Regulation of Platelet Plasma Membrane Ca\textsuperscript{2+}-ATPase by cAMP-dependent and Tyrosine Phosphorylation\textsuperscript{*}

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As a consequence of its central role in the regulation of calcium metabolism in the platelet, the plasma membrane Ca\textsuperscript{2+}-ATPase (PMCA) was assessed for cAMP-dependent and tyrosine phosphorylation. Addition of forskolin or prostaglandin E1, agents known to elevate platelet cAMP and calcium efflux, to platelets pre-labeled with \textsuperscript{32}P\textsubscript{PO}\textsubscript{4} resulted in the direct phosphorylation of platelet cAMP and calcium efflux, to platelets pre-labeled with \textsuperscript{32}P\textsubscript{PO}\textsubscript{4} resulted in the direct phosphorylation of platelet PMCA. Similarly, addition of the catalytic subunit of protein kinase A to platelet plasma membranes resulted in a 1.4-fold stimulation of activity. Thus, the previously reported inhibition of platelet activation by elevated intracellular cAMP may be accomplished in part by inhibition of PMCA, likely resulting in a decrease in intracellular calcium.

Treatment with thrombin evoked tyrosine phosphorylation of platelet PMCA, while PMCA from resting platelets exhibited little tyrosine phosphorylation. Phosphorylation of platelet plasma membranes by pp60\textsuperscript{src} resulted in 75\% inhibition of PMCA activity within 15 min. Similarly, membranes isolated from thrombin-treated platelets exhibited 40\% lower PMCA activity than those from resting platelets. Phosphorylation of erythrocyte ghosts and purified PMCA by pp60\textsuperscript{src} also resulted in up to 75\% inhibition of Ca\textsuperscript{2+}-ATPase activity, and inhibition was correlated with tyrosine phosphorylation. Sequencing of a peptide obtained after \textsuperscript{32}P labeling of purified erythrocyte PMCA in vitro showed that tyrosine 1176 of PMCA\textsubscript{b} is phosphorylated by pp60\textsuperscript{src} \textsuperscript{1734}. These results indicate that tyrosine phosphorylation of platelet PMCA may serve as a positive feedback to inhibit PMCA and increase intracellular calcium during platelet activation.

Ca\textsuperscript{2+} signaling is an integral component of activation in platelets (1). The intracellular Ca\textsuperscript{2+} concentration is regulated by Ca\textsuperscript{2+}-ATPases and Ca\textsuperscript{2+} channels located in both the plasma membrane and in the dense tubular system, a modified endoplasmic reticulum in the platelet (2). Strong agonists such as thrombin promote release of Ca\textsuperscript{2+} from internal stores and influx through the plasma membrane. Conversely, platelet antagonists such as prostaglandin E1 that elevate platelet cAMP, reduce cytosolic Ca\textsuperscript{2+} levels. Since the plasma membrane Ca\textsuperscript{2+}-ATPase (PMCA)\textsuperscript{2} is the main agent of Ca\textsuperscript{2+} removal at resting Ca\textsuperscript{2+} concentrations (2), it is a key point for regulation of platelet Ca\textsuperscript{2+} metabolism. The activity of PMCA has been shown to be activated by several mechanisms including Ca\textsuperscript{2+}/calmodulin, protein kinases A and C, acidic phospholipids, and proteolytic attack (3). In fact, Johnson et al. (4) showed indirectly that cAMP increases the rate of Ca\textsuperscript{2+} extrusion in platelets. However, direct phosphorylation of the platelet PMCA has not been reported previously.

In addition to increased cytosolic Ca\textsuperscript{2+}, platelet activation is also accompanied by tyrosine phosphorylation of several proteins including focal adhesion kinase (pp125\textsuperscript{FAK}) and the non-receptor tyrosine kinase pp60\textsuperscript{src} followed by their association with the cytoskeleton (5, 6). Ca\textsuperscript{2+} appears to be intimately involved in both tyrosine phosphorylation and cytoskeletal re-arrangement in the platelet (7). It has recently been demonstrated that the IP\textsubscript{3} receptor can be phosphorylated on tyrosine residues leading to increased release of Ca\textsuperscript{2+} from internal stores (8). We report here that tyrosine phosphorylation of PMCA inhibits its activity further enhancing generation of the calcium signal.

