Biochem. J. (1990) 268, 213–217 (Printed in Great Britain)

Rat brain inositol 1,4,5-trisphosphate 3-kinase

Ca^{2+}-sensitivity, purification and antibody production

Kazunaga TAKAZAWA, Manuela LEMOS, Anne DELVAUX, Claudine LEJEUNE, Jacques E. DUMONT and Christophe ERNEUX

Institute of Interdisciplinary Research (IRIBHN), School of Medicine, Free University of Brussels (U.L.B.), Campus Erasme (Bât.C), Route de Lennik 808, B-1070 Brussels, Belgium

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 ($Ins P_4$). $Ins P_3$ 3-kinase was purified from rat brain by Blue-Sepharose, phosphocellulose and calmodulin (CaM)–Sepharose affinity chromatography. The purified enzyme was stimulated by Ca^{2+}/CaM by 3–6-fold as compared with the activity measured in the presence of EGTA. Rat brain $Ins P_3$ 3-kinase activity was associated with two silver-stained bands of about equal activity which migrated with an apparent M_r of 50000 on SDS/polyacrylamide gels. $Ins P_3$ 3-kinase activity from rat brain could be immunoprecipitated by an antiserum against the SDS/PAGE-purified 50000-M_r protein doublet. $Ins P_3$ kinase activity from bovine brain and the $Ins P_3$ 5-phosphatase activity from rat brain were not immunoprecipitated. On Western blot, the human brain crude $Ins P_3$ 3-kinase reacted specifically, but less strongly than the rat brain enzyme, with the antiserum.

INTRODUCTION

An increase in cellular inositol 1,4,5-trisphosphate ($Ins P_3$) concentration has been shown to mobilize intracellular Ca^{2+} (Berridge & Irvine, 1989). $Ins P_3$ 3-kinase catalyses the synthesis of a second potential regulator of cellular Ca^{2+} homeostasis, namely inositol 1,3,4,5-tetrakisphosphate ($Ins P_4$; see Irvine *et al.*, 1988; Petersen, 1989). The $Ins P_3$ 3-kinase of most cells is Ca^{2+}/calmodulin (CaM)-sensitive (Biden *et al.*, 1987; Morris *et al.*, 1987; Ryu *et al.*, 1987; Yamaguchi *et al.*, 1988; Johanson *et al.*, 1988; Takazawa *et al.*, 1988; Daniel *et al.*, 1988; Li *et al.*, 1989). Moreover, crude hepatic $Ins P_3$ 3-kinase activity can be stimulated after preincubation of intact cells with a cyclic AMP analogue and vasopressin (Biden *et al.*, 1988), suggesting that the enzyme is highly regulated both in acellular preparations and in intact cells. The $Ins P_3$ 3-kinase from bovine brain could be renatured after SDS/PAGE and was associated with three protein bands with apparent M_r values of 52000, 38000 and 35000 (Takazawa *et al.*, 1989). As the specific activity of $Ins P_3$ 3-kinase in the crude soluble fraction from rat brain was about ten times higher than in bovine brain, we purified the rat brain enzyme in order to prepare antibodies. Rat brain $Ins P_3$ 3-kinase was associated with two silver-stained bands of about equal activity which migrated with an apparent M_r of 50000 on SDS/polyacrylamide gels.

MATERIALS AND METHODS

Materials

Materials for assay and purification of the $Ins P_3$ 3-kinase from bovine and rat brain were as previously reported (Takazawa *et al.*, 1989). Protein A–Sepharose CL-4B was from Pharmacia. ^{125}I-labelled Protein A (50 mCi/mg), Rainbow marker and Hyperfilm-MP were from Amersham. Anti-rabbit IgG was from Sigma. Goat anti-(rabbit IgG)–alkaline phosphatase conjugate, 5-bromo-4-chloro-3-indolyl phosphate and Nitro Blue Tetrazolium were from Promega. Nitrocellulose membrane filters were from Schleicher & Schuell. Human brain frontal cortex was a neurosurgical sample kindly provided by Dr. J. J. Vaderhaeghen, Laboratory of Anatomo Pathology, Free University of Brussels.

