Purification and biochemical properties of a high-molecular-mass inositol 1,4,5-trisphosphate 3-kinase isoenzyme in human platelets

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The phosphorylation of inositol 1,4,5-trisphosphate (InsP_3) to inositol 1,3,4,5-tetrakisphosphate (InsP_4) is catalysed by InsP_3 3-kinase. A method is presented for a rapid purification of the enzyme from human platelets. The purified enzyme was identified as a polypeptide of M_r 69000–70000 after SDS/PAGE. It had a specific activity of $1.45 \pm 0.1 \mu\text{mol/min per mg}$, and the degree of stimulation by Ca^{2+} /calmodulin was 17-fold at saturating cal-

modulin and $10 \mu\text{M}$ free Ca^{2+} . The K_m for InsP_3 and for ATP was $2.0 \mu\text{M}$ and 2.5 mM respectively. Human platelet InsP_3 3-kinase was not recognized by immunodetection with anti-(InsP_3 3-kinase A) or anti-(InsP_3 3-kinase B) antibodies. These data provide the first biochemical evidence for the existence of a novel InsP_3 3-kinase isoenzyme in human platelets, which is distinct from previously reported InsP_3 3-kinase A and InsP_3 3-kinase B.

INTRODUCTION

Inositol 1,4,5-trisphosphate (InsP_3) 3-kinase catalyses the phosphorylation of InsP_3 , which has been shown to mobilize intracellular Ca^{2+} , to inositol 1,3,4,5-tetrakisphosphate (InsP_4), a potential modulator of Ca^{2+} homeostasis (Berridge and Irvine, 1989; Irvine, 1992).

A cDNA clone encoding rat brain Ca^{2+} /calmodulin (CaM)-sensitive InsP_3 3-kinase has been isolated (Choi et al., 1990; Takazawa et al., 1990b) and expressed in *Escherichia coli*. The encoded protein of 459 amino acids has a calculated M_r of 50868, equalling estimates made with the native rat brain enzyme (Takazawa et al., 1990a,b). This enzyme is now referred to as InsP_3 3-kinase A. Using the rat cDNA as a probe, Takazawa and colleagues isolated and expressed human InsP_3 3-kinase A from a human hippocampus cDNA library (which presented 93 % sequence identity with the rat brain InsP_3 3-kinase A amino acid sequence; Takazawa et al., 1991a) and a novel InsP_3 3-kinase isoenzyme, referred to as InsP_3 3-kinase B (Takazawa et al., 1991b). The expressed InsP_3 3-kinase B was more sensitive to Ca^{2+} /CaM (i.e. stimulation factor of 7–10-fold) compared with expressed InsP_3 3-kinase A (2–3-fold stimulated).

Previous reports have suggested that InsP_3 3-kinase might be a substrate for protein kinase C in human platelets (King and Rittenhouse, 1989), rat hepatocytes (Biden et al., 1988) and human lymphocytes (Imboden and Pattison, 1987). However, there was little characterization of the enzyme from these cells: it is not known whether it corresponds to either InsP_3 3-kinase A or B, or a distinct isoenzyme. In this study, we have isolated a novel InsP_3 3-kinase isoenzyme in human platelets. This enzyme has been identified both by silver staining and by regeneration of enzyme activity as a M_r 69000–70000 protein.

MATERIALS AND METHODS

Materials

[2- ^3H] InsP_3 (sp. radioactivity 3.3 Ci/mmol) was obtained from Du Pont–New England Nuclear Corp. InsP_3 , calpain inhibitors I and II, CaM, Triton X-100 and pepstatin were from Boehringer. CaM–Sepharose 4B and Protein A–Sepharose were from

Pharmacia LKB Biotechnology. Benzamidine, leupeptin and anti-(rabbit IgG) were from Sigma. Pefabloc was from Pentapharm A.G. (Basle, Switzerland). Goat anti-(rabbit IgG)–alkaline phosphatase conjugate was from Promega, and nitrocellulose membrane filters were from Schleicher and Schuell. All other chemicals were of the highest available grade.

