Platelet Membrane Calmodulin-Stimulated Calcium–Adenosine Triphosphatase

Altered Activity in Essential Hypertension

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SUMMARY **Platelet free Ca^{2+} concentration has been found to be elevated in essential hypertension and to correlate with blood pressure level. Free cytoplasmic calcium concentration is determined by calcium influx, pooling, and efflux. The present study found a Ca^{2+}-ATPase in platelet membranes that has a high affinity for Ca^{2+} ($K_m \sim 1$ μM), is inhibited by low concentrations of orthovanadate ($K_i \sim 1$ μM), and can be stimulated by calmodulin ($K_m \sim 5$ nM). The absolute increase in calmodulin-stimulated Ca^{2+}-ATPase activity was not different between normotensive and hypertensive subjects; however, the degree of stimulation of Ca^{2+}-ATPase activity at saturating calmodulin concentrations apparently was diminished in calmodulin-deficient membranes from subjects with established essential hypertension (40%) as compared to that in normotensive subjects of similar age (135%; $p < 0.001$). Affinities for calmodulin and Ca^{2+} were comparable between the two groups, while the capacity for Ca^{2+}-ATPase activity (basal and calmodulin-stimulated) was markedly greater (1.5- to 1.8-fold) in both native and calmodulin-deficient membranes from hypertensive subjects. On the other hand, the defective calcium efflux pump activity, as assessed by a decreased degree of calmodulin stimulation, may have contributed to elevated cytoplasmic calcium concentrations and the associated enhanced hormone sensitivity in platelets from essential hypertensive subjects. This may represent an adaptive negative feedback control mechanism to protect the cell against Ca^{2+} overload.**
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KEY WORDS • **hypertension** • **human platelet membranes** • **Ca^{2+}-ATPase** • **calmodulin activation**

PLATELETS from untreated persons with essential hypertension have been shown to have an elevated basal free cytosolic calcium concentration ($[Ca^{2+}]_i$) that is correlated with blood pressure level.[1] Antihypertensive therapy reduces this elevated platelet $[Ca^{2+}]_i$ in hypertensive persons, and this reduction correlates with a decrease in blood pressure[1] and in peripheral vascular resistance.[2] However, the amplified hormone-stimulated elevation in $[Ca^{2+}]_i$ observed in persons with essential hypertension is not corrected by antihypertensive therapy,[3] and this finding suggests a latent, reduced activity of a calcium extrusion mechanism.[4]

Intracellular calcium overload has also been reported for several other cell types from essential hypertensive persons and spontaneously hypertensive rats (SHR).[4–6] Abnormalities in the plasma membranes of vascular smooth muscle,[7, 8] cardiomyocytes,[8] erythrocytes,[9–11] adipocytes,[4, 12] hepatocytes, and synaptosomes[9, 13] have been demonstrated in SHR. These studies have also revealed a decrease in the calcium-binding ability on the inner surface of the plasma membrane, which gives rise to the possibility of a widespread membrane defect in SHR and in human essential hypertension. Studies on cellular calcium handling in erythrocytes[1, 14] and platelets[3] from essential hypertensive subjects and SHR have suggested that the membrane defect relates to calcium efflux mechanisms.

The calmodulin-regulated, ATP-fueled calcium pump in the plasma membrane has been assumed to be involved in maintaining low intracellular calcium concentrations in intact cells[15] by pumping calcium from the cytosolic compartment to the extracellular space against the electrochemical gradient.[15, 16] Defective calmodulin-stimulated calcium transport has been sug-

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gested in erythrocytes of persons with essential hypertension[14] and of SHR[11] and in brain microsomes of SHR.[13] Postnov et al.[14] postulated that decreased plasma membrane calcium pump activity is responsible for cellular calcium overload in hypertension.

To provide evidence for a calmodulin-stimulated, Ca^{2+}-dependent ATPase in the plasma membrane of human platelets, we compared the properties of this enzyme in platelet membranes from normotensive subjects and subjects with essential hypertension. The relative causal or consequential importance of altered Ca^{2+}-ATPase activity in platelets from hypertensive subjects also was assessed.

