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$_2$ 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 ± 0.1 μmol/min per mg, and the degree of stimulation by Ca$^{2+}$/calmodulin was 17-fold at saturating calmodulin and 10 μM free Ca$^{2+}$. The $K_m$ for InsP$_2$ and for ATP was 2.0 μM and 2.5 mM respectively. Human platelet InsP$_2$ 3-kinase was not recognized by immunodetection with anti-(InsP$_2$ 3-kinase A) or anti-(InsP$_2$ 3-kinase B) antibodies. These data provide the first biochemical evidence for the existence of a novel InsP$_2$ 3-kinase isoenzyme in human platelets, which is distinct from previously reported InsP$_2$ 3-kinase A and InsP$_2$ 3-kinase B.

INTRODUCTION
Inositol 1,4,5-trisphosphate (InsP$_3$) 3-kinase catalyses the phosphorylation of InsP$_2$, which has been shown to mobilize intracellular Ca$^{2+}$, to inositol 1,3,4,5-tetrakisphosphate (InsP$_4$), a potential modulator of Ca$^{2+}$ homoestasis (Berridge and Irvine, 1989; Irvine, 1992).

A cDNA clone encoding rat brain Ca$^{2+}$/calmodulin (CaM)-sensitive InsP$_2$ 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$_2$ 3-kinase A. Using the rat cDNA as a probe, Takazawa and colleagues isolated and expressed human InsP$_2$ 3-kinase A from a human hippocampus cDNA library (which presented 93% sequence identity with the rat brain InsP$_2$ 3-kinase A amino acid sequence; Takazawa et al., 1991a) and a novel InsP$_2$ 3-kinase isoenzyme, referred to as InsP$_2$ 3-kinase B (Takazawa et al., 1991b). The expressed InsP$_2$ 3-kinase B was more sensitive to Ca$^{2+}$/CaM (i.e. stimulation factor of 7–10-fold) compared with expressed InsP$_2$ 3-kinase A (2–3-fold stimulated).

Previous reports have suggested that InsP$_2$ 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 characterisation of the enzyme from these cells: it is not known whether it corresponds to either InsP$_2$ 3-kinase A or B, or a distinct isoenzyme. In this study, we have isolated a novel InsP$_2$ 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-$^3$H]InsP$_2$ (sp. radioactivity 3.3 Ci/mmol) was obtained from Du Pont–New England Nuclear Corp. InsP$_2$, 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$_2$ 3-kinase assay was performed at 37°C for 8 min in a 50 μl 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 μg/ml Pefabloc, 5 μM leupeptin, 50 mM benzamidine, 10 μg/ml calpain inhibitors I and II, 1500 c.p.m. of [3$^3$H]InsP$_2$, 5 μM InsP$_2$ 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 μM. The InsP$_2$ 3-phosphatase assay at 30 μM InsP$_2$ was performed as described previously (Takazawa et al., 1990a) in an incubation medium supplemented with protease inhibitors (50 μg/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$_2$ 3-kinase B in E. coli

The expression of plasmid lh3 MB1 encoding InsP$_2$ 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$_2$ 3-kinase activity after SDS/PAGE
The enzyme preparation was made in 62 mM Tris/Cl (pH 6.8), 3% SDS, 5% 2-mercaptoethanol and 10% glycerol (loading buffer), and immediately run on SDS/PAGE (8% gel) at 240 V.

Abbreviations used: CaM, calmodulin; InsP$_2$, inositol 1,4,5-trisphosphate.

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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 µg/ml leupeptin, 50 mM benzamidine and 10 µg/ml calpain inhibitors I and II. After 12 h at 4°C, each fraction was centrifuged (13000 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 1/ 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 [(1.5–2.0) × 10^9 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 µg/ml leupeptin, 50 mM benzamidine, 25 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 13000 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 µg/ml 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 column 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 CPGDGRVGPQGNQR, 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 M₆-69000–70000 InsP₃ 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).