Assay of $Ins P_3$ 3-kinase and $Ins P_3$ 5-phosphatase activities

Incubation mixtures (0.1 ml) for assay of $Ins P_3$ 3-kinase from rat brain contained 84 mM-Hepes/NaOH (pH 7.5), 1 mg of bovine serum albumin/ml 1 mM-ATP, 20 mM-$MgCl_2$, 0–2.5 mM-2,3-bisphosphoglycerate (depending on the presence or absence of $Ins P_3$ 5-phosphatase activity at various purification steps), 12 mM-2-mercaptoethanol, 1 mM-EGTA, 0.1 μM-CaM, diluted enzyme solution, [^{32}P]$Ins P_3$ (1000 c.p.m./assay) and unlabelled $Ins P_3$ to a final concentration of 10 μM. $CaCl_2$ was added to adjust the free Ca^{2+} concentration to 10 μM. The reaction was initiated by adding the enzyme, followed by incubation for 10 min at 37 °C. The reaction was terminated by adding 1 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 and eluted as previously described (Takazawa *et al.*, 1988).

For the $Ins P_3$ 5-phosphatase assay, the incubation mixture contained 84 mM-Hepes/NaOH (pH 7.5), 1 mg of bovine serum albumin/mg, 20 mM-$MgCl_2$, 12 mM-2-mercaptoethanol and 10 μM-[^{32}P]$Ins P_3$. After incubation at 37 °C for 10 min, the reaction was terminated by addition of 1 ml of ice-cold 0.2 M-ammonium formate/0.1 M-formic acid, and the mixture was applied to a 0.2 ml Dowex column. [^{32}P]P_i, the product of Ins(1,4,5)[5-^{32}P]P_3 5-phosphatase, was eluted with 5 ml of 0.2 M-ammonium formate/0.1 M-formic acid (Erneux *et al.*, 1987).

Purification of soluble $Ins P_3$ 3-kinase from rat brain

All operations were carried out at 4 °C. Fresh brains from 40 Sprague–Dawley male rats (200–250 g body weight) were homogenized using a Teflon/glass homogenizer in 3 vol. of 20 mM-Tris/HCl (pH 7.5)/1 mM-EDTA/0.25 M-sucrose/24 mM-2-mercaptoethanol containing protease inhibitors (0.4 mM-phenylmethanesulphonyl fluoride and 5 μM-leupeptin). The homogenate was centrifuged at 35000 *g* for 60 min, and the supernatants were pooled and stored. The particulate fraction was resuspended in 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 crude soluble fraction was applied to a Blue-Sepharose column (5 cm × 10 cm). The column was washed with 400 ml of 20 mM-Tris/HCl

Abbreviation used: CaM, calmodulin.

(pH 7.5)/1 mM-EDTA/0.02% Triton X-100/24 mM-2-mercaptoethanol/protease inhibitors (buffer A) made 0.8 M-NaCl. The enzyme was eluted with 2.5 M-NaCl and 1% Triton X-100 in buffer A. The sample was dialysed overnight against 1.5 litres of 10 mM-Tris/HCl (pH 7.5)/10 mM-KCl/0.01% Triton X-100/12 mM-2-mercaptoethanol/protease inhibitors (buffer B), with two changes of buffer, and applied to a phosphocellulose column (1.4 cm × 13 cm) which was equilibrated in buffer B. The column was washed with buffer B 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 B. The pooled peak fractions (about 30 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 50 ml of 20 mM-Tris/HCl (pH 7.5)/0.2 mM-$CaCl_2$/0.4 M-NaCl/0.5% Triton X-100/12 mM-2-mercaptoethanol/protease inhibitors (buffer C), then with 75 ml of buffer C without Triton X-100, and subsequently eluted with 100 ml of 20 mM-Tris/HCl (pH 7.5)/2 mM-EGTA/12 mM-2-mercaptoethanol/protease inhibitors (buffer D). InsP_3 3-kinase activity was eluted in buffer D containing 0.2% SDS. Pooled peak fractions were concentrated to approx. 1 ml using a PM10 ultrafiltration membrane. Protein concentration was determined by the procedure of Peterson (1977).