In the present work we have assessed direct phosphorylation of platelet PMCA in vivo after exposure to thrombin, an agonist, or prostaglandin E1, an antagonist. In addition, the effects of both cAMP-dependent and tyrosine kinase-mediated phosphorylation of platelet PMCA in vivo on Ca\textsuperscript{2+}-ATPase activity were measured. Finally, in vitro tyrosine phosphorylation of erythrocyte PMCA was used to determine the phosphorylated tyrosine residues. Purified erythrocyte PMCA was used as a model for platelet PMCA since we demonstrate in this work that the platelet contains the same isoforms of PMCA as found in the erythrocyte, PMCA\textsubscript{1} and PMCA\textsubscript{4} (9).

EXPERIMENTAL PROCEDURES

Materials—Outdated human platelet concentrates and whole blood were obtained from the Louisville Chapter of the American Red Cross and from the Central Kentucky Blood Center (Lexington, KY). Polyclonal antibodies against human erythrocyte PMCA were raised in rabbits as described previously (10), and monoclonal anti-PMCA antibodies (9) were kindly provided by Dr. Ernesto Carafoli (Swiss Federal Institute of Technology, Zurich, Switzerland). Mouse monoclonal anti-phosphotyrosine (PY-20) (PY-20) was purchased from Transduction Laboratories (Lexington, KY). All secondary antibodies, electrophoresis and Western blotting reagents were obtained from Bio-Rad. Thrombin, prostaglandin E1, ATP, phospholipase C, NADH, pyruvate kinase, lactate dehydrogenase, catalytic subunit of cAMP-dependent kinase, and egg yolk phosphatidylcholine were purchased from Sigma. Triton X-100 and protein A-agarose were products from Pierce. Genistein was purchased from Calbiochem. \textsuperscript{32}P-Labeled Na\textsubscript{3}PO\textsubscript{4} and ATP were products from DuPont NEN. Endoproteinase Lys-C was purchased from Boehringer Mannheim.

The abbreviations used are: PMCA, plasma membrane Ca\textsuperscript{2+}-ATPase; TES, 2-\{2-hydroxy-1,1-bis(hydroxymethyl)ethyl\}aminoethanesulfonic acid; HPLC, high pressure liquid chromatography.
Plasma Membrane Calcium Pump Tyrosine Phosphorylation

Purified recombinant pp60src and protein phosphatase type 2A were products from Upstate Biotechnology Inc. (Lake Placid, NY).

**Platelet and Erythrocyte Plasma Membrane Preparation—**Platelet plasma membranes were prepared by the glycerol lysis method described by Harmon et al. (15), except that membranes from lysed platelets were isolated in 4°C within 15 min of collection from 50 ml of blood in a Beckman SW-28 rotor at 26,000 rpm for 5 h (13). Membranes collected at 0–33% sucrose interface were concentrated by centrifugation at 100,000 g for 30 min in 0.5 ml of buffer containing 20 mM HEPES buffer, pH 7.4, containing 1 mM EGTA, 10 μg/ml leupeptin, and 0.1% (w/v) glycerol, washed three times in the same buffer, and incubated in 3% (w/v) glycerol in liquid nitrogen, and stored at −70 °C.

**Erythrocyte PMCA assay—**Samples were obtained with anti-PMCA antibodies by Western blotting according to Steck and Kant (14). Final membrane pellets were suspended in 0.1 ml of TES buffer at pH 7.4 containing 0.1 M KCl, 20% (w/v) glycerol, 10 mM MgCl2, 2 mM dithiothreitol, 0.5 mM sodium orthovanadate, 2 mM dithiothreitol, 2 mM EGTA, and 0.5 mg/ml phosphatidylcholine. Reactions were terminated by rapid chromatography (5 min) of 50-μl assay mixtures on 5-ml Sephadex G-50 columns equilibrated with 20 ml of HEPES buffer, 3 mM MgCl2, 0.05 mM CaCl2, 20 mM EGTA, 2 mM dithiothreitol, 0.05 mM TlCl, 50 mM Tris at pH 7.0, 0.1% (w/v) NaCl, and 20% (w/v) glycerol, frozen and stored at −70 °C.