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**Figure 1** Separation of human platelet InsP₃ 3-kinase by CaM–Sepharose affinity chromatography

Crude InsP₃ 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 CaCl₂); B, 0.2 mM CaCl₂/0.4 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. InsP₃ 3-kinase was assayed at 5 µM InsP₃ in the presence of both 0.1 µM CaM and 10 µM free Ca²⁺. Protein content is indicated by (●). This elution profile is representative of three experiments from three different preparations.
The Km for InsP^3 was the protein, exhibited kinase stimulation in the presence of free Ca^{2+} or 1 mM EGTA (free Ca^{2+} below 1 mM) 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.

Table 1  Purification of human platelet InsP^3 3-kinase

<table>
<thead>
<tr>
<th>Step</th>
<th>Volume (ml)</th>
<th>Protein (mg)</th>
<th>Total activity (nmol/min)</th>
<th>Specific activity (nmol/min per mg)</th>
<th>Purification (fold)</th>
<th>Yield (%)</th>
<th>Stimulation by CaM (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude soluble</td>
<td>58</td>
<td>754</td>
<td>121</td>
<td>0.16</td>
<td>1</td>
<td>100</td>
<td>2–3</td>
</tr>
<tr>
<td>CaM-Sepharose</td>
<td>3</td>
<td>0.12</td>
<td>89.5</td>
<td>746</td>
<td>4662</td>
<td>74</td>
<td>7–8</td>
</tr>
</tbody>
</table>

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 μM InsP^3 range at 5 mM ATP in the presence of 0.1 μM CaM and 10 μM 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 ± 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-Sepharose was assayed in the presence of various concentrations of CaM and 0.1 μM ( ), 1 μM ( ) or 10 μM ( ) free Ca^{2+}. InsP^3 concentration was 5 μM. Results are means ± S.D. of triplicates. The experiment was performed four times.

The K_m for InsP^3 of the purified enzyme was 2.0 μM (Figure 2). The K_m for ATP was 2.5 mM at 5 μM InsP^3 (results not shown). Maximal stimulation by Ca^{2+}/CaM of human platelet InsP^3 3-kinase was at 1 μM CaM (at 1 or 10 μM free Ca^{2+}); the enzyme exhibited a specific activity of 1.45 ± 0.1 μmol/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 μM free Ca^{2+}.

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 Mr~67000–71000 fraction (Figure 4a). The Mr~67000–71000 InsP^3 3-kinase was stimulated about 8-fold in the presence of 10 μM free Ca^{2+} and 0.1 μM CaM. A minor Mr~35000–38000 fraction could be identified in the gel: this lower-
Table 672
SDS/PAGE
Mr-67000-71
of
triplicates.

silver
anti-(rat platelets in stained band is indicated 0.25 (7.5 5 5 Localization of purified human platelet
Ig recovery was represented
with 0.3 nmol/min) was loaded on to the same gel for silver staining. The Mr-69000–70000 silver-stained band is indicated by an arrow. The two active Mr-67000–71000 fractions from the separation in (a) were pooled, concentrated and re-applied on to a second SDS/PAGE gel (8%) for silver staining. The Mr-69000–70000 silver-stained band is indicated by an arrow. Apparent Mr values were determined by using Mr standards in a parallel lane.

Table 2 Immunoprecipitation of InsP3 3-kinase activities from human platelets and rat brain

<table>
<thead>
<tr>
<th>InsP3 3-kinase activity (%)</th>
<th>Human platelet</th>
<th>Rat brain</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preimmune serum (10 μl)</td>
<td>100±3</td>
<td>100±0.3</td>
</tr>
<tr>
<td>Immune serum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.0 μl</td>
<td>99±5</td>
<td>104±6</td>
</tr>
<tr>
<td>0.3 μl</td>
<td>103±4</td>
<td>43±9</td>
</tr>
<tr>
<td>1.0 μl</td>
<td>100±1</td>
<td>24±1</td>
</tr>
<tr>
<td>5.0 μl</td>
<td>100±4</td>
<td>17±2</td>
</tr>
<tr>
<td>10.0 μl</td>
<td>97±5</td>
<td>9±3</td>
</tr>
</tbody>
</table>

Mr peak was much less Ca2+/CaM-sensitive (stimulation factor of only 2-fold in the separation shown in Figure 4a). This Mr-35000–38000 fraction was observed three times in five experiments, but never represented more than 7% of the Mr-67000–71000 InsP3 3-kinase activity. The data are specific for the human platelet enzyme, as rat brain InsP3 3-kinase showed a major Mr-48000–51000 fraction (Figure 4b, and Takazawa et al., 1990a). The Mr-48000–51000 fraction of rat brain was stimulated only 2-fold by Ca2+/CaM.