Analytical procedures

InsP_3 3-kinase assay was performed at 37°C for 8 min in a $50 \mu\text{l}$ reaction mixture containing 84 mM Hepes/NaOH (pH 7.4), 5 mM ATP, 1 mg/ml BSA, 1 mM EGTA, 20 mM MgCl_2 , 7.5 mM 2,3-bisphosphoglycerate, 12 mM 2-mercaptoethanol, 50 $\mu\text{g/ml}$ Pefabloc, 5 μM leupeptin, 50 mM benzamidine, 10 $\mu\text{g/ml}$ calpain inhibitors I and II, 1500 c.p.m. of [^3H] InsP_3 , 5 μM InsP_3 and 5–15 μl of enzyme. In assays performed in the presence of Ca^{2+} /CaM, 0.1 μM CaM was added as well as CaCl_2 to adjust the free Ca^{2+} concentration to $10 \mu\text{M}$. The InsP_3 5-phosphatase assay at 30 μM InsP_3 was performed as described previously (Takazawa et al., 1990a) in an incubation medium supplemented with protease inhibitors (50 $\mu\text{g/ml}$ Pefabloc, 5 μM leupeptin and 50 mM benzamidine). Apparent K_m values were measured by using a non-linear least-squares curve-fitting program (Marquardt–Levenberg algorithm). Immune complexes were detected on Western blots, as described previously (Takazawa et al., 1990a). Protein concentrations were measured as described by Petersen (1977).

Expression of InsP_3 3-kinase B in *E. coli*

The expression of plasmid h3 MB1 encoding InsP_3 3-kinase B was performed as previously described (Takazawa et al., 1991b), after addition of 5 mM isopropyl β -thiogalactopyranoside for 3 h.

Regeneration of InsP_3 3-kinase activity after SDS/PAGE

The enzyme preparation was made in 62 mM Tris/HCl (pH 6.8), 3 % SDS, 5 % 2-mercaptoethanol and 10 % glycerol (loading buffer), and immediately run on SDS/PAGE (8 % gel) at 240 V

for 4 h at 4 °C (Takazawa et al., 1990a). After electrophoresis, the gel was cut into 2.5–5 mm sections at 4 °C. Each slice was homogenized in 0.25 ml of an ice-cold extraction buffer containing 84 mM Hepes/NaOH (pH 7.4), 1 mM EDTA, 12 mM 2-mercaptoethanol, 25% (w/v) sucrose, 0.1% Triton X-100, 50 µg/ml Pefabloc, 5 µM leupeptin, 50 mM benzamidine and 10 µg/ml calpain inhibitors I and II. After 12 h at 4 °C, each fraction was centrifuged (13 000 *g* for 10 min) and the supernatant was assayed for activity in the presence of 1% Triton X-100 and for 30 min. Recovery of enzyme activity was calculated by using the enzyme activity loaded on the gel (measured under similar conditions) as 100%.