SDS/polyacrylamide-gel electrophoresis

Crude or purified InsP_3 3-kinase was added to 62 mM-Tris/HCl (pH 6.8)/3% SDS/5% 2-mercaptoethanol/10% glycerol, and immediately run (without boiling) on an SDS/polyacrylamide slab gel (8–10% polyacrylamide) (Laemmli, 1970). We used gels of 5 or 13 cm length. After electrophoresis at 250 V, the gel was cut into two parts: one part was silver-stained (Merrill & Goldman, 1984), and the other was stained with 0.3 M-zinc acetate (Dzandu *et al.*, 1988). The sensitivity of this method is almost the same as for silver staining, and the procedure is fully reversible. Stained protein bands were excised from the wet gel with a razor blade, and washed three times in 250 mM-EDTA/250 mM-Tris (pH 9.0) for 5 min each to chelate Zn^{2+} and three times in 20 mM-Tris/HCl (pH 7.5). Each slice was homogenized in 0.5 ml of 84 mM-Hepes/NaOH (pH 7.5)/0.1% Triton X-100/12 mM-2-mercaptoethanol/25% sucrose. After 12 h of incubation at 4 °C, fractions were centrifuged (4000 *g*, 30 min) and the supernatants were assayed for activity in the presence of 1% Triton X-100.

Preparation of antigen and antibody production

Purified InsP_3 3-kinase (approx. 200 μg of protein) was applied to preparative SDS/polyacrylamide gels. Proteins were revealed by soaking the gel in 0.3 M-zinc acetate. The polypeptide of 50000-M_r was excised, washed as above and homogenized in 1 ml of phosphate-buffered saline (1.37 mM-NaCl/2.7 mM-KCl/1.5 mM-KH_2PO_4/8.1 mM-Na_2HPO_4, pH 7.5). Half of the homogenate was mixed 1:1 with Freund's complete adjuvant and injected into the popliteal lymph nodes of an adult female New Zealand White rabbit (Sigel *et al.*, 1983). After 4 weeks, the other half of the homogenate was mixed with Freund's incomplete adjuvant and injected into multiple intradermal sites on the back. Blood was withdrawn 10 days after the booster injection.

Immunoprecipitation

The Protein A–Sepharose used for immunoadsorption studies was prepared by adding Protein A–Sepharose (0.1 g) to 1 ml of 10 mM-Tris/HCl (pH 8.0)/140 mM-NaCl/0.025% NaN_3/0.1% Triton X-100/0.1% BSA (buffer E). Protein A–Sepharose was pretreated with anti-(rabbit IgG) antibodies by the addition of 140 μg of anti-rabbit IgG. This was kept overnight at 4 °C and washed with 6 × 1 ml of buffer E, centrifuged and resuspended in 1 ml of buffer E.

For immunoprecipitation experiments (Springer, 1987), InsP_3 3-kinase preparations were incubated for 1.5 h at 4 °C in the presence of various quantities of serum in a final volume of 0.1 ml. A 0.05 ml portion of Protein A–Sepharose prepared as described above was added to each tube and incubation was continued for 1.5 h at 4 °C. After adding 0.05 ml of buffer E, the tubes were centrifuged and the supernatants were assayed for InsP_3 3-kinase and 5-phosphatase activities.

Blotting and immunodetection of proteins

After separation by gel electrophoresis, proteins were transferred to a nitrocellulose membrane for 16 h at 60 V and 4 °C (Towbin *et al.*, 1979). Rabbit serum against rat brain InsP_3 3-kinase or preimmune serum were used at a 1:250–1:2000 dilution. ^{125}I-labelled Protein A was used as a secondary reagent for development by autoradiography (Burnette, 1981). Alternatively, immune complexes on blots were detected using alkaline-phosphatase-conjugated anti-rabbit IgG and corresponding colorimetric methods (Kincaid, 1988).

RESULTS AND DISCUSSION

Purification of the rat brain 50000-M_r InsP_3 3-kinase

We have previously shown that bovine brain InsP_3 3-kinase can be easily renatured after SDS/PAGE, thus permitting the identification of the enzyme/protein band on SDS gels (Takazawa *et al.*, 1989). This technique was used to tentatively identify the InsP_3 3-kinase in crude preparations of rat brain. In the typical experiment shown in Fig. 1, crude InsP_3 3-kinase, which migrated with an apparent M_r value of 50000, was Ca^{2+}/CaM-sensitive. Electrophoresis after each purification step of the InsP_3