Subjects and Methods

The study included 18 healthy normotensive volunteers (5 women, 13 men; mean age, 37 years; range, 27–51 years) and 19 subjects with established essential hypertension (7 women, 12 men; mean age, 41 years; range, 19–57 years). Secondary forms of hypertension were ruled out on the basis of normal chest radiographs, urinalysis values, and blood chemogram values as well as normal plasma catecholamine levels and normal renin activity. Hypertensive subjects had not received any antihypertensive therapy for at least 1 month before the study, and all had a casual blood pressure of 160/100 mm Hg or more on several occasions. The normotensive subjects were not taking any medication and had no known family history of hypertension for two generations. Their casual blood pressure was consistently 140/85 mm Hg or less. The subjects' blood pressure was measured after 10 minutes in the seated position using a mercury sphygmomanometer (diastolic pressure, Korotkoff sound V). Informed consent was obtained from all subjects.

Materials

Unless otherwise specified, all reagents were obtained from Sigma Chemical, St. Louis, MO, USA; Merck AG, Darmstadt, West Germany; Fluka AG, Buchs, Switzerland; and Boehringer, Mannheim, West Germany, and were of the highest purity grade available.

Isolation of Platelets and Preparation of Native Membranes

After an overnight fast during which smoking was not allowed, 60 ml of blood was drawn from the supine subjects between 0800 and 0900 by venous puncture, using 0.36% sodium citrate as the anticoagulant. Platelets from each subject were isolated separately using low speed centrifugation and washing procedures as described previously.[17] The contamination of the washed platelet preparation by erythrocytes and leukocytes was less than 0.005% and 0.15% respectively. Platelets were suspended in ice-cold lysis buffer containing 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM Tris-hydrochloride (pH 7.0 at 20°C) and frozen in liquid nitrogen. After thawing at room temperature, the platelet lysate was centrifuged at 39,000 *g* for 10 minutes at 4°C. The pellet was washed twice by resuspension (using a tight fitting Potter homogenizer, Braun, Melsingen, West Germany) in 50 ml of lysis buffer and centrifugation at 39,000 *g* for 10 minutes at 4°C. The membrane pellet (0.3–0.5 mg protein) was finally resuspended in 1.0 ml of storage buffer containing 130 mM KCl, 0.5 mM $MgCl_2$, 1.0 μM $CaCl_2$, 2.0 mM dithiothreitol, and 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane-sulfonic acid (HEPES)-NaOH (pH 7.4) and maintained at 4°C until use.

Preparation of Calmodulin-Deficient Membranes

Membranes were rendered calmodulin-deficient using hypertonic/ethylene glycol bis (β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) procedures essentially as described by Caroni and Carafoli.[18] A 600-μl aliquot of suspended membranes was incubated for 20 minutes at 4°C in 25 ml of a preincubation buffer containing 0.6 M KCl, 4 mM EGTA, and 20 mM HEPES-NaOH (pH 7.4). The membrane suspension was centrifuged at 39,000 *g* for 10 minutes at 4°C. Membranes were then consecutively washed, by suspension and centrifugation, once with preincubation buffer and twice with 20 M HEPES-NaOH (pH 7.4). Calmodulin content of membranes was measured using a NEN-calmodulin radioimmunoassay kit (New England Nuclear GmbH, Dreieich, West Germany). Samples were prepared for radioimmunoassay according to the heating procedures (5 minutes, 90°C) described by the manufacturers. The calmodulin content of native membranes ($n = 6$) was 18.9 ± 2.5 ng/mg protein and was 5.1 ± 2 ng/mg protein for EGTA-treated membranes ($n = 6$).