**Ca2+-ATPase Activity—**ATPase activity was assayed using a system in which ATP hydrolysis is coupled to Na+/H+ exchange by pyruvate kinase using phosphoenolpyruvate and pyruvate kinase (16). Purified erythrocyte PMCA was assayed in 0.1 ml of buffer containing 3% (w/v) glycerol, 0.5 mg/ml phosphatidylcholine, and the coupled assay components. The difference in the rate of ATP hydrolysis in the presence and absence of 10 mM EGTA was used to calculate the Ca2+-ATPase activity. Assay of erythrocyte ghosts and platelet plasma membranes was performed in 0.01 ml of TES buffer at pH 7.5, containing 0.1 mM MgCl2, 5 mM ATP, 50 mM MgCl2, 0.01 mM CaCl2, and coupled assay components.

**Labeling of Whole Platelets with [32P]PO4 and Immunoprecipitation of PMCA—**Platelet concentrates (5 × 109 cells/ml) in citrate anticoagulant were centrifuged at 5,000 × g and resuspended in the same volume of Tyrode’s buffer (2 ml) containing 10 mM HEPES buffer at pH 7.4, 0.1 mM asparagine, 0.2 units/ml apyrase, and 2 mM EGTA. [32P]Na2PO4 (0.2 μCi) was added, and the platelets were gently mixed for 2 h at room temperature. At the end of the incubation period, the labeled platelets were pelleted by centrifugation at 5,000 × g for 3 min and resuspended in 0.1 ml of the same added components used for [32P] labeling except that EGTA was omitted when prostaglandin E1 (5 μM) or forskolin (1 μM) was added. After a 2-min incubation period at room temperature, the platelets were pelleted by centrifugation at 5,000 × g for 3 min and were resuspended in 100,000 × g for 3 min. Following reaspiration in 100 μl of 10 mM Tris at pH 7.2, 5 mM dithiothreitol, and 10% (w/v) glycerol and reconcentration for 3 min in a final washed pellet was resuspended in 50 μl of the centrifugation buffer and assayed immediately for Ca2+-ATPase activity.

**Western Blotting—**Proteins were electrophoresed on 7.5% SDS-polyacrylamide gels (17) followed by electrophoretic transfer to nitrocellulose membranes at 100 V in Tris-glycine buffer at pH 8.3 containing 10% methanol. After transfer, the blots were washed in TTBS (30 mM Tris at pH 7.5, 150 mM NaCl, and 0.1% Tween 20 containing 1 mM sodium o-ovanadate) and blocked at room temperature for 1 h in 5% (w/v) nonfat dry milk dissolved in this TTBS solution. After washing two times in TTBS for 10 min, primary antibody dissolved at the appropriate dilution in TTBS containing 2% (w/v) bovine serum albumin was incubated with the membrane for 1 h. The membrane was washed three times with four times in TTBS and then incubated in the appropriate secondary antibody conjugated to horseradish peroxidase at a dilution of 1:20,000 for 1 h. The membranes were washed as described after the primary antibody incubation and exposed to chemiluminescence reagents (Amersham Corp.). Immunoreactive proteins were visualized by exposure of the membrane to x-ray film. PMCA protein bands were obtained by PMCA phosphorylation with PY20 (anti-phosphotyrosine), and specific isoforms of PMCA with anti-PMCA 1N, 2N, 3N, and 4N (9). In some experiments membranes were stripped of bound antibody and probed with a different primary antibody after incubation of the membrane in 62.5 mM Tris at pH 6.8, containing 2% (w/v) SDS and 100 mM 2-mercaptoethanol at 60 °C for 1 h. The blots were then washed with TTBS, and the Western blotting procedure was repeated. The mass of PMCA present was determined by Western blotting known amounts of pure erythrocyte PMCA on the same gel with unknowns followed by ECL-based immunodetection and quantitation of scans of the resulting lumigrams using SigmaScan software.

**pp60src-dependent Phosphorylation and Ca2+-ATPase Activity Measurements—**Purified erythrocyte PMCA was phosphorylated with 0.4 units pp60src/μg PMCA at 30 °C in 5 μg/ml Triton X-100, 20 mM HEPES buffer at pH 7.2, 130 mM NaCl, 31 mM MgCl2, 0.1 mM ATP, 23 mM MnCl2, 2 mM sodium orthovanadate, 2 mM dithiothreitol, 2 mM EGTA, and 0.5 mg/ml phosphatidylcholine. Reactions were terminated by rapid chromatography (5 min) of 50-μl assay mixtures on 5-ml Sephadex G-50 columns equilibrated with 20 μl of HEPES buffer, 3 mM MgCl2, 0.05 mM CaCl2, 20 mM EGTA, 2 mM dithiothreitol, 0.5 mM TlCl, 50 mM Tris at pH 7.0, 0.1% (w/v) NaCl, and 20% (w/v) glycerol, frozen and stored at −70 °C.