Purification procedure for platelet InsP_3 3-kinase

Washed human platelets were prepared from 100 human blood units (Centre de Transfusion, Hôpital Erasme, Brussels) after blood collection from healthy volunteers in $\frac{1}{3}$ vol. of acid/citrate/dextrose (1.5% citric acid, 2.5% trisodium citrate, 2% dextrose). Platelet-rich plasma was prepared by two successive centrifugations at 500 *g* for 15 min at room temperature. The plasma was carefully removed, and platelets were sedimented at 1800 *g* for 10 min before resuspending and washing in 10 ml of an isotonic buffer containing 10 mM Hepes/NaOH (pH 6.2), 2.7 mM KCl, 1 mM MgCl_2 , 135 mM NaCl, 12 mM NaHCO_3 , 5.5 mM dextrose, 0.3% BSA and 13 mM trisodium citrate (Macphee et al., 1988). After a further centrifugation at 1800 *g* for 10 min, the platelet pellet was resuspended $[(1.5\text{--}2.0) \times 10^9 \text{ platelets/ml}]$ in an ice-cold buffer containing 50 mM Tris/HCl (pH 8.0), 1 mM EDTA, 10% sucrose, 12 mM 2-mercaptoethanol, 50 µg/ml Pefabloc, 5 µM leupeptin, 50 mM benzamidine, 20 µg/ml pepstatin and 10 µg/ml calpain inhibitors I and II. The platelets were lysed by three successive cycles of freeze–thawing, followed by centrifugation at 13 000 *g* for 10 min. The supernatant (58 ml of crude soluble fraction supplemented with 1% Triton X-100) was applied in the presence of 0.5 mM CaCl_2 to a 13 ml CaM–Sepharose column which had been equilibrated in 50 mM Tris/HCl (pH 7.5), containing 12 mM 2-mercaptoethanol, 0.2 mM CaCl_2 , 0.1 M NaCl, 0.5% Triton X-100, 50 µg/ml Pefabloc, 5 µM leupeptin, 10 µg/ml calpain inhibitors I and II and 50 mM benzamidine (buffer A) at 4 °C. The column was washed with 130 ml of buffer A in which the concentration of NaCl was increased to 0.4 M. The column was finally washed with 70 ml of buffer A containing 2 mM EGTA in the absence of CaCl_2 and Triton X-100. InsP_3 3-kinase activity was eluted with 100 ml of buffer A containing 2 mM EGTA and 1% Triton X-100 in the absence of CaCl_2 . The active fractions were pooled, concentrated with an Amicon cell to a final volume of 3 ml and immediately stored in batches at –70 °C.

Peptide synthesis and antibody production

The peptides LPGGPTGMARPGGARC, corresponding to part of the N-terminal region of the human InsP_3 3-kinase A sequence [to raise an anti-(peptide A) antibody], and CPPGDRVGVQP-GNSR, corresponding to part of the N-terminal region of the human InsP_3 3-kinase B sequence [to raise an anti-(peptide B) antibody] were synthesized at The Microchemical Facility at the Babraham Institute (U.K.). Briefly, peptide synthesis was performed by fluorenylmethoxycarbonyl chemistry. Peptides were purified by gel filtration in Bio-Gel P-2 gel (Bio-Rad) in 67% acetic acid and conjugated to the purified protein derivative of tuberculin (Maloy, 1991). A 1 mg portion of each antigen was injected three times into two rabbits (New Zealand White). By e.l.i.s.a., the anti-(peptide A) and anti-(peptide B) antibodies

recognized specifically the immunizing antigen at dilutions up to $1:10^4$.

Immunoprecipitation of InsP_3 3-kinase activity

Enzyme preparation (5–40 µl) was mixed with different volumes of anti-(InsP_3 3-kinase A or B) polyclonal antibodies (0–10 µl) and agitated for 1.5 h in a final volume of 50 µl. A 25 µl portion of Protein A–Sepharose prepared as described by Takazawa et al. (1990a) was added to each tube and incubation was continued for 1.5 h at 4 °C. After centrifugation, each supernatant was assayed for InsP_3 3-kinase activity.

RESULTS

Purification of human platelet M_r 69 000–70 000 InsP_3 3-kinase

In order to identify human platelet InsP_3 3-kinase by regeneration of enzyme activity after SDS/PAGE (Takazawa et al., 1990a), the enzyme was purified by CaM–Sepharose affinity chromatography. InsP_3 3-kinase activity from a crude soluble fraction of human platelets was shown to interact directly with CaM–Sepharose. This allowed separation of InsP_3 5-phosphatase activity, all of which was detected in the flow-through (results not shown), from InsP_3 3-kinase activity, which was subsequently eluted in the absence of Ca^{2+} . The elution profile was optimized in the presence of a high detergent concentration (1% Triton X-100) (Takazawa et al., 1990a) and different protease inhibitors, such as calpain inhibitors (I and II). A typical elution profile is shown in Figure 1. We calculated the purification factor to be approx. 4500-fold, with a recovery of 75% (Table 1), whereas 25% was lost during washing of the column. Specific activity at 5 µM InsP_3 in the presence of 10 µM free Ca^{2+} and 0.1 µM CaM was 0.75 µmol/min per mg of protein. InsP_3 3-kinase activity was stimulated about 8-fold by Ca^{2+} /CaM (Table 1).