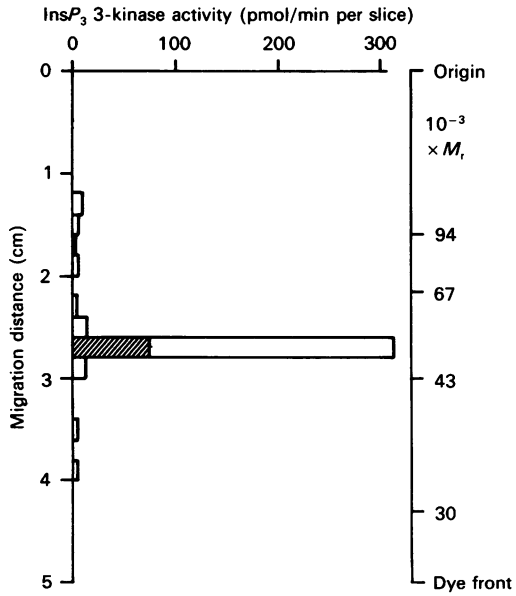

Fig. 1. SDS/PAGE of crude InsP_3 3-kinase from rat brain

A 200 μg portion of crude soluble fraction from rat brain was applied to an SDS/10%-polyacrylamide 5 cm slab gel. After electrophoresis, one lane was cut into 2 mm slices, homogenized and assayed as described in the Materials and methods section. The apparent M_r value was determined by using M_r standards in a parallel lane. InsP_3 3-kinase activity was assayed at 10 μM-InsP_3 in the presence of 0.1 μM-CaM and 10 μM free Ca^{2+} (□) or 1 mM-EGTA (▨; free Ca^{2+} below 1 nM). Total recovery of activity was 27% when assayed in the presence of Ca^{2+}/CaM. Values are means of duplicates.

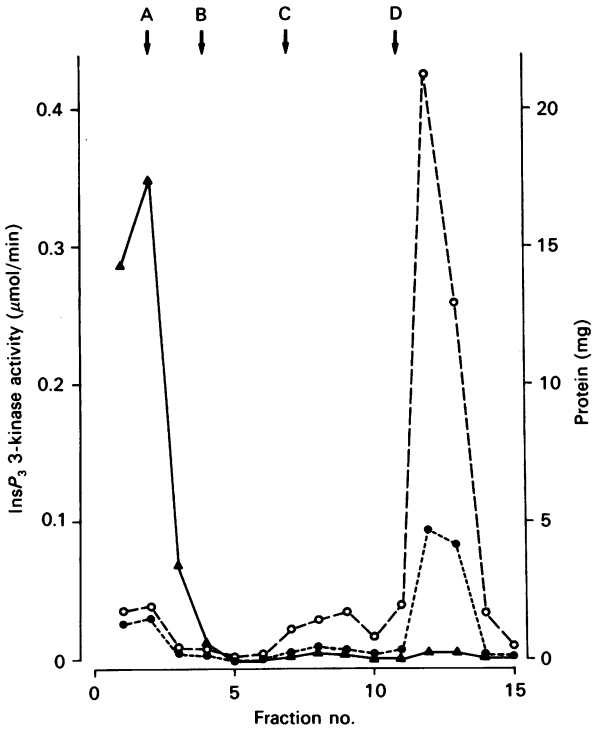

Fig. 2. Separation of InsP_3 3-kinase by CaM–Sepharose affinity chromatography

InsP_3 3-kinase (21 mg in 30 ml), partially purified by Blue–Sepharose and phosphocellulose chromatography, was applied to CaM–Sepharose as described in the Materials and methods section. The column was eluted as indicated by the arrows: A, 0.2 mM-$CaCl_2$/0.4 M-NaCl/0.5% Triton X-100; B, 0.2 mM-$CaCl_2$/0.4 M-NaCl; C, 2 mM-EGTA/0.1 M-NaCl; D, 2 mM-EGTA/0.2% SDS. The volume of fractions 1–10 was 25 ml, and that of later fractions was 16 ml. Basal (●) and Ca^{2+}/CaM-stimulated (○) activities were determined as in Fig. 1. Protein content is indicated as a continuous line (▲). Total recovery of enzyme activity was 28% when assayed in the presence of Ca^{2+}/CaM. This profile is one representative experiment out of five.