**Purification of Erythrocyte PMCA—**Calmodulin-free erythrocyte ghosts were prepared from outdated whole blood and PMCA purified by calmodulin-Sepharose affinity chromatography according to the method of Niggli et al. (15). The final preparation exhibited a major band at −140 kDa and a minor band at −100 kDa on SDS-polyacrylamide gels. The specific activity of the purified material was 3.8 nmol of ATP hydrolyzed/min/mg, and a 2.2-fold stimulation of activity was accomplished by Western blotting as described below. The presence of PMCA was monitored at 214 nm with a mobile phase of 0.06% trifluoroacetic acid.
**RESULTS**

**Cyclic AMP-dependent Phosphorylation of Human Platelet PMCA**—The work of Johnson et al. (4) demonstrated that an increase in platelet cAMP results in increased efflux of Ca^{2+}, suggesting that the platelet PMCA is stimulated by cAMP-dependent phosphorylation. Neyes et al. (18) demonstrated that both sarcolemmal and erythrocyte PMCAs are phosphorylated by cAMP-dependent kinase resulting in stimulation of Ca^{2+}-ATPase activity. Furthermore, Johnson and Haynes (19) showed that the Na-Ca^{2+} exchanger makes a negligible contribution to platelet calcium efflux at resting calcium concentrations, thus implying that the main effect of cAMP on platelet Ca^{2+} efflux is on PMCA. Consequently, we attempted to demonstrate that elevated cAMP leads to direct phosphorylation of PMCA in the platelet. The result of increasing cytosolic platelet cAMP with prostaglandin E1 or forskolin is shown in Fig. 1. Platelets were labeled with [32P]PO_4 and then treated with forskolin or prostaglandin E1 prior to immunoprecipitation of PMCA. The results demonstrate that both agents cause a significant increase in incorporation of [32P], presumably resulting from cAMP-dependent phosphorylation. The effect of cAMP-dependent phosphorylation on Ca^{2+}-ATPase activity was determined using purified platelet plasma membranes. Addition of protein phosphatase type 2A inhibited Ca^{2+}-ATPase activity, and subsequent addition of the catalytic subunit of cAMP-dependent protein kinase resulted in 1.4-fold stimulation (Table I). These results confirm that platelet PMCA can be stimulated by cAMP-dependent phosphorylation and show that the protein in isolated membranes is partially phosphorylated. Studies presented in a later section demonstrate that PMCA1 and -4 are the two isoforms present in human platelets, and these are most likely PMCA1b and -4b.

**Tyrosine Phosphorylation of PMCA in Human Platelets in Vivo**—During platelet activation, several proteins become specifically phosphorylated on tyrosine residues and associate with the platelet cytoskeleton. Since it is not known if platelet PMCA is associated with the cytoskeleton in resting platelets, it was necessary to look for PMCA in the cytoskeleton of resting platelets to determine a baseline for PMCA distribution between the membrane and cytoskeleton. The results shown in Fig. 2 demonstrate that PMCA is not associated with the cytoskeleton or other Triton X-100-insoluble material in the resting platelet. The band labeled “heavy chain” represents non-specific binding of the primary or secondary antibodies to the heavy chain of IgG used to immunoprecipitate PMCA.

**Immunoprecipitation of PMCA from resting platelets.**—Platelets (1.5 ml of a suspension containing 5 × 10^{10} cells/ml) were labeled with [32P]PO_4 and then suspended in Tyrode’s buffer containing aspirin, apyrase, and prostaglandin E1 (5 units/ml) for 5 min followed by immunoprecipitation as described under “Experimental Procedures.” Forskolin and prostaglandin E1 were omitted in the control. The immunoprecipitates were submitted to SDS-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane. Radioactivity was detected by phosphorimaging, and then immunodetection was performed with anti-PMCA (5F10). PMCA was visualized by chemiluminescence.

**TABLE I**

| Addition to membranes | Specific activity |
|-----------------------|------------------|
| No addition | 12.6 ± 3.4 (n = 4) |
| Phosphatase | 9.2 ± 0.9 (n = 4) p < 0.035 |
| Phosphatase then kinase | 14.7 ± 1.1 (n = 4) p < 0.002 |

a Phosphatase treated compared with untreated membranes.

b Phosphatase treated then kinase treated compared with phosphatase treated.