Assay for Mg^{2+}-ATPase and Calmodulin-Stimulated Ca^{2+}-ATPase Activities in Platelet Membranes

The Ca^{2+}-ATPase and Mg^{2+}-ATPase activities were determined essentially under the conditions described by Niggli et al.[19] Although we have omitted the spectrophotometric coupled enzyme system in favor of colorimetric quantitation, ATPase activities were comparable. Both native and calmodulin-deficient membrane preparations were assayed in triplicate for ATPase activity in a buffer containing 120 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, 20 mM *N*-tris(hydroxymethy-2-aminoethane-sulfonic acid (TES)-NaOH (pH 7.5 at 37°C), and either 0.1 mM $CaCl_2$ or 1 mM EGTA. The incubation mixtures included 50 μM trifluoperazine or varying concentrations (5×10^{-9}–10^{-6} M) of calmodulin, as indicated in the legends to tables and figures. The reaction was initiated by adding 20 μl of membrane suspension (4–10 μg protein) to 380-μl incubation mixtures that had been prewarmed for 5 minutes at 37°C (final incubation volume, 400 μl). The incubation was continued at 37°C for 15 minutes. Under the described assay conditions, ATPase activities were linear with time and proportional to enzyme concentration. Less than 5% of the ATP was hydrolyzed after 15 minutes at 37°C. Reactions were terminated by adding 300 μl of stop buffer (10 parts 3 M sodium acetate, pH

4.1, to 1 part 37% formaldehyde).[20] Inorganic phosphate released from ATP was measured by the colorimetric method of Rathburn and Betlach,[20] except that absorbance was determined at 680 nm instead of 735 nm. Appropriate controls were routinely included to correct for nonenzymatic ATP hydrolysis and for possible inorganic phosphate contamination of membranes and solutions.

The Mg^{2+}-ATPase activity was estimated from assays performed in the absence of $CaCl_2$. The Ca^{2+}-ATPase activity was determined by subtracting activity measured in the absence of $CaCl_2$ from the activity measured in the presence of $CaCl_2$. The ATPase activities are expressed as nanomoles of inorganic phosphate released from ATP per milligram of membrane protein per minute at 37°C. In the case of calmodulin-stimulated Ca^{2+}-ATPase, activities measured in the presence of varying concentrations of calmodulin are also expressed relative to activity measured in the absence of added calmodulin, which was taken as 100%.

Measurement of the Calcium Affinity of Platelet Membrane Ca^{2+}-ATPase

Calcium-magnesium buffers for calibration were made up with calcium-magnesium-EGTA recipes calculated on the basis of apparent dissociation constants for calcium-EGTA and magnesium-EGTA of 318 nM and 14.4 mM, respectively, at pH 7.5 and 37°C. The apparent dissociation constants were calculated from the absolute stability constants and enthalpies tabulated by Bartfai.[21] Free calcium concentrations are given in the text as pCa values, which represent the negative logarithm of the molar free calcium concentration. The Ca^{2+}-sensitivity was determined in native and calmodulin-deficient membranes under the following conditions: 120 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, 2 mM EGTA, 20 mM TES-NaOH (pH 7.5 at 37°C), and in the presence or absence of varying free calcium concentrations (3.3×10^{-9} M–1.01×10^{-4} M). Assays performed on calmodulin-deficient membranes also included 0.1 μM calmodulin. Experimental procedures and calculation of Ca^{2+}-ATPase activities were performed as described in the preceding section. Indices of Ca^{2+} affinity are represented in the text as the negative logarithm of the molar free Ca^{2+} concentration required to obtain half-maximal stimulation of basal Ca^{2+}-ATPase activity (pCa_{50}).

Determination of Protein Concentrations

After solubilization of membrane suspensions in 0.2% Triton X-100 (60 minutes at room temperature), protein concentrations were determined according to the colorimetric method of Bradford[22] using bovine serum albumin as the standard. Standards included the appropriate concentration of Triton X-100 to correct for increased background absorbance.

Statistical Analysis

Statistical analysis was performed using the Student's t test for paired and unpaired data, where n equals number of subjects. Values are given as means ± SEM, and the level of significance was taken as a p value less than 0.05. Curves indicating the activation of Ca^{2+}-ATPase by calmodulin were analyzed individually by the weighted nonlinear regression method of De Lean et al.[23] using a four-parametric logistic function. The K_m represents the concentration of Ca^{2+} or calmodulin needed to half maximally stimulate enzyme activity. The K_i represents the concentration of orthovanadate causing half-maximal inhibition of enzyme activity.