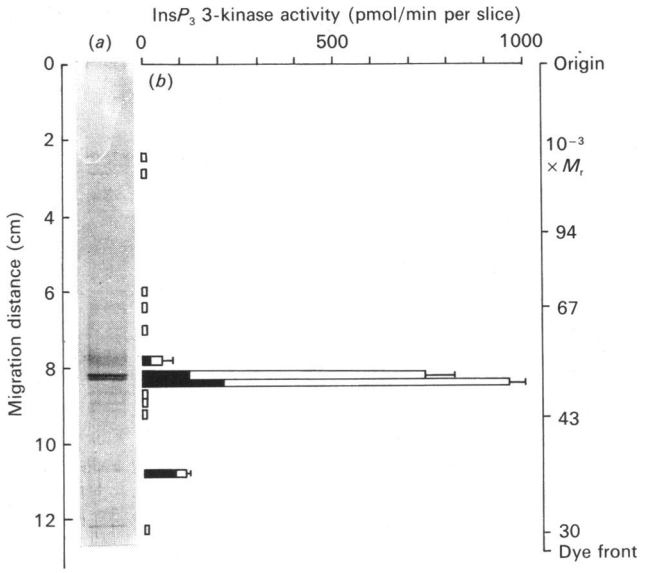

Fig. 3. Localization of purified InsP_3 3-kinase activity after SDS/PAGE

Purified InsP_3 3-kinase (2.8 μg of protein and 6.6 nmol/min enzyme activity) after CaM–Sepharose chromatography was transferred to an SDS/8% polyacrylamide 12 cm slab gel. After electrophoresis, the gel was cut into two parts; one was silver-stained (*a*), and the other was soaked in zinc acetate (*b*) and activity was assayed in each protein band. Basal (■) and Ca^{2+}/CaM-stimulated (□) activity were determined as in Fig. 1. Values are means of triplicates ± S.D.

3-kinase gave a peak of enzyme activity with the same M_r (results not shown). The rat brain InsP_3 3-kinase was purified by Blue-Sepharose, phosphocellulose and CaM–Sepharose affinity chromatography, with slight modifications compared with our previous report (Takazawa *et al.*, 1989). CaM–Sepharose separation was optimized by the use of a buffer containing 0.2 mM-$CaCl_2$ and 0.5% Triton X-100 to wash the column and to minimize non-specific adsorption; InsP_3 3-kinase was eluted afterwards in a buffer containing 2 mM-EGTA and 0.2% SDS (Fig. 2). At the final step, 0.2 mg of protein was obtained from 40 brains (Table 1). We calculated the final purification to be approx. 400-fold, with a recovery of 6%. As SDS (0.2%) inhibited InsP_3 3-kinase by 50% (results not shown), we underestimated the degree of enzyme purification and final specific activity. When the purified material was analysed on SDS/polyacrylamide gels, InsP_3 3-kinase was associated with two major silver-stained bands which migrated with an apparent M_r value of 50000. The two activities were about equal in three different preparations and were stimulated to the same extent (3–5-fold) by Ca^{2+}/CaM (Fig. 3). The low activity at about M_r 35000 could be seen in several preparations. It was not Ca^{2+}/CaM-sensitive (Fig. 3), in contrast with the 35000-M_r InsP_3 3-kinase from bovine brain (Takazawa *et al.*, 1989). As with the bovine brain InsP_3 3-kinase, Ca^{2+}/CaM acted on the $V_{max.}$ of the enzyme (Fig. 4). The K_m of the enzyme for InsP_3 estimated in the absence of 2,3-bisphosphoglycerate was 11 μM. Distinct kinetics and differences in Ca^{2+}/CaM sensitivity were reported by Johanson *et al.* (1988) in rat brain. Disagreement in the data probably resulted from the purification methods, particularly the use of appropriate detergents: the elution from CaM–Sepharose was

Table 1. Purification of rat brain InsP_3 3-kinase

InsP_3 3-kinase activity was assayed at 10 μM-InsP_3 in the presence of 10 μM free Ca^{2+} and 0.1 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 preparation representative of four.