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Platelet plasma membranes were dephosphorylated with protein phosphatase 2A by preincubating membranes (70 μg of total protein) with 0.5 units of phosphatase in 10 mM TES buffer containing 1 mM dithiothreitol and 1 mM EDTA for 10 min at 30 °C prior to assaying for Ca^{2+}-ATPase activity. When kinase treatment followed phosphatase treatment, 63 units of catalytic subunit of cAMP-dependent protein kinase were added to the membranes along with 0.1 mM ATP, 50 mM K_2PO_4, and 10 mM MgCl_2, and the membranes were incubated for an additional 10 min.

**Effects of in Vitro Phosphorylation of PMCA with Purified pp60^{src}**—Phosphorylation of PMCA by both cAMP-dependent state. Activation of platelets with thrombin or spontaneous aggregation (labeled ADP because of the involvement of secreted ADP in spontaneous aggregation) resulted in tyrosine phosphorylation of PMCA as shown in Fig. 3. Considerably less tyrosine phosphorylation was observed in platelets treated with prostaglandin E1 to block spontaneous aggregation. Furthermore, the tyrosine kinase inhibitor genistein totally eliminated all tyrosine phosphorylation and prevented spontaneous aggregation. Stripping and reprobing the immunoblot membrane with monoclonal 5F10 anti-PMCA showed that differences in tyrosine phosphorylation were not simply due to the difference in amounts of PMCA applied to the SDS gel.

The results in Fig. 3 indicate that PMCA is directly phosphorylated on tyrosine residues during platelet activation. The kinetics of tyrosine phosphorylation of PMCA after addition of thrombin are shown in Fig. 4. Increased phosphorylation induced by thrombin was seen at 5 min, and by 10 min the level of phosphorylation returned nearly to the baseline level observed in the absence of thrombin.

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Fig. 1. cAMP-mediated phosphorylation of platelet PMCA in vivo. Platelets (5 × 10^{10} cells/ml) were labeled with [32P]Na_3PO_4 and then treated with either forskolin (1 mM) or prostaglandin E1 (PGE1) (5 μM) for 5 min followed by immunoprecipitation as described under “Experimental Procedures.” Forskolin and prostaglandin E1 were omitted in the control. The immunoprecipitates were submitted to SDS-polyacrylamide gel electrophoresis and blotted onto a nitrocellulose membrane. Radioactivity was detected by phosphorimaging, and then immunodetection was performed with anti-PMCA (5F10). PMCA was visualized by chemiluminescence.

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Fig. 2. Immunoprecipitation of PMCA from resting platelets. Platelets (1.5 ml of a suspension containing 5 × 10^{10} cells/ml) resuspended in Tyrode’s buffer containing aspirin, apyrase, and prostaglandin E1 were solubilized with 0.4% Triton X-100 containing EGTA and protease inhibitors (see “Experimental Procedures”). After centrifugation at 16,000 × g, the Triton-insoluble material was solubilized in 1% SDS and diluted 4-fold with Triton solubilization buffer. PMCA was then immunoprecipitated with monoclonal anti-PMCA, and the protein A-PMCA complex was solubilized in SDS and electrophoresed; Western blotting was then performed with anti-PMCA 5F10. Antibody binding was visualized by chemiluminescence. PMCA indicates a lane loaded with 1.0 μg of purified erythrocyte PMCA, and cytoskeleton indicates the lane containing the immunoprecipitate from the Triton-insoluble fraction.
Plasma Membrane Calcium Pump Tyrosine Phosphorylation

Fig. 3. Tyrosine phosphorylation of platelet PMCA. Platelets (1.5 ml of a suspension containing 5 × 10^10 cells/ml) were resuspended in Tyrode’s buffer containing thrombin (5 units/ml), no additions (labeled ADP due to the occurrence of spontaneous aggregation accompanied by secretion of ADP), genistein (0.5 mM), or prostaglandin E1 (1.0 μM) for 5 min at room temperature, and immunoprecipitation was then carried out as described in Fig. 2. Western blots were first probed with anti-phosphotyrosine (PY20), stripped, and reprobed with anti-PMCA (5F10).