Figure 1 Separation of human platelet InsP_3 3-kinase by CaM–Sepharose affinity chromatography

Crude InsP_3 3-kinase was applied to a CaM–Sepharose column as described in the Materials and methods section. The column was eluted as indicated by the arrows: A, sample load (0.5 mM CaCl_2); B, 0.2 mM CaCl_2 /0.4 M NaCl/0.5% Triton X-100; C, 2 mM EGTA/0.1 M NaCl; D, 2 mM EGTA/0.1 M NaCl/1% Triton X-100. InsP_3 3-kinase was assayed at 5 µM InsP_3 in the presence of both 0.1 µM CaM and 10 µM free Ca^{2+} (●). Protein content is indicated by (△). This elution profile is representative of three experiments from three different preparations.

Table 1 Purification of human platelet InsP_3 3-kinase

InsP_3 3-kinase activity was assayed at $5 \mu\text{M}$ InsP_3 in the presence of $0.1 \mu\text{M}$ CaM and $10 \mu\text{M}$ free Ca^{2+} or 1 mM EGTA (free Ca^{2+} below 1 nM) for basal activity. Total and specific activities are given in the presence of Ca^{2+} /CaM. Results are from one representative preparation out of three.

Step	Volume (ml)	Protein (mg)	Total activity (nmol/min)	Specific activity (nmol/min per mg)	Purification (fold)	Yield (%)	Stimulation by CaM (fold)
Crude soluble	58	754	121	0.16	1	100	2–3
CaM–Sephadex	3	0.12	89.5	746	4662	74	7–8

Figure 2 Substrate/velocity relationship of purified human platelet InsP_3 3-kinase activity

The data are shown as a direct plot in the 0 – $30 \mu\text{M}$ InsP_3 range at 5 mM ATP in the presence of $0.1 \mu\text{M}$ CaM and $10 \mu\text{M}$ free Ca^{2+} . As the purified enzyme was not contaminated with InsP_3 5-phosphatase activity, the assay was performed in the absence of 2,3-bisphosphoglycerate. Results are means $\pm$ S.D. of quadruplicates. The experiment was performed four times.

Figure 3 CaM-concentration/response curve of purified human platelet InsP_3 3-kinase activity

Human platelet InsP_3 3-kinase activity purified after CaM–Sephadex was assayed in the presence of various concentrations of CaM and $0.1 \mu\text{M}$ ($\square$), $1 \mu\text{M}$ (∇) or $10 \mu\text{M}$ ($\bullet$) free Ca^{2+} . InsP_3 concentration was $5 \mu\text{M}$. Results are means $\pm$ S.D. of triplicates. The experiment was performed four times.

The K_m for InsP_3 of the purified enzyme was $2.0 \mu\text{M}$ (Figure 2). The K_m for ATP was 2.5 mM at $5 \mu\text{M}$ InsP_3 (results not shown). Maximal stimulation by Ca^{2+} /CaM of human platelet InsP_3 3-kinase was at $1 \mu\text{M}$ CaM (at 1 or $10 \mu\text{M}$ free Ca^{2+}): the enzyme exhibited a specific activity of $1.45 \pm 0.1 \text{ nmol/min per mg}$ of protein, and stimulation was approx. 17-fold when compared with the activity determined in the presence of 1 mM EGTA (Figure 3). Half-maximal stimulation was at 80 nM CaM at 1 or $10 \mu\text{M}$ free Ca^{2+} .

