Tyrosine Phosphorylation of Human Platelet Plasma Membrane Ca\(^{2+}\)-ATPase in Hypertension

K.A. Blankenship, C.B. Dawson, G.R. Aronoff, W.L. Dean

Abstract—Intracellular Ca\(^{2+}\) is increased in the platelets of hypertensive individuals. Previously, we demonstrated that platelet plasma membrane Ca\(^{2+}\)-ATPase (PMCA) activity inversely correlates with diastolic blood pressure and that inhibition of this Ca\(^{2+}\) pump could explain the elevation of cytosolic Ca\(^{2+}\) in hypertension. More recently, we discovered that PMCA is phosphorylated on tyrosine residues during thrombin-stimulated platelet aggregation and that this phosphorylation causes inhibition of PMCA activity. In the present work, we tested the hypothesis that tyrosine phosphorylation of PMCA in hypertensive patients could account for the observed inhibition of the Ca\(^{2+}\) pump. Platelets were obtained from untreated hypertensive and normotensive volunteers. PMCA was immunoprecipitated from solubilized platelets, and tyrosine phosphorylation was quantified by chemiluminescence of immunoblots treated with anti-phosphotyrosine. PMCA content was measured on the same immunoblots by stripping and reprobing with anti-PMCA. Phosphorylation was reported as normalized phosphotyrosine chemiluminescence per nanogram PMCA (mean±SE). The average PMCA tyrosine phosphorylation for 15 normotensive subjects was 0.53±0.09, whereas the average for 8 hypertensive individuals was 1.82±0.25 (P<0.0005, Mann-Whitney U test). Age, gender, and systolic blood pressure did not correlate with PMCA phosphorylation. These results suggest that PMCA in platelets of hypertensive individuals is inhibited because of tyrosine phosphorylation, resulting in increased platelet intracellular Ca\(^{2+}\), hyperactive platelets, and increased risk of heart attack and stroke. (Hypertension. 2000;35:103-107.)

Key Words: Ca\(^{2+}\)-transporting ATPase ■ calcium ■ hypertension, essential ■ phosphorylation ■ platelets

Hypertension is a risk factor for thrombotic events.\(^1\) Platelets, being activated by increased cytosolic Ca\(^{2+}\), are instrumental in the initiation of thrombosis.\(^2\) Platelets of hypertensive individuals exhibit enhanced sensitivity to agonists\(^3\) and often have increased basal intracellular Ca\(^{2+}\) levels.\(^4,5\) Furthermore, a positive, continuous, linear relation exists between the magnitude of blood pressure and the incidence of coronary heart disease.\(^6\) Increased platelet Ca\(^{2+}\) in hypertension contributes to higher platelet activity and subsequent risk for thrombosis. The mechanism responsible for this increase in platelet Ca\(^{2+}\) is not known.

Previously, we reported differential phosphorylation of the inositol trisphosphate receptor–regulated Ca\(^{2+}\) release from platelet internal membranes.\(^7\) We also observed a time-dependent increase in tyrosine phosphorylation of platelet plasma membrane Ca\(^{2+}\)-ATPase (PMCA) on stimulation with thrombin, which was correlated with decreased pump activity.\(^8\) In the present study, we tested the hypothesis that tyrosine phosphorylation of PMCA in hypertensive patients could account for the observed inhibition of the Ca\(^{2+}\) pump in hypertension.\(^9\) We analyzed this phenomenon in platelets from normotensive and untreated hypertensive individuals; this analysis required development of methodologies to determine relative levels of tyrosine phosphorylation of PMCA and to normalize these in terms of total PMCA present. This allowed for correction of experimental variability in immunoprecipitation. We found that PMCA in platelets of hypertensive individuals exhibits enhanced tyrosine phosphorylation.