Fig. 4. Time course of tyrosine phosphorylation of PMCA in thrombin-stimulated platelets. Platelets were pelleted and resuspended in Tyrode’s buffer at 5.5 × 10^10 cells/ml. Either thrombin (1 unit/ml) or EGTA (2 mM) was added (0 min), and aliquots were removed and solubilized with Triton X-100 at the indicated times as described under “Experimental Procedures.” Immunoprecipitated PMCA was electrophoresed and blotted onto a nitrocellulose membrane. The membrane was first probed with anti-phosphotyrosine followed by chemiluminescence detection. The nitrocellulose membrane was then stripped with SDS and reprobed with anti-PMCA (5F10). Film was scanned and analyzed using SigmaGel. The peak area of the phosphotyrosine signal was then divided by the area determined for PMCA to yield the phosphotyrosine/PMCA ratio for each time point. The ratio presented on the axis does not reflect the absolute ratio of phosphotyrosine to PMCA bands.

PMCA Isoforms in Human Platelets—Since purified erythrocyte PMCA is an excellent source for identification of phosphorylated tyrosine residues, it is essential to determine the isoform content of the platelet. Erythrocytes contain the two housekeeping forms of PMCA, 1 and 4. Probing purified platelet membranes with antibodies specific for the N termini of the same two isoforms as erythrocytes and lack PMCA2 and -3. Thus erythrocyte PMCA is an excellent model for determination of tyrosine phosphorylation sites. More detailed analysis of the mRNA expression of different PMCA isoforms in platelets and megakaryocytes using reverse transcription-polymerase chain reaction analysis (20) have given similar results and found the isoforms to be PMCA1a and -4b.

Site(s) of Phosphorylation of PMCA by pp60^src—To determine the tyrosine residues phosphorylated in PMCA, we labeled purified erythrocyte PMCA in vitro with ^32P by phosphorylation with pp60^src and [γ-^32P]ATP. After overnight labeling of 1 nmol of purified erythrocyte PMCA with 90 units of pp60^src, the phosphorylated protein was purified by SDS-polyacrylamide gel electrophoresis and blotted onto polyvinylidene difluoride membrane as described under “Experimental Procedures.” The labeled protein was reduced and alkylated and then digested with the endoproteinase Lys-C. HPLC analysis of the generated peptides is shown in Fig. 8. A single radioactive peptide peak containing approximately 26 pmol of ^32P was detected, and this peptide was isolated and subjected to automated Edman sequence analysis as described under “Experimental Procedures.” Extensive losses of ^32P-containing material occurred during sample processing, so the recovery of this peptide was not quantitative. The sequence obtained, shown in Table II, corresponded to residues 1162–1177 in hPMCA4b (24), a splice variant of PMCA4 found in most tissues (20). The tyrosyl residue at position 1176 was not detected, indicating that it probably is phosphorylated. Tyr-1176 is preceded by a glutamic acid residue 4 residues N-terminal to the tyrosine as has been observed for many tyrosine phosphorylation sites but is missing the usual lysine or arginine residue 7 residues N-terminal to the tyrosine (29). Since this tyrosine phosphorylation site is C-terminal to the calmodulin binding domain, it

protein kinase and protein kinase C stimulates PMCA activity (3). The effect of tyrosine phosphorylation on platelet PMCA was assessed by the addition of pp60^src phosphorylation to purified platelet plasma membranes. This is a reasonable model for platelet-tyrosine phosphorylation since pp60^src is the most abundant non-receptor tyrosine kinase in the platelet and is associated with the plasma membrane (21). The results shown in Fig. 5A demonstrate that platelet plasma membrane Ca2+-ATPase activity was rapidly inhibited by tyrosine phosphorylation. Within 15 min, the platelet PMCA activity was reduced by 75% of the initial activity ( □ ). Furthermore, membranes isolated from thrombin-treated platelets also exhibited inhibition of PMCA activity. Membranes were isolated from thrombin- (5 min) or EGTA-treated platelets by sonication and differential centrifugation and assayed for Ca2+-ATPase activity in the presence of 200 nM thapsigargin to inhibit internal membrane Ca2+-ATPase activity (22). Membranes obtained from EGTA-treated platelets exhibited PMCA activity of 4.1 ± 0.9 nmol/min/mg protein, while thrombin treatment yielded membranes with an activity of 2.5 ± 0.5, an inhibition of 40% with p < 0.021 (two-tail Student’s t test).