Step	Protein (mg)	Total activity (μmol/min)	Specific activity (μmol/min per mg)	Purification (-fold)	Yield (%)	Stimulation by CaM (-fold)
Crude soluble	1664	15.2	0.00913	1	100	1.7
Blue-Sepharose	278	6.16	0.0221	2.4	40.5	1.8
Phosphocellulose	21.1	4.17	0.198	21.7	27.5	1.8
CaM–Sepharose	0.237	0.872	3.68	403	5.7	5.0

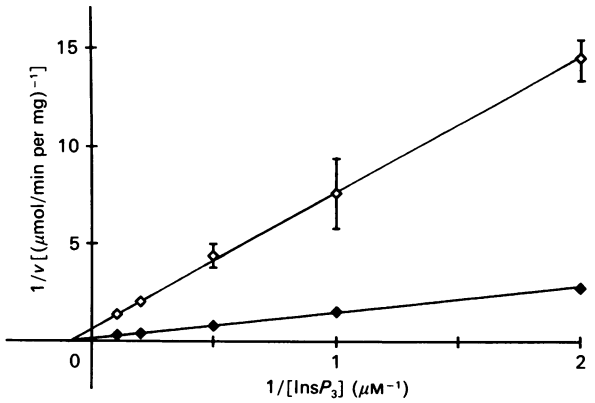

Fig. 4. Substrate–velocity relationships of InsP_3 3-kinase activity

The data are shown as a double-reciprocal plot with the InsP_3 concentration in the 0.5–10 μM range. Samples of enzyme were assayed in the presence of 0.1 μM-CaM plus 10 μM free Ca^{2+} (◆) or 1 mM-EGTA (◇). As the purified enzyme was not contaminated with InsP_3 5-phosphatase activity, the assay was done in the absence of 2,3-bisphosphoglycerate. The extrapolated K_m value for InsP_3 was 11 μM, whereas the $V_{max.}$ was 9.52 and 1.54 μmol/min per mg of protein in the presence and absence respectively of Ca^{2+}/CaM. Results are means of triplicates ± S.D. Where the S.D. values are not indicated, they are smaller than the symbols.

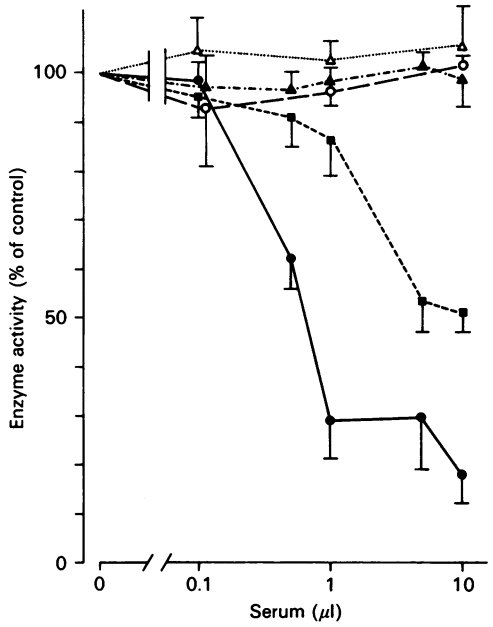

Fig. 5. Immunoprecipitation of InsP_3 3-kinase and 5-phosphatase activities

Crude soluble fractions from rat brain (●), bovine brain (▲) and human brain (■) were immunoprecipitated with immune serum as described in the Materials and methods section. After the addition of Protein A–Sepharose and centrifugation, InsP_3 3-kinase (●, ▲, ■) or 5-phosphatase (△) was assayed in the supernatant. Immunoprecipitation of InsP_3 3-kinase from rat brain crude soluble fraction with preimmune serum (○) was also determined. The total InsP_3 3-kinase activities were 0.48 nmol/min in rat brain, 0.50 nmol/min in bovine brain and 1.51 nmol/min in human brain. Total InsP_3 5-phosphatase activity was 0.47 nmol/min in rat brain. Results are means of triplicates ± S.D.

performed in a buffer containing 0.2% SDS (Fig. 2) in order (1) to increase the recovery from the affinity column (Takazawa *et al.*, 1989) and (2) to stabilize the 50000-M_r protein doublet. We