Methods

Materials

Outdated human platelet concentrates (5×10\(^{10}\) cells/mL) were obtained from the Louisville Chapter of the American Red Cross. Purified erythrocyte PMCA was isolated from whole human blood as previously described.\(^8\) Rabbit preimmune serum and polyclonal antibodies against purified human erythrocyte PMCA were produced by Advanced ChemTech. Mouse monoclonal anti-PMCA antibody, clone 5F10, was purchased from Affinity BioReagents. Monoclonal horseradish peroxidase–conjugated anti-phosphotyrosine PY20-HRPO was purchased from Transduction Laboratories. Goat antimouse IgG secondary antibody, electrophoresis, and immunoblotting reagents were obtained from Bio-Rad. Immobilized protein A–agarose was purchased from Pierce. Nonfat dry milk used for blocking Western blots was manufactured by Nestle. Chemiluminescence reagents were purchased from NEN Life Sciences. Kodak XAR-5 film was used to visualize immunoblots. All other reagents were purchased from Sigma Chemical Co.
Subject Selection
Normotensive volunteers (n=15) were recruited from the University of Louisville medical campus. Hypertensive subjects (n=8) with diastolic blood pressures ≥90 mm Hg were not on medication and had been newly diagnosed at local blood pressure screenings. Recruitment was nonbiased with respect to age (22 to 59 years of age), gender, race, or socioeconomic status and followed the protocol previously established.9

Isolation of Platelets
As previously described,9 the initial 3 mL of blood drawn was discarded, and the following 40 mL was collected into acidic citrate anticoagulant to prevent activation. This was divided in half so that duplicate samples could be processed. Platelet-rich plasma was prepared by centrifugation of whole blood at 175g for 15 minutes. Platelets were pelleted at 2000g for 10 minutes and solubilized with Triton X-100 for further processing.

Preparation of Thrombin-Activated Platelet Lysate
Aliquots of 2 mL of outdated human platelet concentrates in citrate anticoagulant were centrifuged at 500g for 2 minutes at room temperature to remove erythrocytes. Platelets were collected by centrifugation at 5300g for 3 minutes. Pellets were gently resuspended in 1.5 mL Tyrode’s buffer containing 10 mmol/L HEPES, 0.1 mmol/L acetylsalicylic acid, and 0.2 U/mL apyrase. At time zero, either 2 mmol/L EGTA or 5 U/mL thrombin was added, and platelets were collected by centrifugation at 5300g at 4 minutes after treatment. Platelet pellets were resuspended in 250 µL solubilization buffer made up of 0.4% (vol/vol) Triton X-100, 30 mmol/L Tris, 0.15 mol/L NaCl, 10 mmol/L EGTA, 10 µg/mL each of leupeptin, antipain, and pepstatin A, and 1 mmol/L each of sodium orthovanadate, dithiothreitol, and phenylmethylsulfonyl fluoride. All subsequent steps were performed at 4°C on a rotary mixing device. Platelets were incubated for 1 hour, and insoluble materials were removed by centrifugation at 8300g for 10 minutes. Lysates were frozen and stored for quantification of tyrosine phosphorylation as described below.

Immunoblotting and Quantification
SDS-solubilized immunoprecipitates9 (40 µL) and controls (SDS-PAGE–prestained high-range standards, 100 ng purified erythrocyte PMCA, and 10 µg thrombin-stimulated platelet lysates containing tyrosine-phosphorylated pp125FAK) were loaded on 7.5% SDS-PAGE gels.10 After electrophoresis, proteins were transferred to 0.45-

duplicate samples was analyzed as a function of blood pressure, age, gender, and race.

Statistics
The Mann-Whitney U test and stepwise regression analysis were used for statistical analysis of the data. Significance was set at a value of P<0.05.

Results
To demonstrate the linearity of chemiluminescence signals, increasing quantities of erythrocyte PMCA and tyrosine-phosphorylated pp125FAK in solubilized thrombin-stimulated platelets were electrophoresed, immunoblotted, and quantified as described in Methods. In Figure 1, PMCA and FAK phosphotyrosine quantities were plotted against pixel number, a measure of the strength of the chemiluminescence signal, for film exposure times of 1 and 3 minutes. Both PMCA and FAK demonstrated a linear relation between quantity applied and signal strength. For either given length of exposure, the signal-to-mass ratio was consistent over the linear range of the film for PMCA (25 to 150 ng) and for FAK in solubilized platelets (2.5 to 15 µL). As expected, slopes of the lines were not equal for different exposure times.

