Inositol Polyphosphate 4-phosphatase Is Inactivated by Calpain-mediated Proteolysis in Stimulated Human Platelets*

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Inositol polyphosphate 4-phosphatase (4-phosphatase), an enzyme that catalyzes the hydrolysis of the 4-position phosphate of phosphatidylinositol 3,4-bisphosphate, was shown to be a substrate for the calcium-dependent protease calpain in vitro and in stimulated human platelets. Stimulation of platelets with the calcium ionophore, A23187, resulted in complete proteolysis of 4-phosphatase and a 75% reduction in enzyme activity. Thrombin stimulation of platelets resulted in partial proteolysis of 4-phosphatase and a 41% reduction in enzyme activity \( (n = 8, \text{range of 36–51\%}) \). In addition, preincubation with the calpain inhibitor, calpeptin, suppressed the accumulation of phosphatidylinositol 3,4-bisphosphate in thrombin-stimulated platelets by 36% \( (n = 2, \text{range 35–37\%}) \). These data suggest that the calpain-mediated inhibition of 4-phosphatase is involved in the phosphatidylinositol 3,4-bisphosphate accumulation in thrombin-stimulated platelets.

The activation of phosphatidylinositol 3-kinases (PtdIns 3-kinase) is an essential element of receptor-mediated signal transduction that results in the rapid accumulation of two potential lipid second messengers, phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P_2) and phosphatidylinositol 3,4,5-trisphosphate (PtdIns(3,4,5)P_3) \( (1–3) \). Although the intracellular targets of these lipids are unknown, both have been shown to activate calcium-independent isozymes of protein kinase C (9). Recently, PKB/Akt has been shown to be regulated by calpain-mediated proteolysis, including protein phosphotyrosine phosphatase 1B \( (16) \), phospholipase C-\( \beta_1 \) \( (17) \), and integrin \( \beta_3 \) \( (18) \).

In this study, we demonstrate that 4-phosphatase is a substrate for calpain and is inhibited by calpain-mediated proteolysis in vitro and in stimulated human platelets. In addition, we show that calpain inhibition suppresses the accumulation of PtdIns(3,4)P_2 in thrombin-stimulated human platelets. These results suggest a role for 4-phosphatase in the regulation of intracellular PtdIns(3,4)P_2 levels.

**EXPERIMENTAL PROCEDURES**

**Materials**—Bovine thrombin, A23187, Triton X-100, dithiothreitol, prostaglandin \( E_\_3 \), and acetylsalicylic acid were purchased from Sigma. Pefabloc® SC and leupeptin were purchased from Boehringer Mannheim. Porcine erythrocyte calpain type I and human recombinant calpastatin were purchased from Calbiochem. Calpeptin was purchased from LC Laboratories. \( [32P] \)H_3PO_4 was purchased from ICN Biomedicals. Ins(3,4)P_2 and \( [3H] \)Ins(3,4)P_2 were prepared as described previously \( (11) \).

In Vitro Calpain Treatment of Recombinant 4-phosphatase—Six histidine-tagged recombinant human 4-phosphatase was expressed in *Escherichia coli* using the 6HisTrc vector (Clontech), and recombinant protein was purified on a nickel-nitrilotriacetic acid-agarose column (Qiagen). Recombinant six-histidine-tagged 4-phosphatase (100 ng/ml) was incubated with various amounts of type I porcine calpain in 20 mM Hepes \( (pH 7.5) \), 100 mM NaCl, 2 mM EDTA, 3 mM CaCl_2, and 1 mM dithiothreitol for 10 min at 37°C. Reactions were stopped by the addition of 10 mM EDTA and 1 \( \mu \)g of calpastatin/ml. Platelet Preparation and Stimulation—Washed platelets were prepared from plasma obtained from healthy donors as described previously \( (19) \) with the modifications that the platelet-rich plasma was incubated at 37°C for 20 min with 1 mM acetylsalicylic acid and 10 \( \mu \)M prostaglandin \( E_\_1 \), prior to centrifugation. Platelets (10^9/ml) were stimulated by 1 \( \mu \)M A23187 or 1 unit of thrombin/ml in 15 mM Tris \( (pH 7.4) \), 140 mM NaCl, 5.5 mM glucose, and 2.5 mM CaCl_2 (platelet aggregation buffer) unless otherwise indicated. Platelets were stirred at 37°C using a aggerator (Payton).