Results

Ca^{2+}-ATPase Activity in Platelet Membranes of Normotensive and Essential Hypertensive Subjects

Table 1 shows the specific activities of Ca^{2+}-ATPase in platelet plasma membranes determined before and after preincubation with EGTA to strip the membranes of endogenous calmodulin. Without this preincubation there was no Ca^{2+}-ATPase response to exogenous calmodulin in either normotensive or hypertensive subjects. With calmodulin-deficient membranes, the degree of activation of Ca^{2+}-ATPase by 0.5 μM calmodulin was lower (1.2- to 1.4-fold stimulation) in hypertensive subjects than in normotensive subjects (2- to 2.7-fold stimulation; $p < 0.001$). A comparison of the specific activities of membrane Ca^{2+}-ATPase indicated that the capacity for Ca^{2+}-ATPase activity was higher in hypertensive than in normotensive subjects ($p < 0.05$; see Table 1). This difference was apparent regardless of whether or not the membranes had been pretreated with EGTA or whether the assays were conducted in the presence or absence of exogenous calmodulin.

TABLE 1. *Effect of Calmodulin on Platelet Membrane (Native and Calmodulin-Deficient) Ca^{2+}-ATPase in Normotensive and Hypertensive Subjects*

	Ca^{2+}-ATPase activity (P_i released, nmol/mg/min)			
	No preincubation		After preincubation	
Subjects	Basal	Plus calmodulin	Basal	Plus calmodulin
Normotensive (n = 13)	27.0 ± 3.8	30.4 ± 4.7	26.4 ± 3.6	55.1 ± 7.2*
Hypertensive (n = 14)	49.3 ± 7.0†	47.5 ± 7.6†	66.2 ± 8.3‡	90.8 ± 10.4*,†

Values are means ± SEM. P_i = inorganic phosphate.
*$p < 0.001$, difference between Ca^{2+}-ATPase activities assayed in the absence or presence of 0.5 μM calmodulin; †$p < 0.05$, ‡$p < 0.001$, compared with normotensive subjects.

It should be noted that basal (no added calmodulin) Ca^{2+}-ATPase activities within each group were not significantly different before and after preincubation (see Table 1). This finding may be attributed in part to the 50% loss of protein following preincubation and subsequent centrifugation procedures (data not shown). Such membrane "purification" was also evidenced by the elevated level of Ca^{2+}-ATPase activity at saturating calmodulin (0.5 μM) concentrations in calmodulin-deficient membranes (see Table 1).

Influence of Trifluoperazine on Platelet Membrane ATPase Activities

In both normotensive and hypertensive subjects the Ca^{2+}-ATPase of untreated membranes was markedly inhibited (40–60%; $p < 0.05$) if assays were performed in the presence of trifluoperazine (Table 2). The Ca^{2+}-ATPase sensitivity to inhibition by trifluoperazine was abolished in membranes that had been preincubated in the presence of EGTA (data not shown). The Mg^{2+}-ATPase activity of untreated platelet membranes from both groups was not influenced by trifluoperazine (see Table 2). A similar Mg^{2+}-ATPase trifluoperazine insensitivity was observed for preincubated membranes (data not shown). While the basal and calmodulin-stimulated Ca^{2+}-ATPase capacities were greater in membranes from essential hypertensive subjects than in those from normotensive subjects (see Tables 1 and 2), the specific activity of membrane Mg^{2+}-ATPase did not differ between the two groups (see Table 2). In addition, calmodulin per se did not influence the activity of the Mg^{2+}-ATPase in either group (data not shown). In most cases (either group) the preincubation and subsequent centrifugation procedures resulted in an increased (1.3- to 2-fold) membrane Mg^{2+}-ATPase–specific activity when compared with that of untreated membranes (data not shown).