Figure 4 SDS/PAGE of CaM–Sephadex-purified InsP_3 3-kinase from human platelets and crude soluble fraction from rat brain

InsP_3 3-kinase activity was measured after electrophoresis at 4°C as described in the Materials and methods section. Each lane was cut into 2.5 or 5 mm slices, and each slice was homogenized in extraction buffer. A $10 \mu\text{l}$ portion of each fraction was assayed at $5 \mu\text{M}$ InsP_3 and in the presence of 1% Triton X-100 for 30 min . Basal ($\blacksquare$) and Ca^{2+} /CaM-stimulated ($\square$) activity were determined under the same conditions as in Table 1. Apparent M_r values were determined by using M_r standards in a parallel lane. (a) $0.8 \mu\text{g}$ of purified InsP_3 3-kinase from human platelets with an activity of 0.27 nmol/min applied on the gel (assayed as outlined above in the presence of Ca^{2+} /CaM). (b) $13 \mu\text{g}$ of protein of crude soluble rat brain InsP_3 3-kinase with an activity of 0.27 nmol/min . Total recoveries of enzyme activity were 30% in (a) and 46% in (b). Results are from one representative experiment out of five.

Identification of human platelet InsP_3 3-kinase after SDS/PAGE

Purified human platelet enzyme was identified by regeneration of enzyme activity after SDS/PAGE: enzyme activity was associated with a major M_r -67 000–71 000 fraction (Figure 4a). The M_r -67 000–71 000 InsP_3 3-kinase was stimulated about 8-fold in the presence of $10 \mu\text{M}$ free Ca^{2+} and $0.1 \mu\text{M}$ CaM. A minor M_r -35 000–38 000 fraction could be identified in the gel: this lower-

Figure 5 Localization of purified human platelet InsP_3 3-kinase activity after SDS/PAGE

(a) Purified human platelet InsP_3 3-kinase (9.5 μg of protein and enzyme activity of 3.6 nmol/min that was applied on to the gel assayed as in Figure 4) was separated by SDS/PAGE (7.5 cm gel, 8% acrylamide) to identify the active M_r 67 000–71 000 fraction. Total recovery of enzyme activity at M_r 67 000–71 000 was 31%. (b) The same enzyme preparation (0.8 μg of protein and enzyme activity of 0.3 nmol/min) was loaded on to the same gel for silver staining. The M_r 69 000–70 000 silver-stained band is indicated by an arrow. (c) The two active M_r 67 000–71 000 fractions from the separation in (a) were pooled, concentrated and re-applied on to a second SDS/PAGE gel (8%) for silver staining. The M_r 69 000–70 000 silver-stained band is indicated by an arrow. Apparent M_r values were determined by using M_r standards in a parallel lane.

Table 2 Immunoprecipitation of InsP_3 3-kinase activities from human platelets and rat brain

Purified human platelet enzyme was immunoprecipitated with various quantities (0–10 μl) of anti-(rat brain M_r 50 000 InsP_3 3-kinase A) antiserum as described in the Materials and methods section. A crude soluble fraction of rat brain was taken as positive control. Immunoprecipitation of InsP_3 3-kinase activity with preimmune serum (10 μl) was also determined, and corresponded to 100%. The total InsP_3 3-kinase activity at 5 μM InsP_3 present per tube was 0.15 and 0.25 nmol/min for human platelet and rat brain enzyme respectively. Results are means $\pm$ S.D. of triplicates.

	InsP_3 3-kinase activity (%)	
	Human platelet	Rat brain
Preimmune serum (10 μl)	100 $\pm$ 3	100 $\pm$ 0.3
Immune serum		
0.0 μl	99 $\pm$ 5	104 $\pm$ 6
0.3 μl	103 $\pm$ 4	43 $\pm$ 9
1.0 μl	100 $\pm$ 1	24 $\pm$ 1
5.0 μl	100 $\pm$ 4	17 $\pm$ 2
10.0 μl	97 $\pm$ 5	9 $\pm$ 3

M_r peak was much less Ca^{2+} /CaM-sensitive (stimulation factor of only 2-fold in the separation shown in Figure 4a). This M_r 35 000–38 000 fraction was observed three times in five experiments, but never represented more than 7% of the M_r 67 000–71 000 InsP_3 3-kinase activity. The data are specific for the human platelet enzyme, as rat brain InsP_3 3-kinase showed a major M_r 48 000–51 000 fraction (Figure 4b, and Takazawa et al., 1990a). The M_r 48 000–51 000 fraction of rat brain was stimulated only 2-fold by Ca^{2+} /CaM.