The specificity of the horseradish peroxidase–conjugated anti-phosphotyrosine antibody PY20-HRPO was verified by immunoblotting tyrosine-phosphorylated PMCA that was immunoprecipitated from thrombin-treated platelets with PY20-HRPO in the presence of increasing concentrations of exogenous O-phospho-L-tyrosine. The immunoblotted phosphotyrosine signal declines in a dose-dependent manner with inclusion of exogenous O-phospho-L-tyrosine (Figure 2). Coincubation of PY20-HRP with 10 µmol/L phosphoserine

Figure 1. Relation between chemiluminescence response and the quantity of immunobioted PMCA or phosphotyrosine. Purified human erythrocyte PMCA (C) and (D) or thrombin-activated platelet (plt) extracts (+) were electrophoresed and blotted onto nitrocellulose sheets as described in Methods. After treatment with the appropriate primary and secondary antibodies, chemiluminescence signals were determined as described in Methods. The pixel total (left y axis, PMCA) corrected for background chemiluminescence is shown for 1-minute (C) and 3-minute (D) exposures vs nanograms of PMCA loaded on the polyacrylamide gel. Similarly, the phosphotyrosine chemiluminescence signal (right y axis, FAK) on pp125FAK is shown vs the number of microliters (x axis value x=0.1) of thrombin-stimulated plt extract loaded on the polyacrylamide gel.
Figure 2. Effect of phosphoserine (PS), phosphothreonine (PT), and O-phospho-L-tyrosine (PY) on detection of PMCA-phosphotyrosine. PMCA was immunoprecipitated from thrombin-activated platelets, electrophoresed, and blotted onto nitrocellulose sheets as described in Methods. Chemiluminescence of PMCA after exposure to PY20 (anti-PY) was determined in the absence and presence of the concentrations of PS, PT, and PY indicated on the x-axis.

or phosphothreonine did not affect PY20-HRPO detection of tyrosine phosphorylation on immunobLOTS (Figure 2).

Platelet PMCA was immunoprecipitated from the blood of normotensive and nontreated hypertensive individuals and evaluated for levels of phosphotyrosine and PMCA as described in Methods. Representative immunoblots are shown in Figure 3A and 3B. In Figure 3A, immunoprecipitates (from normotensive and hypertensive volunteers) and thrombin-activated platelets were blotted with anti-phosphotyrosine. Numerous tyrosine-phosphorylated proteins are present in thrombin-activated platelets, including the prominently labeled FAK at 125 kDa. Bands corresponding to PMCA at 135 kDa were also labeled by the antibody. Results of stripping and reprobing with anti-PMCA are shown in Figure 3B. A prominent band appears at 135 kDa in the immunoprecipitates corresponding to PMCA. Figure 3C shows the results of a control experiment designed to determine the identity of the lower molecular weight bands recognized by anti-PMCA. Lanes IP1 and IP2 (Figure 3C) are immunoprecipitates from the same platelet sample exhibiting significant amounts of PMCA and smaller polypeptides recognized by anti-PMCA. Lane IP-Plt (Figure 3C) shows the results of a control experiment designed to determine the identity of the nonspecific binding to immunoglobulins used for immunoprecipitation. Similarly, in Figure 3B, bands other than those appearing at the molecular weight of PMCA (135 kDa) result from the breakdown of PMCA (≈100-kDa band seen in PMCA standard and volunteer immunoprecipitates) and nonspecific binding to the immunoglobulins used for immunoprecipitation.