Inhibition of Platelet Membrane Ca^{2+}-ATPase by Orthovanadate

Figure 1 shows that platelet membrane Ca^{2+}-ATPase was sensitive to orthovanadate: half-maximal inhibition was observed at approximately 0.8 μM and 1.0 μM orthovanadate for native and calmodulin-deficient membranes, respectively. Although maximal inhibition was observed at 3 μM orthovanadate, about 25% of the total Ca^{2+}-ATPase–specific activity of membrane preparations was retained, even at 100 μM orthovanadate. Neither K_i (orthovanadate) values nor

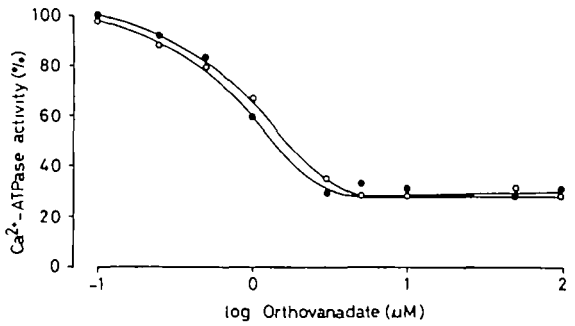

FIGURE 1. *Inhibition of platelet membrane Ca^{2+}-ATPase by orthovanadate. Native (○) and calmodulin-deficient (●) membranes were assayed for Ca^{2+}-ATPase activity in the presence of orthovanadate. Reaction medium contained 120 mM KCl, 5 mM $MgCl_2$, 2 mM EGTA, 1 mM ATP, 20 mM TES-NaOH (pH 7.5 at 37°C) and varying concentrations of orthovanadate, with or without $CaCl_2$ (final free concentration of 2.5 μM). Assays on calmodulin-deficient membranes also contained 0.1 μM calmodulin. The Ca^{2+}-ATPase activity was obtained by subtracting values obtained in the absence of Ca^{2+} and presence of orthovanadate from those obtained in the presence of Ca^{2+} and orthovanadate. One hundred percent activity corresponds to Ca^{2+}-ATPase activity measured in the absence of orthovanadate.*

residual Ca^{2+}-ATPase–specific activities at maximally inhibiting concentrations of orthovanadate were different between normotensive and hypertensive subjects (data not shown).

Ca^{2+} Sensitivity of Platelet Membrane Ca^{2+}-ATPase

The affinity of native membrane Ca^{2+}-ATPase for Ca^{2+} was not different between hypertensive and normotensive subjects (Figure 2). A similar observation was made when calmodulin-deficient membranes were assayed in the presence of saturating concentrations of calmodulin (data not shown). In the absence of added calmodulin, the latter membrane preparations required greater Ca^{2+} concentrations for half-maximal activation of Ca^{2+}-ATPase ($pCa_{50} \sim 6.0$ for both normotensive and hypertensive subjects; data not shown). In accordance with data presented in Tables 1 and 2, the capacity for Ca^{2+}-ATPase activity was higher ($p < 0.05$) in hypertensive subjects (V_{max} = 96 ± 12 nmol P_i/mg/min) than in normotensive subjects (V_{max} = 58

TABLE 2. *Effect of Trifluoperazine on Native Platelet Membrane Ca^{2+}-ATPase and Mg^{2+}-ATPase Activities*

Subjects	Ca^{2+}-ATPase activity (P_i released, nmol/mg/min)		Mg^{2+}-ATPase activity (P_i released, nmol/mg/min)	
	−TFP	+TFP (50 μM)	−TFP	+TFP (50 μM)
Normotensive (n = 13)	27.0 ± 3.8	19.8 ± 2.4*	65.9 ± 8.4	51.3 ± 9.7
Hypertensive (n = 14)	49.3 ± 7.0	30.6 ± 6.3*	56.1 ± 11.7	54.2 ± 22.5

Values are means ± SEM. P_i = inorganic phosphate; TFP = trifluoperazine.
*$p < 0.05$, difference between ATPase activities assayed either in the presence or absence of TFP.

