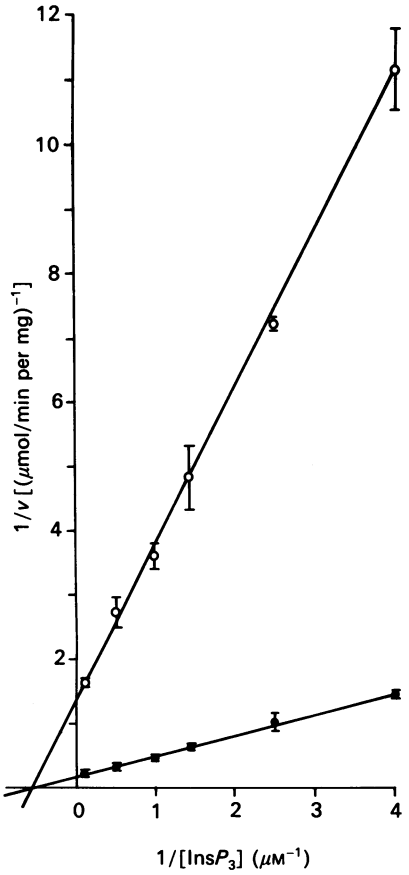

Fig. 5. Substrate/velocity relationships of $InsP_3$ 3-kinase activity

The data are shown as a double-reciprocal plot against $InsP_3$ concentration in the 0.25–10 μM range. Samples of enzyme were assayed in the presence of both 1 μM-CaM and 100 μM free Ca^{2+} (●) or 1 mM-EGTA (○). The extrapolated K_m value for $InsP_3$ was 1.8 μM, whereas $V_{max.}$ was 5.41 and 0.71 μmol/min per mg of protein in the presence and absence of Ca^{2+}/CaM respectively.

activities in rat brain (Johanson *et al.*, 1988; Morris *et al.*, 1988). However, in the study by Johanson *et al.* (1988), the $InsP_3$ 3-kinase was stimulated approx. 2-fold by CaM at saturating concentration of $InsP_3$, and the enzyme characterized by Morris *et al.* (1988) was not CaM-sensitive. Our own data indicate that: (1) Ca^{2+}-

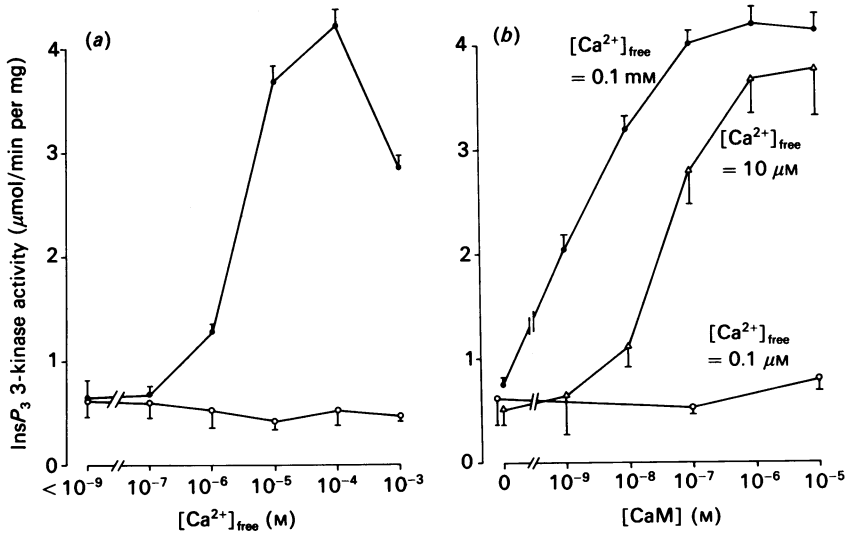

Fig. 4. Ca^{2+} (*a*) and CaM (*b*) concentration/response curves for $InsP_3$ 3-kinase activity

$InsP_3$ 3-kinase activity purified after CaM–Sepharose was assayed (*a*) in the absence (○) or presence (●) of 1 μM-CaM and (*b*) at 0.1 mM, 10 μM and 0.1 μM free Ca^{2+}. $InsP_3$ concentration was 10 μM. Results are means of triplicates ± S.D.

sensitivity could be observed in both rat and bovine brain homogenate (Takazawa *et al.*, 1988); (2) the effect was more pronounced with bovine brain; (3) the effect was maintained after a 28000-fold increase in specific activity (Table 1, Figs. 1 and 2). Disagreement in the data about the size and CaM-sensitivity are not yet understood, but could result from assay conditions, the purification methods or the existence of multiple isoenzymes, as has been demonstrated for the CaM-sensitive cyclic nucleotide phosphodiesterase (Beavo, 1988; Rossi *et al.*, 1988).