Normalized phosphotyrosine signals were expressed as a function of immunoprecipitated PMCA mass, and the average of each individual’s 2 samples was plotted against their diastolic blood pressures in Figure 4. The average PMCA tyrosine phosphorylation for 15 normotensives was 0.53 ± 0.09 (mean ± SE), whereas the average for 8 hypertensives was 1.82 ± 0.25 (mean ± SE). This difference is statistically significant at P < 0.0005 by the Mann-Whitney U test. With PMCA phosphorylation as the dependent variable and age, gender, and systolic and diastolic blood pressures as independent variables, multiple regression analysis with a backward model-building technique showed that the only independent variable that was a predictor of phosphorylation ratio was diastolic blood pressure. The model was as follows:
phosphorylation ratio = \(-2.58 + \text{diastolic blood pressure} \times 0.0436\). The \(P\) value was 0.002.

**Discussion**

Intracellular \(\text{Ca}^{2+}\) concentration is regulated by \(\text{Ca}^{2+}\)-ATPases and \(\text{Ca}^{2+}\) channels located in both the plasma membrane and the dense tubules, a modified endoplasmic reticulum in the platelet.\(^{11}\) Agonists promote release of \(\text{Ca}^{2+}\) from internal stores and influx through the plasma membrane. Because PMCA is the main agent of extracellular \(\text{Ca}^{2+}\) transport at resting \(\text{Ca}^{2+}\) concentrations,\(^{11}\) it is a key point for regulation of platelet \(\text{Ca}^{2+}\) metabolism. Another \(\text{Ca}^{2+}\) pump, located in the dense tubules, sequesters intracellular \(\text{Ca}^{2+}\) to be released on activation via the inositol 1,4,5-trisphosphate pathway.\(^{12}\) These 2 pumps work to maintain low basal intracellular \(\text{Ca}^{2+}\) concentrations.

Multiple hypotheses have been presented to explain the observed increase in hypertensive platelet \(\text{Ca}^{2+}\). One would suspect that either more \(\text{Ca}^{2+}\) is entering the platelet or less is being extruded or properly sequestered within the dense tubules. In support of the influx hypothesis, \(\text{Ca}^{2+}\) channel blockers have been successfully used to treat hypertension.\(^{13,14}\) Reduction of peripheral resistance is the primary mechanism by which \(\text{Ca}^{2+}\) channel blockers control hypertension, but reduction of platelet \(\text{Ca}^{2+}\) influx could also contribute to protection from thrombotic events.\(^{14,15}\) The \(\text{Ca}^{2+}\) channel blocker nifedipine was used by Ahn et al\(^{16}\) to reduce \(\text{Ca}^{2+}\) in the platelets of hypertensives. Similarly, isradipine was reported to decrease platelet aggregation,\(^{17}\) even specifically at the site of atherosclerotic lesions.\(^{18}\) Evidence also indicates that changes occur in the activity of platelet \(\text{Ca}^{2+}\) transporters in hypertension. Lowered \(\text{Ca}^{2+}\) efflux by platelet PMCA as a result of reduced pump activity was demonstrated in individuals with essential hypertension by Gulati et al\(^{19}\) and by us.\(^{9}\) Furukawa et al\(^{20}\) demonstrated that treatment of washed platelets with an inhibitor of the \(\text{Ca}^{2+}\) pump present in the plasma of hypertensive patients resulted in increased platelet aggregation and intracellular \(\text{Ca}^{2+}\) levels. This supports earlier work by Takaya et al\(^{21}\) suggesting that the intracellular buildup of platelet \(\text{Ca}^{2+}\) in hypertensives results from inhibition of \(\text{Ca}^{2+}\)-ATPase activity. A report by Resink et al\(^{22}\) countered these, however, by showing the PMCA in hypertensive individuals to be stimulated. Their work contrasts with studies on humoral factors in which the ability to modulate intracellular free \(\text{Ca}^{2+}\) was proposed to be via inhibition of the \(\text{Ca}^{2+}\)-ATPase.\(^{23,24}\) Although there is not yet complete consensus on the role of PMCA in hypertension, most of the studies do suggest that in hypertension, impairment of pump activity contributes to heightened levels of \(\text{Ca}^{2+}\) and response to agonists.