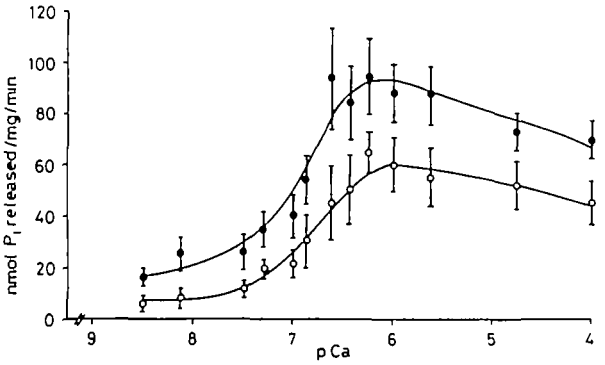

FIGURE 2. *The Ca^{2+}-sensitivity of platelet membrane Ca^{2+}-ATPase: comparison between five normotensive (○) and five essential hypertensive (●) subjects. Native membranes were assayed for Ca^{2+}-ATPase activity in the presence of varying free calcium concentrations (pCa), as described in Methods. Values are means ± SEM. P_i = inorganic phosphate.*

± 9 nmol P_i/mg/min). Inhibition of Ca^{2+}-ATPase activity occurred at Ca^{2+} concentrations greater than 10 μM (see Figure 2). In this respect, the reduced specific activities of native membrane Ca^{2+}-ATPase obtained from experiments in Tables 1 and 2 (as compared to data in Figure 2) may be explained by the use of an inhibitory Ca^{2+} concentration (100 μM) in these assays, whereas V_{max} values obtained from Ca^{2+} activation experiments were derived from activities measured at approximately 1 μM.

Stimulation of Ca^{2+}-ATPase Activity by Calmodulin in Calmodulin-Deficient Platelet Membranes of Normotensive and Essential Hypertensive Subjects

Membranes from the platelets of normotensive and hypertensive subjects were rendered calmodulin-deficient and assayed for calmodulin-stimulated Ca^{2+}-ATPase activity in the presence of increasing concentrations of calmodulin and at a saturating concentration of calcium. The affinity of membrane Ca^{2+}-ATPase for calmodulin was not significantly different between normotensive (4.93 ± 1.20 nM) and hypertensive subjects (5.87 ± 1.16 nM; Figure 3). In terms of specific activity values (nmol P_i released/mg/min) it was apparent that the absolute increase in calmodulin-stimulated Ca^{2+}-ATPase activity was similar (~ 25 nmol P_i released/mg/min) for both groups (see Figure 3). The higher basal value (no added calmodulin) consistently measured in hypertensive subjects was not due to a preferential retention of calmodulin during the described depletion procedures, since the activation profiles and K_m (for calmodulin) values were similar to those obtained for normotensive subjects (see Figure 3). Furthermore, the residual calmodulin content of membranes after depletion was not different between normotensive and hypertensive subjects (6–8 ng/mg protein). Analysis of the data in terms of percent activation by calmodulin indicated that the degree of stimulation of Ca^{2+}-ATPase by calmodulin was