The results shown in Fig. 5A also demonstrate that PMCA in erythrocyte ghosts is rapidly inhibited by pp60^src, reaching 60% inhibition by 1 h ( ▲ ). Purified PMCA is also inhibited to a similar extent, but the process is much slower, possibly because of the lack of membranes or the presence of the detergent Triton X-100 ( ▼ ). The inhibition of activity seen, at least in the case of the purified PMCA, was not due to loss of ability to interact with calmodulin. As shown in Fig. 5B, the phosphoryrosine-containing form of PMCA bound as well, if not better, to CaM-Sepharose in the presence of Ca2+ as compared with the dephospho-form.

Fig. 6 shows that there is excellent correlation between tyrosine phosphorylation of purified erythrocyte PMCA and the inhibition of Ca2+-ATPase activity seen in Fig. 5. pp60^src is autophosphorylated, and this precedes tyrosine phosphorylation of PMCA as expected (23). The band below PMCA has a molecular mass of approximately 90 kDa and is a proteolysis product of PMCA as shown by Western blotting with the anti-PMCA antibody (data not shown).

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P. C. Brandt and W. L. Dean, unpublished observations.
might be expected to affect calmodulin binding. However, treatment of purified PMCA with pp60src did not affect the ability of PMCA to bind to calmodulin-Sepharose as shown in Fig. 5B. The affinity of calmodulin for the phosphotyrosine form of PMCA was further assessed by measuring calmodulin stimulation of calmodulin-free erythrocyte ghosts after treatment with pp60src. If tyrosine phosphorylation inhibited PMCA by lowering the affinity for calmodulin, then a higher concentration of calmodulin would be expected to alleviate the inhibition. Membranes were treated with pp60src or pp60Src storage buffer (25 mM HEPES buffer, pH 7.0, containing 50% (v/v) glycerol, 0.1% (v/v) Nonidet P-40, and 1.0 mM dithiothreitol) for 30 min at 30 °C as described for Fig. 5A, and PMCA activity was assayed in the absence or presence of 50, 250, 1000, and 3000 μM calmodulin. Calmodulin stimulated PMCA activity 2-fold compared with the activity in the absence of added calmodulin at all four calmodulin concentrations. The inhibition caused by pp60Src (based on the comparison between pp60Src-treated membranes and the buffer only control) was the same (30%) in the absence of calmodulin as for the four calmodulin concentrations used in this experiment. Thus the inhibition of PMCA activity could not be overcome by a 60-fold increase in calmodulin concentration. This result strengthens the conclusion reached from Fig. 5B that tyrosine phosphorylation does not affect calmodulin binding to PMCA.

The stoichiometry of phosphorylation was determined by comparison of 32P incorporation with the mass of PMCA calculated by scanning of Coomassie Blue-stained gels as described under “Experimental Procedures.” Labeling for 6 hours at room temperature yielded incorporation of 5 pmol of phosphate into 11 pmol of purified PMCA. Since the erythrocyte contains approximately 70% PMCA4 (9, 18) and we only detected a single labeled peptide in PMCA4, this corresponds to 65% incorporation into tyrosine 1176. This result is in reasonable agreement with the 72% inhibition of PMCA activity observed after 6 h of phosphorylation (Fig. 4). It should be noted that analysis of trypsin digests of
PMCA plays a central role in platelet Ca\(^{2+}\) metabolism. PMCA and the Na\(^+\)-Ca\(^{2+}\) exchanger are the only proteins capable of removing Ca\(^{2+}\) from the cell. Since published data indicate that the Na\(^+\)-Ca\(^{2+}\) exchanger does not contribute significantly to platelet Ca\(^{2+}\) efflux at resting Ca\(^{2+}\) levels, then PMCA must have the primary role of maintaining the resting level of cytoplasmic Ca\(^{2+}\). Regulation of the activity of PMCAs is an integral and essential component of calcium-signaling pathways. To date, all reported regulatory signals (i.e. calmodulin binding and reversible phosphorylation) have been reported to activate pump activity. However, unlike most other calmodulin-regulated enzymes, PMCAs have a substantial basal unstimulated activity. Thus, activation results in increases of basal activity of 2–10-fold at most. We report here that tyrosine phosphorylation of PMCA4 in platelets leads to substantial, if not complete, inhibition of its Ca\(^{2+}\)-ATPase activity below the basal level. This would allow for a more rapid increase in cellular calcium during initial response phase in the platelet as well as to a much greater differential effect of activators compared with the inhibited state. Because of the important role of Ca\(^{2+}\) in platelet activation, these changes in PMCA activity could have significant effects on intracellular Ca\(^{2+}\) levels and platelet function. The present work clearly supports the central role of PMCA in platelet Ca\(^{2+}\) metabolism since we demonstrated platelet antagonists stimulate the pump, whereas agonists inhibit its activity. Thus kinases provide positive feedback control of Ca\(^{2+}\) metabolism during activation and inhibition of platelet function.