A sample of purified enzyme was separated by SDS/PAGE (Figure 5a): the two active Mr-67000–71000 fractions were associated with a silver-stained band which had an apparent Mr of 69000–70000 (Figure 5b). The silver-stained Mr-50000–51000 band did not correspond to any InsP3 3-kinase activity, as identified by regeneration of enzyme activity (Figure 5a). When the two active fractions of Mr-67000–71000 (Figure 5a) were pooled and re-applied on to a second SDS gel, a single Mr-69000–70000 silver-stained band could be seen, with no evidence of proteolysis (Figure 5c).

Immunological characterization of human platelet InsP3 3-kinase

Anti-(rat brain Mr-50000 InsP3 3-kinase A) serum (Takazawa et al., 1990a) was not able to immunoprecipitate human platelet InsP3 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. Immunoprecipitation of InsP3 3-kinase activity with preimmune serum (10 μl) was also determined, and corresponded to 100%. The total InsP3 3-kinase activity at 5 μM InsP3 present per tube was 0.15 and 0.25 nmol/min for human platelet and rat brain enzyme respectively. Results are means ± S.D. oftriplicates.

Mr-50000-71

66 kDa
50 kDa

Dye front

Figure 5 Localization of purified human platelet InsP3 3-kinase activity after SDS/PAGE

(a) Purified human platelet InsP3 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 Mr-67000–71000 fraction. Total recovery of enzyme activity at Mr-67000–71000 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 Mr-69000–70000 silver-stained band is indicated by an arrow. (c) The two active Mr-67000–71000 fractions from the separation in (a) were pooled, concentrated and re-applied on to a second SDS/PAGE gel (8%) for silver staining. The Mr-69000–70000 silver-stained band is indicated by an arrow. Apparent Mr values were determined by using Mr standards in a parallel lane. Results are from one representative experiment out of five.

Figure 6 Immunoblotting of InsP3 3-kinase activities from rat brain, human platelet and recombinant InsP3 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 InsP3, purified human platelet InsP3 3-kinase (lanes 2 and 5; 1.7 μg, with an activity of 0.58 nmol/min) and crude bacterial lysate of recombinant InsP3 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 Mr-50000 InsP3 3-kinase A (50 kDa) and the Mr-63000 InsP3 3-kinase B (63 kDa) are designated by arrows. The Mr-66000 band was also recognized by the pre-immune serum (see the Results section). Apparent Mr values were determined by using Mr standards in a parallel lane. Results are from one representative experiment out of five.

DISCUSSION

The cloning of a human isoenzyme referred to as InsP3 3-kinase B provided the first molecular evidence for the existence of InsP3 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 61000 in liver and 93000 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-$M_r$ (69000–70000) 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 $M_r$-50000 InsP$_3$ 3-kinase A (Takazawa et al., 1990a,b). (1) The human platelet InsP$_3$ 3-kinase presented a higher antibody 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-($M_r$-50000 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-($M_r$-63000 human InsP$_3$ 3-kinase B) antibodies. Our data therefore suggest the presence of a novel high-molecular-mass isoenzyme in human platelets that is distinct from InsP$_3$ 3-kinase isoenzymes A and B.

Lin et al. (1993) recently reported the purification of a major active $M_r$-36000 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 $M_r$-36000 polypeptide probably resulted from proteolysis of a higher-molecular-mass InsP$_3$ 3-kinase. We could speculate that the $M_r$-36000 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 $\alpha$-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/d Dephosphorylation mechanisms. Further studies are required to elucidate these mechanisms.

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**REFERENCES**


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