Preparation of Platelet Lysates and 4-phosphatase Activity Assay—Platelet lysates were prepared by the addition of 0.5 ml of 2% Triton X-100, 20 mM Hepes \( (pH 7.5) \), 10 mM EDTA, 10 \( \mu \)g of leupeptin/ml, and 0.1 mM EGTA/1 mM Pefabloc® SC to a platelet suspension (300 000 platelets/\mu l) and sonicated on ice. Platelet lysates were clarified by 5 min centrifugation at 13 000 g. The resulting supernatant was used for protein assays and 4-phosphatase activity assays.
nescence reagents (Pierce) and Biomax™ Film (Eastman Kodak Co.).

TBS-T and 4-phosphatase was detected by Supersignal™ chemiluminescence reagents (Pierce) and Biomax™ Film (Eastman Kodak Co.).

Membranes were washed with TBS-T and then incubated with 1:4000 diluted anti-rabbit IgG-Horseradish peroxidase (Amersham Corp.) in TBS for 1 h at room temperature. Membranes were then washed with TBS-T and then incubated with 1:2000 dilution of rabbit anti-(C-terminal peptide antiserum (lane 1), stimulated with 1 unit/ml thrombin (lane 2), stimulated with 1 unit/ml thrombin after pretreatment with 30 μM calpeptin (lane 3), in the presence of 0.5 mM RGDS (lane 4) or 2 mM EDTA (lane 5). For the immunoblot in A, the 39-kDa fragment was detected with an exposure five times longer than that used to detect the 105-kDa 4-phosphatase. The error bars in B indicate the range of enzyme activity values. The 4-phosphatase specific activity of lysates from unstimulated platelets was $6 \times 10^{-4} \mu$mol/min/mg of protein.

1 mM Pefabloc® to a 0.5-ml suspension of platelets ($10^9$/ml). Platelet lysates were assayed for 4-phosphatase activity using [$^{3}H$]Ins(3,4)P$_2$ as substrate as described previously (11).

4-Phosphatase Immunoblotting—Platelet suspensions were boiled for 4 min in SDS loading buffer containing 50 mM Tris (pH 6.8), 2% SDS, 10% β-mercaptoethanol, 0.05% bromphenol blue, 10 mM EDTA, and 10 μg of leupeptin/ml. Samples were separated by SDS-polyacrylamide gel electrophoresis using a 10% polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were blocked with 5% powdered milk, 0.05% Tween 20 in Tris-buffered saline (TBS) for 1 h at room temperature and then incubated with 1:2000 dilution of rabbit antiserum directed against the 4-phosphatase C-terminal peptide in TBS containing 0.05% Tween 20 (TBS-T) for 1 h at room temperature. Membranes were washed with TBS-T and then incubated with 1:4000 diluted anti-rabbit IgG-Horseradish peroxidase (Amersham Corp.) in TBS-T for 1 h at room temperature. Membranes were then washed with TBS-T and 4-phosphatase was detected by Supersignal™ chemiluminescence reagents (Pierce) and Biomax™ Film (Eastman Kodak Co.).

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FIG. 1. Immunoblot analysis and enzyme activity of recombinant inositol-polyphosphate 4-phosphatase-treated with calpain in vitro. 100 ng/ml of six His-tagged human 4-phosphatase was treated for 10 min at 37 °C with the amounts of type II calpain indicated. Proteolysis was detected by immunoblot analysis using a C-terminal peptide antiserum (A), and the effect of proteolysis on enzyme activity was determined using Ins(3,4)P$_2$ as substrate (B).

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FIG. 2. Immunoblot analysis and enzyme activity of inositol-polyphosphate 4-phosphatase from calcium ionophore-stimulated human platelets. The anti-4-phosphatase immunoblot (A) and enzyme activity using Ins(3,4)P$_2$ as substrate (B) was determined for unstimulated human platelets stimulated with 1 mM A23187 for 0 min (lane 1), 1 min (lane 2), 2 min (lane 3), 5 min (lane 4), 10 min (lane 5), 10 min following pretreatment with 30 μM calpeptin (lane 6), and 10 min in the presence of 2 mM EDTA (lane 7). The 4-phosphatase specific activity of lysates from unstimulated platelets was $6 \times 10^{-4} \mu$mol/min/mg of protein.