Half-maximal stimulation of InsP_3 3-kinase was determined at 3 μM free Ca^{2+} in the presence of 1 μM-CaM. In the absence of CaM, the enzyme was not stimulated (Fig. 4*a*). In confirmation of the report of Ryu *et al.* (1987), the CaM concentration/response curve was shifted to lower concentrations of CaM as Ca^{2+} was increased: as shown in Fig. 4(*b*), half-maximal stimulation was at 2 nM-CaM at 0.1 mM-Ca^{2+} and at 40 nM-CaM at 10 μM-Ca^{2+}.

The purified InsP_3 3-kinase followed Michaelis–Menten kinetics and was stimulated by Ca^{2+}/CaM. K_m values for InsP_3 were 1–2 μM. The mechanism of activation by CaM appears to be an increase in enzyme maximal velocity without a substantial change in K_m for InsP_3. Fig. 5 illustrates a 6-fold increase in $V_{max.}$ of purified InsP_3 3-kinase and no change in K_m for InsP_3. The same type of mechanism has been described in rat heart for the InsP_3 3-kinase (Renard & Poggioli, 1987) and for the bovine brain CaM-sensitive cyclic nucleotide phosphodiesterase with cyclic AMP as substrate (Kincaid & Vaughan, 1988). Thus these allosteric changes of enzyme kinetics appeared to be essentially the same for the two CaM-regulated enzymes. Distinct kinetics were reported by Johanson *et al.* (1988) in rat brain. As previously, disagreement in the data could result from the existence of isoenzymes or from the methods used to purify the enzyme.

Identification of InsP_3 3-kinase on SDS/polyacrylamide gels

Preliminary experiments indicated that the anionic detergent SDS strongly inhibited InsP_3 3-kinase activity. At 0.1 % SDS, the enzyme was totally inactive. However, this effect could be partially reversed provided that the assay was performed in the presence of the non-ionic detergent Triton X-100 at 1 % (results not shown). It was verified and thus concluded that the activity could be assayed after the addition of 'sample buffer'. The reason for this is not totally understood, but it could result from the formation of mixed anionic/non-ionic detergent micelles (Robinson *et al.*, 1980). A similar method was used in the characterization of phosphatidylinositol kinase from *Saccharomyces cerevisiae* (Belunis *et al.*, 1988). InsP_3 3-kinase was purified by CaM–Sepharose followed by phosphocellulose chromatography, dissolved in 'sample buffer' and subjected to electrophoresis (Fig. 6). The gel was cut into 1.5 mm segments and assayed for activity after incubation for 12 h at 4 °C. InsP_3 3-kinase was detected as three peaks, which migrated with apparent M_r values of approx. 52000, 38000 and 35000. The relatedness of the three proteins is clearly indicated by the ability of Ca^{2+}/CaM to stimulate the enzyme (Fig. 6*a*). The degree of activation by Ca^{2+}/CaM, which was 7-fold before electrophoresis, was substantially increased after SDS/polyacrylamide-gel electrophoresis:

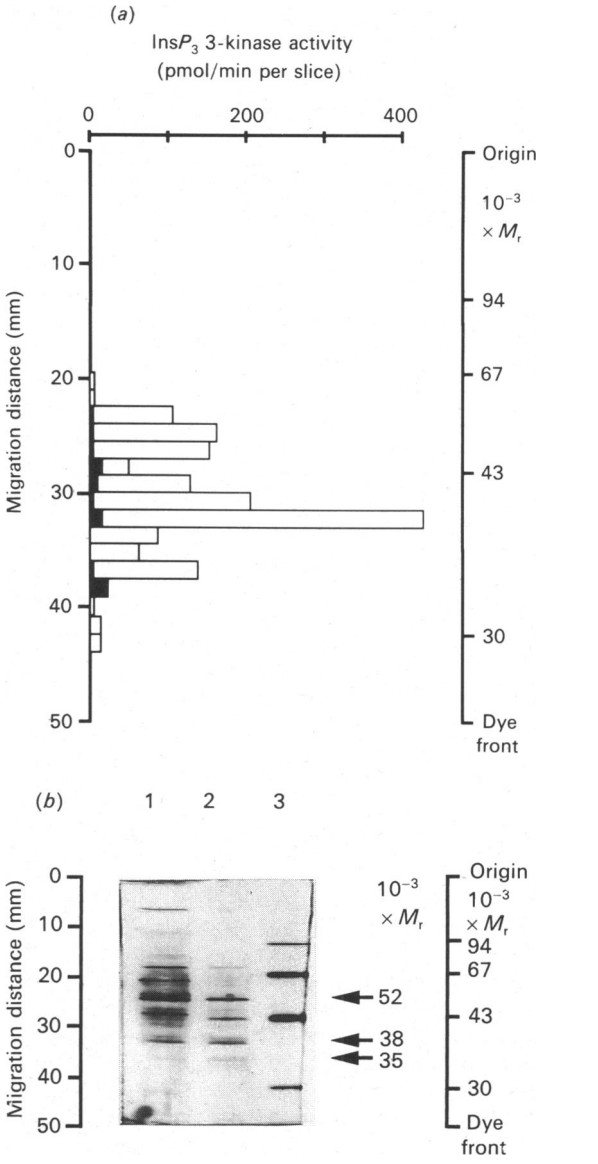

Fig. 6. SDS/polyacrylamide-gel electrophoresis of purified InsP_3 3-kinase

(*a*) Localization of InsP_3 3-kinase activity after electrophoresis. One lane was cut into 1.5 mm slices, homogenized and assayed as described in the Materials and methods section. InsP_3 concentration was 10 μM. Basal (■) and CaM-stimulated (□) activity was determined in the same conditions as in Fig. 1. (*b*) Silver-stained gel from the same electrophoresis. Lane 1 contains 0.7 μg of the InsP_3 3-kinase after CaM–Sepharose chromatography (Fig. 1). Lane 2 contains 0.4 μg of the purified enzyme after phosphocellulose II chromatography (Table 1), and lane 3 contains protein standards, consisting of 0.09 μg of phosphorylase *b* (M_r 94000), 0.12 μg of bovine serum albumin (M_r 67000), 0.21 μg of ovalbumin (M_r 43000) and 0.12 μg of carbonic anhydrase (M_r 30000).

activity was stimulated about 31-, 28-, and 26-fold for the M_r-52000, -38000 and -35000 peaks respectively, at 10 μM-InsP_3 (Table 2). We have verified that this increase follows the same kinetics as shown in Fig. 5 (i.e. by an increase in $V_{max.}$ of the enzyme; results not shown). Silver staining after electrophoresis in a parallel lane indicated the presence of three bands in this region (Fig. 6*b*). Other

Table 2. InsP_3 3-kinase activity after SDS/polyacrylamide-gel electrophoresis

InsP_3 3-kinase extracted from the three bands (i.e. M_r 35000, 38000 and 52000) was assayed at 10 μM-InsP_3 in the presence of EGTA (basal activity) or of 0.1 mM-free Ca^{2+} and 1 μM-CaM (stimulated activity). Enzyme activity was expressed as pmol/min. Values are means of triplicates ± S.D.

InsP_3 3-kinase activity (pmol/min)	Protein M_r: 35 000	38 000	52 000
Basal	5±2	15±5	5±3
Stimulated	135±5	425±28	143±6
Activity ratio	26	28	31

bands migrated with apparent M_r values of 70000 and 42000 with little enzyme activity.

We drew the following conclusions from the gel-electrophoresis experiments. (*a*) Purified InsP_3 3-kinase could be identified on SDS gels; this was established by the demonstration of enzyme activity in the gel region corresponding to that of the stained proteins. (*b*) Three peaks of activity have been separated: activity of each was much increased in the presence of Ca^{2+}/CaM. Isoenzymes of the CaM-sensitive cyclic nucleotide phosphodiesterase have been reported (Hansen & Beavo, 1986; Beavo, 1988). Our data could indicate that isoenzymes of the InsP_3 3-kinase have been separated on SDS gels, although proteolysis of a single protein has not been ruled out. (*c*) Taken together with the f.p.l.c.-gel-filtration experiment (Fig. 3), our data suggest that bovine brain InsP_3 3-kinase is a monomer.

In conclusion, an important property of CaM interaction that is often observed with target enzymes is the Ca^{2+}-dependence of the interaction (Krinks *et al.*, 1984). We have performed a series of experiments with the bovine brain InsP_3 3-kinase which support this conclusion: over 95% of the kinase activity was adsorbed by CaM–Sepharose and could be released in a buffer containing 2 mM-EGTA provided that the elution was carried out in the presence of Triton X-100. In our hands, detergent was required to prevent poor enzyme recoveries and much lower (i.e. 20-fold) specific activities of the kinase after CaM–Sepharose chromatography. Moreover, the bovine brain InsP_3 3-kinase is clearly a Ca^{2+}/CaM-sensitive enzyme, as shown in all purification steps and after SDS/polyacrylamide-gel electrophoresis.

InsP_3 3-kinase is a target enzyme of cellular regulation: not only is it responsible for InsP_4 production (a potential second messenger in cellular Ca^{2+} homoeostasis; see Irvine *et al.*, 1988), but it controls the rate of InsP_3 metabolism. The mechanism of stimulation of InsP_3 3-kinase by Ca^{2+} could account for the rapid formation of InsP_4 in stimulated cells (Irvine *et al.*, 1986; Zilberman *et al.*, 1987; Hughes *et al.*, 1988; Shears, 1989).

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