We demonstrated directly that PMCA is phosphorylated when platelets are treated with agents known to elevate cAMP (Fig. 1). Furthermore, treatment of isolated plasma membranes with the catalytic subunit of cAMP-dependent kinase and protein phosphatase type 2A. These results directly confirm the conclusions of Johnson et al. (4) that cAMP stimulates PMCA in the platelet. This stimulation should lead to lower intracellular Ca\(^{2+}\) and a decreased propensity for activation. In addition, it appears that PMCA in membranes isolated from resting platelets is partially phosphorylated as demonstrated by the loss of activity upon dephosphorylation.

Activation of platelets by a strong agonist such as thrombin results in tyrosine phosphorylation of several proteins followed by their incorporation into the Triton-insoluble cytoskeleton. This process is at least partially regulated by Ca\(^{2+}\) (6, 7, 27). The concomitant tyrosine phosphorylation of PMCA would aid in coordination of this process. Here we show for the first time that platelet PMCA is phosphorylated on a tyrosine residue during platelet activation and that this phenomenon is inhibited by the tyrosine kinase inhibitor genistein (Fig. 3). This phosphorylation does not appear to be accompanied by association of PMCA with the Triton-insoluble cytoskeleton since the ATPase is not associated with the cytoskeleton in resting platelets (Fig. 2) and does not decrease in the Triton-soluble fraction after activation (Fig. 3). In addition the kinetics of PMCA phosphorylation are quite similar to that reported for pp125\(^{FAK}\) (7).

Even more important is the demonstration that tyrosine phosphorylation inhibits PMCA. Addition of pp60\(^{src}\)-inhibited PMCA activity in purified platelet membranes 75% in 15 min at room temperature. Furthermore, platelet activation by
thrombin decreased PMCA activity in isolated membranes by 40%. The results of pp60src treatment of purified erythrocyte PMCA indicate that the kinase preparation is not contaminated with proteinases, since there is no indication of degradation of PMCA (Fig. 6). Although the rate of kinase-mediated inhibition of PMCA is much slower in the purified membrane-free preparation, the final level of inhibition is the same in erythrocyte ghosts as the purified preparation. Although these results do not prove that pp60src is the actual kinase responsible for PMCA phosphorylation during activation, this kinase is the most abundant non-receptor tyrosine kinase in the platelet and is associated with the plasma membrane in resting platelets (21).

Although a potential pp60src consensus tyrosine phosphorylation site (25) is present in all PMCA1 isoforms at Tyr-596, our tryptic phosphopeptide mapping and sequence analyses yielded no evidence that this isoform was phosphorylated pp60src in vitro. PMCA4 isoforms lack a consensus tyrosine phosphorylation site, but PMCA4b was nevertheless phosphorylated on Tyr-1176, a site lacking the basic residue 7 residues N-terminal of the tyrosine but exhibiting the predicted acidic residue, glutamic acid, 4 residues N-terminal to Tyr-1175. It is possible that PMCA1 was phosphorylated on the predicted tyrosine and that it was missed because PMCA1 is only 30% or less of the total PMCA of the erythrocyte. In platelet plasma membranes, PMCA activity was inhibited approximately 80% by tyrosine phosphorylation, even though PMCA4 is only approximately 50% of the total PMCA (Fig. 6). This suggests that both isoforms may be phosphorylated in platelet membranes. The results presented here are in agreement with the recent observation that the tyrosine phosphatase inhibitor, phenylarsine oxide, increases Ca\(^{2+}\) influx at the plasma membrane in endothelial cells (28) at phenylarsine oxide concentrations that we have subsequently shown have no effect on the activity of PMCA itself.\(^3\) This suggests that control of Ca\(^{2+}\) influx by reversible phosphorylation by threonine, serine, and tyrosine kinases may be a general regulatory mechanism in many diverse cell types.

\(^3\) T. C. Vanaman, unpublished observations.

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