A sample of purified enzyme was separated by SDS/PAGE (Figure 5a): the two active M_r 67 000–71 000 fractions were associated with a silver-stained band which had an apparent M_r of 69 000–70 000 (Figure 5b). The silver-stained M_r 50 000–51 000 band did not correspond to any InsP_3 3-kinase activity, as

Figure 6 Immunoblotting of InsP_3 3-kinase activities from rat brain, human platelet and recombinant InsP_3 3-kinase B

Crude fraction from rat brain (lanes 1 and 4; 8 μg of protein, with an activity of 0.17 nmol/min at 5 μM InsP_3), purified human platelet InsP_3 3-kinase (lanes 2 and 5; 1.7 μg , with an activity of 0.58 nmol/min) and crude bacterial lysate of recombinant InsP_3 3-kinase B (lanes 3 and 6; 14 μg , with an activity of 0.21 nmol/min) were subjected to SDS/PAGE (8% gels) and then electrophoretically transferred to nitrocellulose. The blots were incubated with anti-(peptide A) serum diluted 1:500 (lanes 1–3) or with anti-(peptide B) serum diluted 1:500 (lanes 4–6). The M_r 50 000 InsP_3 3-kinase A (50 kDa) and the M_r 63 000 InsP_3 3-kinase B (63 kDa) are designated by arrows. The M_r 66 000 band was also recognized by the pre-immune serum (see the Results section). Apparent M_r values were determined by using M_r standards in a parallel lane. Results are from one representative experiment out of five.

identified by regeneration of enzyme activity (Figure 5a). When the two active fractions of M_r 67 000–71 000 (Figure 5a) were pooled and re-applied on to a second SDS gel, a single M_r 69 000–70 000 silver-stained band could be seen, with no evidence of proteolysis (Figure 5c).

Immunological characterization of human platelet InsP_3 3-kinase

Anti-(rat brain M_r 50 000 InsP_3 3-kinase A) serum (Takazawa et al., 1990a) was not able to immunoprecipitate human platelet InsP_3 3-kinase activity (Table 2) or to recognize the platelet enzyme on immunoblots (results not shown). In these experiments, a crude soluble fraction from rat brain was used as positive control. In order to confirm this last result, and to determine whether the platelet enzyme corresponds to InsP_3 3-kinase B, we used antibodies which recognized specific peptide sequences of human InsP_3 3-kinases A and B (see the Materials and methods section). Since none of the new sera worked for immunoprecipitation (results not shown), they were treated by immunoblots: we showed that anti-(peptide A) serum recognized the M_r 50 000 rat brain InsP_3 3-kinase A (Figure 6, lane 1), as previously shown with anti-(M_r 50 000 rat brain InsP_3 3-kinase A) serum (Takazawa et al., 1990a). Moreover, anti-(peptide A) serum was unable to recognize human platelet enzyme and human brain InsP_3 3-kinase B (Figure 6, lanes 2 and 3). On the other hand, anti-(peptide B) serum recognized human brain InsP_3 3-kinase B as an M_r 63 000 protein (Figure 6, lane 6). In contrast, the serum was unable to recognize rat brain InsP_3 3-kinase A and human platelet InsP_3 3-kinase isoenzyme (Figure 6, lanes 4 and 5). The blots, incubated with the corresponding pre-immune sera (dilution 1:500), did not reveal any signal, except the M_r 66 000 band in Figure 6, lane 1 (results not shown).