Experimental variability can be significant for both immunoprecipitation and chemiluminescent quantification of proteins on immunoblots. Typically, 50 to 100 ng of platelet PMCA was immunoprecipitated per 10\(^{11}\) platelets in our experiments. To quantify the mass of immunoblotted platelet PMCA, we needed a standard against which to normalize chemiluminescence signals. Because erythrocytes and platelets express the same 2 isoforms of PMCA,\(^{25}\) we chose purified human erythrocyte PMCA as the standard. A linear relation between erythrocyte PMCA mass, 25 to 150 ng, and chemiluminescence signal strength was observed. This linearity was maintained even with varying lengths of exposure (Figure 1). Erythrocyte PMCA, then, is a valid control, and 100 ng was included as a standard on every SDS-PAGE gel. As noted in Figure 1, the strength of the chemiluminescence signal when plotted against sample mass varied in intensity depending on the duration of film exposure. Erythrocyte PMCA could correct for this variable on immunoblots probed with anti-PMCA but not with anti-phosphotyrosine because purified erythrocyte PMCA is not phosphorylated. We chose to normalize phosphotyrosine signals of PMCA against signals of pp125\(^{5\text{AK}}\) present in thrombin-stimulated platelets. Tyrosine-phosphorylated pp125\(^{5\text{AK}}\) reproducibly exhibited a consistent signal-to-mass ratio over the range of 2.5 to 15 \(\mu\)L solubilized platelets (Figure 1). The linearity of this relation verified the suitability of pp125\(^{5\text{AK}}\) for a standard.

Previously, we observed that PMCA was phosphorylated on activation with thrombin on the basis of immunoblotting with anti-phosphotyrosine antibodies.\(^{8}\) With the inclusion of genistein, a tyrosine kinase inhibitor, phosphorylation of PMCA was ablated. This suggested that the signal recognized by the anti-phosphotyrosine antibody PY20-HRPO was in fact phosphotyrosine and not other residues with cross-reactivity to the antibody. To further validate the specificity of PY20-HRPO, we attempted to block the phosphotyrosine signal on immunoblots by competition with exogenous O-phospho-l-tyrosine. As seen in Figure 2, the phosphotyrosine chemiluminescence signal was competitively eliminated with exogenous O-phospho-l-tyrosine, whereas the phospho amino acids phosphoserine and phosphothreonine had no effect. Taken together with previous results, data in Figure 2 confirm the specificity of the PY20-HRPO antibody and its applicability in quantifying phosphotyrosine levels of PMCA.

The results depicted in Figure 3 show that polypeptides other than the 135-kDa native polypeptide are immunoprecipitated and recognized by PMCA and phosphotyrosine antibodies. This raises the possibility that our polyclonal PMCA antibody immunoprecipitates other proteins with a similar molecular weight. However, we have demonstrated by 2-dimensional electrophoretic analyses that the only 135-kDa PMCA is ablated. This suggested that the signal recognized on activation with thrombin on the basis of immunoblotting was due to phosphotyrosine and not other residues.

**Figure 2** confirms the specificity of the PY20-HRPO antibody and its applicability in quantifying phosphotyrosine levels of PMCA.

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In whole platelets and in both platelet plasma membrane preparations and highly purified erythrocyte PMCA samples, we had earlier observed a rapid significant reduction of \(\text{Ca}^{2+}\)-ATPase activity on tyrosine phosphorylation of PMCA.\(^{8}\) Inhibition of pump activity should result in increased cytosolic \(\text{Ca}^{2+}\) levels and enhanced sensitivity to agonists. Platelets from hypertensives often possess these same characteristics. Our model predicts that PMCA from hypertensives would have increased basal levels of tyrosine phosphorylation compared to normotensives. This was in fact observed (Figure 4). Thus, it appears that a factor in hypertension causes increased tyrosine phosphorylation of platelet
PMCA resulting in inhibition of pump activity and increased cytosolic Ca\(^{2+}\). Illumination of the molecular mechanisms involved in platelet PMCA regulation in hypertension may provide valuable information for the design of new clinical and pharmacological treatment modalities for hypertension.

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References

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