4-Phosphatase Immunoblotting—Platelet suspensions were boiled for 4 min in SDS loading buffer containing 50 mM Tris (pH 6.8), 2% SDS, 10% β-mercaptoethanol, 0.05% bromphenol blue, 10 mM EDTA, and 10 μg of leupeptin/ml. Samples were separated by SDS-polyacrylamide gel electrophoresis using a 10% polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were blocked with 5% powdered milk, 0.05% Tween 20 in Tris-buffered saline (TBS) for 1 h at room temperature and then incubated with 1:2000 dilution of rabbit antiserum directed against the 4-phosphatase C-terminal peptide in TBS containing 0.05% Tween 20 (TBS-T) for 1 h at room temperature. Membranes were washed with TBS-T and then incubated with 1:4000 diluted anti-rabbit IgG-Horseradish peroxidase (Amersham Corp.) in TBS-T for 1 h at room temperature. Membranes were then washed with TBS-T and 4-phosphatase was detected by Supersignal™ chemiluminescence reagents (Pierce) and Biomax™ Film (Eastman Kodak Co.).

FIG. 3. Immunoblot analysis and enzyme activity of inositol-polyphosphate 4-phosphatase from thrombin-stimulated human platelets. The anti-4-phosphatase immunoblot (A) and 4-phosphatase activity using Ins(3,4)P$_2$ as substrate (B) were determined for stirred human platelet that were unstimulated (lane 1), stimulated with 1 unit/ml thrombin (lane 2), stimulated with 1 unit/ml thrombin after pretreatment with 30 μM calpeptin (lane 3), in the presence of 0.5 mM RGDS (lane 4) or 2 mM EDTA (lane 5). For the immunoblot in A, the 39-kDa fragment was detected with an exposure five times longer than that used to detect the 105-kDa 4-phosphatase. The error bars in B indicate the range of enzyme activity values. The 4-phosphatase specific activity of lysates from unstimulated platelets was $6 \times 10^{-4} \mu$mol/min/mg of protein.

4-Phosphatase Immunoblotting—Platelet suspensions were boiled for 4 min in SDS loading buffer containing 50 mM Tris (pH 6.8), 2% SDS, 10% β-mercaptoethanol, 0.05% bromphenol blue, 10 mM EDTA, and 10 μg of leupeptin/ml. Samples were separated by SDS-polyacrylamide gel electrophoresis using a 10% polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were blocked with 5% powdered milk, 0.05% Tween 20 in Tris-buffered saline (TBS) for 1 h at room temperature and then incubated with 1:2000 dilution of rabbit antiserum directed against the 4-phosphatase C-terminal peptide in TBS containing 0.05% Tween 20 (TBS-T) for 1 h at room temperature. Membranes were washed with TBS-T and then incubated with 1:4000 diluted anti-rabbit IgG-Horseradish peroxidase (Amersham Corp.) in TBS-T for 1 h at room temperature. Membranes were then washed with TBS-T and 4-phosphatase was detected by Supersignal™ chemiluminescence reagents (Pierce) and Biomax™ Film (Eastman Kodak Co.).

HPLC Analysis of Glycerophosphorylinositols—Glycerophosphorylinositols were separated on a Partisil SAX column using flow rate of 1 ml/min and a gradient of 0–1 M ammonium phosphate (pH 3.8) consisting of linear gradient from O to 25% solvent B over 60 min followed by a linear gradient to 100% over 50 min (pump A: water, pump B: 1 M ammonium phosphate). [$^{32}$P]PO$_4$-labeled glycerophosphorylinositols derivatives were detected using an A-100 radioactive flow detector (Radiomatic).

Miscellaneous Techniques—Protein concentration was determined
using the Bio-Rad protein assay reagent. Lipid phosphate was determined using the method of Ames and Dubin (21).