DISCUSSION

The cloning of a human isoenzyme referred to as InsP_3 3-kinase B provided the first molecular evidence for the existence of InsP_3 3-kinase isoenzymes (Takazawa et al., 1991b). Previous reports

of the enzyme(s) in rat liver (Conigrave et al., 1992) and pig aortic smooth muscle (Yamaguchi et al., 1988) have suggested the presence of proteins of higher molecular mass compared with InsP_3 3-kinase A. Apparent M_r values of 61 000 in liver and 93 000 in smooth muscle were shown by silver staining. However, the specific activities of the purified enzymes were much lower (at least 10-fold) than those of the purified rat brain enzyme. Therefore, regeneration of enzyme activity after SDS/PAGE adds considerable weight to the existence of high- M_r forms of InsP_3 3-kinase.

We report here the purification and characterization of a novel high- M_r (69 000–70 000) InsP_3 3-kinase isoenzyme in human platelets. This was shown by both regeneration of enzyme activity after SDS/PAGE and silver staining. The addition of calpain inhibitors during the purification procedure was critical for limiting proteolysis and thus stabilizing the native enzyme (Lee et al., 1990; Takazawa et al., 1990a), as calpain has been shown to be a major neutral protease in the cytosol of human platelets (Truglia and Stracjer, 1981; Tsujinaka et al., 1982). Moreover, the use of one-step chromatography also limited proteolysis during purification of InsP_3 3-kinase activity.

Biochemical data for the human platelet InsP_3 3-kinase show that the platelet enzyme is quite distinct from the rat brain M_r -50 000 InsP_3 3-kinase A (Takazawa et al., 1990a,b). (1) The human platelet InsP_3 3-kinase presented a higher molecular mass (see above). (2) The stimulation factor by Ca^{2+} /CaM was higher for the human platelet enzyme than for the rat brain protein, approx. 17-fold and 4-fold respectively, at saturating CaM. (3) The human platelet enzyme was not recognized by anti- $(M_r$ -50 000 rat brain InsP_3 3-kinase A) antibodies. (4) It was also not recognized by immunodetection using antibodies prepared against the N-terminal region of human InsP_3 3-kinase A.

Since the human platelet InsP_3 3-kinase had a higher molecular mass, which may be comparable with that of human InsP_3 3-kinase B, we questioned whether the platelet enzyme would be either InsP_3 3-kinase B or a novel form. It was observed that the platelet enzyme was not recognized by specific anti- $(M_r$ -63 000 human InsP_3 3-kinase B) antibodies. Our data therefore suggest the presence of a novel high-molecular-mass isoenzyme in human platelets which is distinct from InsP_3 3-kinase isoenzymes A and B.

Lin et al. (1993) recently reported the purification of a major active M_r -36 000 polypeptide in human platelets. They obtained a low specific activity (283 nmol/min per mg at saturating CaM) and a low yield of purification (16%). As discussed by Lin et al. (1993), the M_r -36 000 polypeptide probably resulted from proteolysis of a higher-molecular-mass InsP_3 3-kinase. We could speculate that the M_r -36 000 polypeptide could be the catalytic domain of the platelet enzyme, as proteolysis of the rat brain enzyme yielded an active polypeptide in the same molecular-mass range (Takazawa et al., 1990a).

A simple model which accounts for the enhancement of InsP_3 3-kinase activity upon exposure of human platelets to thrombin

would be a direct stimulation of the enzyme by Ca^{2+} (Daniel et al., 1987). However, in addition to Ca^{2+} , human platelet InsP_3 3-kinase can be shown to be a substrate of protein kinase C. This has been studied both directly, on purified enzyme (D. Communi, M. Rider and C. Erneux, unpublished work; Lin et al., 1990), and indirectly, by treatment of human platelets with the protein kinase C agonist β -phorbol 12,13-dibutyrate (King and Rittenhouse, 1989). It is therefore possible that InsP_3 3-kinase activity may be modulated by intracellular Ca^{2+} levels, as shown for the CaM-sensitive cyclic nucleotide phosphodiesterase (Erneux et al., 1985) and, in addition, may be regulated by phosphorylation/dephosphorylation mechanisms. Further studies are required to elucidate these mechanisms.

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