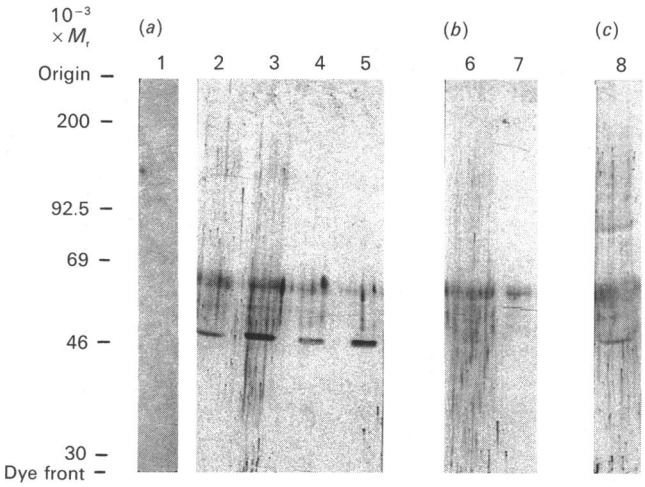

Fig. 6. Western blot of InsP_3 3-kinase activities from rat brain, bovine brain and human brain

InsP_3 3-kinase preparations from rat brain (*a*), bovine brain (*b*) and human brain (*c*) were subjected to SDS/PAGE (8% gels) and electrophoretically transferred to nitrocellulose. The blots were incubated with preimmune serum diluted 1:250 (lane 1) or with immune serum diluted 1:250 (lanes 2–8). Lanes 1 and 2 are crude soluble fraction from rat brain (enzyme activity 0.37 nmol/min), lane 3 is partially purified enzyme from the Blue Sepharose step (0.45 nmol/min), lane 4 from the phosphocellulose step (0.87 nmol/min) and lane 5 from the CaM–Sepharose step (0.16 nmol/min). Lanes 6 and 7 are crude soluble fraction (0.16 nmol/min) and purified InsP_3 3-kinase (3.2 nmol/min) respectively from bovine brain. Lane 8 is crude soluble fraction from human brain (0.33 nmol/min).

thus kept the purified enzyme in a buffer containing 0.2% SDS in frozen portions. When purified in the presence of Triton X-100 instead of SDS, the rat brain InsP_3 3-kinase yielded four active fragments of lower M_r on SDS/polyacrylamide gels (results not shown).

Precipitation of InsP_3 3-kinase activity by anti-(rat brain 50000-M_r protein) antiserum

Rat brain InsP_3 3-kinase was purified as a doublet of apparent M_r 50000 (Fig. 3). In order to obtain antibodies to InsP_3 3-kinase, the purified InsP_3 3-kinase was isolated from preparative SDS/polyacrylamide gels and injected into a rabbit. Serum was tested for its ability to immunoprecipitate InsP_3 3-kinase activity. The antiserum (10 μl) immunoprecipitated 80% of the InsP_3 3-kinase activity present in the crude soluble fraction of rat brain (Fig. 5). InsP_3 5-phosphatase could not be immunoprecipitated. Fig. 6 shows an immunoblot of the rat brain InsP_3 3-kinase at different purification steps in which equal amounts of InsP_3 3-kinase activity were subjected to electrophoresis, and ^{125}I-Protein A was used as secondary reagent. The serum reacted with the 50000-M_r doublet after all purification steps. No signal was obtained with the preimmune serum (Fig. 6, lane 1). Two bands of M_r 50000 were also detected with alkaline-phosphatase-conjugated second antibodies and a 2000-fold dilution of serum. This method lowers the artefactual staining of higher-M_r (52000–60000) bands (results not shown). The antibodies directed against the SDS/PAGE-purified 50000-M_r protein were thus specific for InsP_3 3-kinase.

Immunological cross-reactions between rat brain InsP_3 3-kinase and the bovine and human brain enzymes

The immunological similarities between brain InsP_3 3-kinases were examined using the antiserum. As seen in the Western immunoblot in Fig. 6, the antiserum reacted with the crude

soluble fraction of the human brain enzyme and recognized a 50000-M_r protein band, although interaction was much less than with the rat brain enzyme. Reaction with bovine brain crude soluble fraction was at the detection limit of this assay. There was no reaction with purified InsP_3 3-kinase from bovine brain. Fig. 5 shows that 50% of the human brain InsP_3 3-kinase activity, but none of the bovine brain activity, could be precipitated by the antiserum.