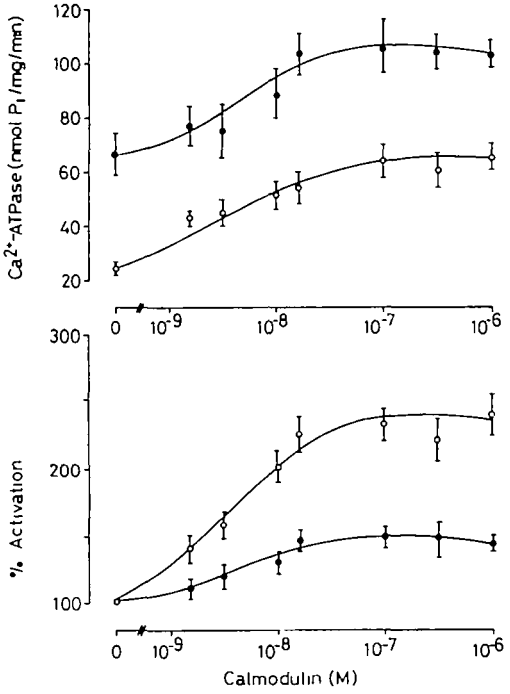

FIGURE 3. *Stimulation of platelet membrane Ca^{2+}-ATPase by calmodulin: comparison between 13 normotensive (○) and 14 essential hypertensive (●) subjects. Calmodulin-deficient membranes were assayed for Ca^{2+}-ATPase activity in the presence of varying concentrations of calmodulin, as described in Methods. Bottom panel. Stimulation by calmodulin is expressed relative to the activity obtained in the absence of calmodulin (taken as 100%). Top panel. These values were derived from measurements of Ca^{2+}-ATPase activities (expressed as P_i released in nmol/mg membrane protein/min). Data are means ± SEM.*

markedly diminished in membranes from hypertensive subjects compared with that from normotensive subjects (see Figure 3). This difference was apparent over the complete range of calmodulin concentrations tested. At saturating concentrations of calmodulin, the V_{max} (% activation) of the calmodulin-stimulated Ca^{2+}-ATPase was significantly lower in membranes from hypertensive subjects (~ 1.4-fold above basal) than in those from normotensive subjects (~ 2.3-fold above basal; $p < 0.001$; see Figure 3 and Table 1).

Discussion

Our results provide evidence that human platelet membranes contain a Ca^{2+}-ATPase that can be stimulated by calmodulin. Stimulation of the Ca^{2+}-ATPase by calmodulin (~ 2.6-fold at saturating calmodulin concentrations) could only be observed in calmodulin-deficient membranes. This finding suggests that the preparations of native (untreated) membranes contain sufficient endogenous calmodulin to maximally stimulate Ca^{2+}-ATPase activity in the presence of calcium alone. The calmodulin dependence of this enzyme is

supported by the observed trifluoperazine inhibition of Ca^{2+}-ATPase activity in native but not in calmodulin-deficient membrane preparations. The observation that removal of endogenous calmodulin does not abolish the activation by calcium alone is in accordance with data on erythrocyte membrane Ca^{2+}-ATPase.[19, 24]

The affinity of the platelet membrane Ca^{2+}-ATPase for calmodulin (~ 5 nM) was similar to that reported for the erythrocyte plasma membrane Ca^{2+}-ATPase pump (~ 2–15 nM).[16, 19, 25] The specific activity of platelet membrane calmodulin-activated Ca^{2+}-ATPase at saturating concentrations of calmodulin and calcium (P_i released, 30–60 nmol/mg/min) was also similar to values reported for erythrocyte membrane calmodulin-stimulated Ca^{2+}-ATPase (P_i released, 30–48 nmol/mg/min).[15, 19, 25] This finding, together with the low contamination of platelet cell preparations by erythrocytes (≤ 0.005%), excludes the possibility that the observed calmodulin-stimulated Ca^{2+}-ATPase activity in platelet membrane preparations arises from contamination by erythrocyte plasma membrane Ca^{2+}-ATPase.

In addition to the calcium extrusion system in external membranes,[26] an alternative calcium pumping system in platelets has been localized to internal membrane structures such as the dense tubular system[26–28] and was reported to be immunochemically similar to the Ca^{2+} pump from skeletal muscle sarcoplasmic reticulum.[29, 30] Dean and co-workers[29, 30] have proposed that the interior membrane Ca^{2+}-ATPase of platelets maintains a low cytoplasmic calcium concentration by pumping calcium into internal compartment(s). This Ca^{2+}-ATPase can be differentiated from the plasma membrane Ca^{2+}-ATPase in that it lacks significant stimulation by calmodulin.[29] The plasma membrane Ca^{2+}-ATPase is inhibited by orthovanadate with a K_i value below 1 μM,[15] whereas the Ca^{2+}-ATPase of internal membrane systems such as the sarcoplasmic reticulum requires about 50 μM orthovanadate for half-maximal inhibition.[31] In our studies, platelet membrane calmodulin-activated Ca^{2+}-ATPase was found to have a K_i for orthovanadate of approximately 1 μM and therefore derives from plasma membrane. Although residual orthovanadate-insensitive Ca^{2+}-ATPase activity indicates the presence of endoplasmic reticulum in membrane preparations, the contribution of this activity to total platelet membrane Ca^{2+}-ATPase activity was similar in normotensive and hypertensive groups.