RESULTS AND DISCUSSION

The presence of PEST sequences in the predicted amino acid sequence of 4-phosphatase suggested that this enzyme might be a substrate for the calcium-dependent protease, calpain (13). As shown in Fig. 1A, immunoblot analysis of recombinant 4-phosphatase treated with calpain in vitro using anti-4-phosphatase C-terminal peptide antiserum indicates that the enzyme is a substrate for calpain. A proteolytic fragment of 104 kDa is detected using this antiserum, indicating that a calpain cleavage site exists near the 4-phosphatase N terminus. However, this 104-kDa fragment is proteolyzed further to fragments that are not detected by this antiserum, indicating the presence of at least one additional cleavage site near the C-terminal epitope. This proteolysis results in a 75% decrease in the activity of recombinant 4-phosphatase (Fig. 1B). The 25% residual 4-phosphatase activity is resistant to calpain treatment with 500 ng/ml (data not shown).

To determine whether 4-phosphatase is a substrate for calpain in vitro, human platelets were stimulated with calcium ionophore A23187, an agonist known to activate platelet calpain in the presence of extracellular calcium (16, 17). As shown in Fig. 2A, immunoblot analysis of lysates prepared from unstimulated platelets stimulated with 1 μM A23187 using anti-4-phosphatase C-terminal peptide antiserum indicates that the full-length 105-kDa 4-phosphatase is rapidly (half-life of 2 min) and completely proteolyzed in the presence extracellular calcium with the generation of a 39-kDa immunoblotting proteolytic fragment (lanes 1–5). In addition, the presence of EDTA (lane 6) or preincubation of platelets with the cell-permeant calpain inhibitor, calpeptin, blocks A23187-stimulated proteolysis of 4-phosphatase (Fig. 2A). This proteolysis of 4-phosphatase correlates with a 75% decrease in the observed enzyme activity in platelet lysates which was prevented by the presence of EDTA or preincubation with calpeptin (Fig. 2B). The remaining 25% of platelet 4-phosphatase activity was not inactivated by 30-min ionophore stimulation (data not shown). The amount of enzyme activity that is resistant to calpain-mediated proteolysis in platelets is similar to that observed when 4-phosphatase is treated with calpain in vitro.

Stimulation of platelets with the physiological agonist, thrombin, is also known to activate platelet calpain via a mechanism that requires both extracellular calcium (17) and platelet aggregation (22). As shown in Fig. 3A, immunoblot analysis of lysates prepared from stirred platelets stimulated for 5 min with 1 unit/ml thrombin indicates that the 4-phosphatase is partially proteolyzed in the presence of extracellular calcium resulting in a 39-kDa immunoblotting proteolytic fragment similar to that observed in ionophore-stimulated platelets (lane 2). This proteolysis was blocked by preincubation with calpeptin (lane 3), the presence of RGDS, a tetrapeptide that prevents platelet aggregation (lane 4), or the presence of EDTA (lane 5). Thrombin stimulation resulted in an average decrease of 41% (n = 8, range of 36–51%) in 4-phosphatase activity observed in platelet lysates, and this activity decrease was blocked by the agents that prevent calpain-mediated proteolysis (Fig. 3B).

To evaluate the possible role of calpain-mediated inactivation of 4-phosphatase on the levels of PtdIns(3,4)P2 in thrombin-stimulated platelets, the effect of calpain inhibition on the accumulation of PtdIns(3,4)P2 was measured. As shown in Fig. 4, preincubation of platelets with calpeptin prior to thrombin stimulation suppressed the accumulation of PtdIns(3,4)P2 by an average of 36% (n = 2, range of 35–37%). A similar suppression of PtdIns(3,4)P2 levels of approximately 40% has been reported previously for platelets stimulated with thrombin in the absence of extracellular calcium (23) or in the presence of RGDS (24, 25), two factors known to prevent calpain activation. PtdIns(3,4)P2 accumulation in thrombin-stimulated platelets is biphasic with a rapid (within 20 s) calcium-independent phase and a slow (after 90 s) calcium-dependent phase (23). It has been proposed that the biphasic rise in PtdIns(3,4)P2 levels is a result of a rapid activation of PtdIns 3-kinase followed by a slower calcium/aggregation-dependent inactivation of a PtdIns(3,4)P2 phosphatase that produces the further sustained rise in PtdIns(3,4)P2 (23, 25). The data reported here are consistent with this model and suggest a mechanism involving the inactivation of 4-phosphatase by calpain-mediated proteolysis to produce the calcium/aggregation-dependent accumulation of PtdIns(3,4)P2 in thrombin-stimulated platelets.