General conclusions

The data presented here and previously (Takazawa *et al.*, 1989) show that InsP_3 3-kinases from both rat and bovine brain are Ca^{2+}/CaM-sensitive enzymes. Identification of the enzyme(s) by SDS/PAGE was confirmed by regeneration of activity. The InsP_3 3-kinase from bovine brain was purified as three proteins of M_r 52000, 38000 and 35000 on SDS/polyacrylamide gels; in contrast, the rat brain enzyme was purified as a doublet of M_r 50000. The bovine brain enzyme is much more sensitive to Ca^{2+}/CaM than the rat brain enzyme. Finally, an antibody to SDS-purified 50000-M_r rat brain protein does not recognize the bovine brain InsP_3 3-kinase. Taken together, our data suggest that different isoenzymes are expressed in bovine brain, rat brain and probably other tissues. Final proof of this hypothesis will be achieved by primary sequence determination of each isoenzyme. Antibodies prepared against the InsP_3 3-kinase should facilitate isolation of a cDNA clone for the rat brain enzyme.

We thank Dr. J. Van der Heyden and Dr. F. Lejeune for help in the preparation of the antiserum, and H. Passareiro for the purification of CaM. We are also grateful to B. Robaye and A. Hepburn for their helpful criticism throughout this work. This project was funded by a grant from the Ministère de la Politique Scientifique (Belgium).

REFERENCES

Berridge, M. J. & Irvine, R. F. (1989) Nature (London) **341**, 197–205

Biden, T. J., Comte, M., Cox, J. A. & Wollheim, C. B. (1987) J. Biol. Chem. **262**, 9437–9440

Biden, T. J., Altin, J. G., Karjalainen, A. & Bygrave, F. L. (1988) Biochem. J. **256**, 697–701

Burnette, N. W. (1981) Anal. Biochem. **112**, 195–203

Daniel, J. L., Dangelmaier, C. A. & Smith, J. B. (1988) Biochem. J. **253**, 789–794

Dzandu, J. K., Johnson, J. F. & Wise, G. E. (1988) Anal. Biochem. **174**, 157–167

Erneux, C., Delvaux, A., Moreau, C. & Dumont, J. E. (1987) Biochem. J. **247**, 635–639

Irvine, R. F., Moor, R. M., Pollock, W. K., Smith, P. M. & Wreggett, K. A. (1988) Phil. Trans. R. Soc. London B **320**, 281–298

Johanson, R. A., Hansen, C. A. & Williamson, J. R. (1988) J. Biol. Chem. **263**, 7465–7471

Kincaid, R. L. (1988) Methods Enzymol. **159**, 627–652

Laemmli, U. K. (1970) Nature (London) **277**, 680–685

Li, G., Comte, M., Wollheim, C. B. & Cox, J. A. (1989) Biochem. J. **260**, 771–775

Merrill, C. R. & Goldman, D. (1984) in Two-Dimensional Gel Electrophoresis of Protein: Methods and Applications (Celis, J. E. & Bravo, R., eds.), pp. 93–109, Academic Press, Orlando, FL

Morris, A. J., Downes, C. P., Harden, T. K. & Michell, R. H. (1987) Biochem. J. **248**, 489–493

Petersen, O. H. (1989) Cell Calcium **10**, 375–383

Peterson, G. L. (1977) Anal. Biochem. **83**, 346–356

Ryu, S. H., Lee, S. Y., Lee, K.-Y. & Rhee, S. G. (1987) FASEB J. **1**, 388–393

Sigel, M. B., Sinha, Y. N. & VanderLaan, W. P. (1983) Methods Enzymol. **93**, 3–12

Springer, T. A. (1987) in Current Protocols in Molecular Biology (Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K., eds.), pp. 10.16.1–10.16.10, John Wiley and Sons, New York

Takazawa, K., Passareiro, H., Dumont, J. E. & Erneux, C. (1988) Biochem. Biophys. Res. Commun. **153**, 632–641

Takazawa, K., Passareiro, H., Dumont, J. E. & Erneux, C. (1989) Biochem. J. **261**, 483–488

Towbin, H., Staehelin, T. & Gordon, J. (1979) Proc. Natl. Acad. Sci. U.S.A. **76**, 4350–4354

Yamaguchi, K., Hirata, M. & Kuriyama, H. (1988) Biochem. J. **251**, 129–134

Received 23 November 1989/1 February 1990; accepted 8 February 1990