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REFERENCES

1. Auger, K. R., Serunian, L. A., Seltoff, S. P., Libby, P., and Cantley, L. C. (1989) Cell 57, 167–175.
2. Traynor-Kaplan, A. E., Thompson, B. L., Harris, A. L., Taylor, P., Omann, G. V., and Sklar, L. A. (1989) J. Biol. Chem. 264, 15668–15673.
3. Jackson, T. R., Stephens, L. R., and Hawkins, P. T. (1992) J. Biol. Chem. 267, 16607–16616.
4. Nakanishi, H., Brewer, K. A., and Exton, J. H. (1993) J. Biol. Chem. 268, 13–16.
5. Toker, A., Meyer, M., Reddy, K. K., Falcó, J. R., Aneja, R., Aneja, S., Parra, A., Burns, D. J., Ballas, L. M., and Cantley, L. C. (1994) J. Biol. Chem. 269, 32358–32367.
6. Toker, A., Bachelet, C., Chen, C.-S., Falcó, J. R., Hartwig, J. H., Cantley, L. C., and Kovacsics, T. J. (1995) J. Biol. Chem. 270, 29525–29531.
7. Zhang, J., Falcó, J. R., Reddy, K. K., Abrams, C. S., Zhao, W., and Rittenhouse, S. E. (1995) J. Biol. Chem. 270, 22907–22910.
8. Burgier, B. M., and Coffer, P. J. (1995) Nature 376, 599–602.
9. Franke, T. F., Yang, S.-I., Chen, T. O., Datta, K., Karadschas, A., Morrison, D. K., Kaplan, D. R., and Tsichlin, P. N. (1995) Cell 81, 727–736.
10. Franke, T. F., Kaplan, D. R., Cantley, L. C., and Toker, A. (1997) Science 275, 665–668.
11. Bansal, V. S., Caldwell, K. K., and Majerus, P. W. (1990) J. Biol. Chem. 265, 1060–10611.
12. Norris, F. A., and Majerus, P. W. (1994) J. Biol. Chem. 269, 8716–8720.
13. Norris, F. A., Auetathvekit, V., and Majerus, P. W. (1995) J. Biol. Chem. 270, 16128–16133.
14. Rogers, S., Wells, R., and Rehbein, M. (1986) Science 234, 364–368.
15. Wang, K. W., Villalobo, A., and Reufogalis, R. D. (1989) Biochem. J. 262, 693–706.
16. Frangioni, J. V., Oda, A., Smith, M., Salzman, E. W., and Neel, B. G. (1993) EMBO J. 12, 4843–4856.
17. Banno, Y., Nakashima, S., Hachiya, T., and Nozawa, Y. (1995) J. Biol. Chem. 270, 4318–4324.
18. de Du, X., Saito, T., Tsuchiya, S., and Iwatsuki, Y. (1995) J. Biol. Chem. 270, 26146–26151.
19. Baenninger, N. L., and Majerus, P. W. (1974) Methods Enzymol. 31, 149–155.
20. Lips, D. L., and Majerus, P. W. (1989) J. Biol. Chem. 264, 19911–19915.
21. Ames, B. N., and Dubin, G. V. (1960) J. Biol. Chem. 235, 769–775.
22. Fox, J. E. B., Taylor, R. G., Taffarel, M., Boyles, J. K., and Goll, D. E. (1993) J. Cell Biol. 120, 1501–1507.
23. Sokolowski, A., King, W. G., and Rittenhouse, S. E. (1992) Biochem. J. 286, 351–357.
24. Sultan, C., Plantavid, M., Bachelet, C., Grondin, P., Breton, M., Mauco, G., Levy-Toledano, S., Caen, J. P., and Chap, H. (1991) J. Biol. Chem. 266, 23554–23557.
25. Rittenhouse, S. E. (1996) Blood 88, 4401–4414.