In subjects with essential hypertension both native and calmodulin-deficient membrane Ca^{2+}-ATPase capacities were higher (~ 2.5 fold) than those in membranes from normotensive subjects. The results relating to orthovanadate-sensitive and orthovanadate-insensitive activity of Ca^{2+}-ATPase activity in platelet membrane preparations suggest that the higher specific enzyme activity observed in hypertensive subjects is unlikely to be due to different relative contributions from endoplasmic reticulum Ca^{2+}-ATPase. Neither the affinity of membrane Ca^{2+}-ATPase for calmodulin nor the absolute increase of calmodulin-activated Ca^{2+}-ATPase activity (P_i released in nmol/mg/min) was significantly different between the two groups; however, when the activation of Ca^{2+}-ATPase in calmodulin-deficient membranes is considered in terms of degree of activation by calmodulin rather than absolute changes in specific activity, responses to calmodulin activation in calmodulin-deficient membranes were blunted in hypertensive subjects (40% activation at V_{max}) compared with those in normotensive subjects (135% activation at V_{max}).

This interpretation is in accordance with data on rates of calcium accumulation in erythrocyte vesicles and brain synaptosomes from hypertensive subjects[14] and SHR.[11, 13] In these studies, a reduced V_{max} for calmodulin-stimulated calcium accumulation, with no alteration in the affinity of the calcium transporting system for calmodulin, was observed in hypertensive states. It was postulated that alterations in calcium pump activity are related to a defect of the calmodulin-ATPase interaction.[11, 14] Our present findings do not clearly demonstrate the existence of this defect. Nevertheless, the interaction of calmodulin and enzymes is modulated by hydrophobic forces,[32] and altered membrane microviscosity in hydrophobic regions has been demonstrated in erythrocytes of SHR[33] and subjects with essential hypertension.[34]

Platelet membrane Ca^{2+}-ATPase had a high affinity constant for Ca^{2+} (K_m = 0.5 μM) when measured in either native or calmodulin-deficient (with added calmodulin) membranes. The Ca^{2+}-affinities were not significantly different between membranes from normotensive and hypertensive subjects. This finding tallies with studies on Ca^{2+} binding and transport in brain microsomes and cardiac sarcolemma from SHR[8, 13] but not with the reported reduction in Ca^{2+} affinity of erythrocyte membrane Ca^{2+} transport in SHR[11] and subjects with essential hypertension.[14]

The increased capacity for platelet membrane Ca^{2+}-ATPase activity in essential hypertension may reflect a mechanism that compensates for a lower degree of stimulation of the calcium transport system by calmodulin. Alternatively, the elevated specific activities in membranes from these subjects may indicate a primed Ca^{2+} transport system that functions to protect against cellular Ca^{2+} overload arising from an increase in stimulatory circulating humoral factors. There is strong evidence to implicate the involvement of the "phosphatidylinositol turnover" phenomenon in regulating platelet cytosolic free Ca^{2+} concentrations and function in response to a variety of substances,[35–37] and Carafoli and colleagues[15, 19, 38] have demonstrated that acidic phospholipids and polyphosphoinositides[16] are powerful activators of purified reconstituted erythrocyte Ca^{2+}-ATPase. Polyphosphoinositides have also been shown to activate rabbit platelet membrane Ca^{2+}-ATPase.[39] This activation is alternative to that induced by calmodulin[15, 19] and has been proposed to play a role in the modulation of the enzyme in the native membrane[38, 40] in addition to calmodulin.

In conclusion, the data presented herein provide evidence for the existence of a calmodulin-activated

Ca^{2+}-ATPase in platelet membranes. Purification and reconstitution of this putative enzyme are necessary to establish unequivocably its identity as a calcium pump mediating calcium efflux. Using either native or calmodulin-deficient membranes, we have shown that the Ca^{2+}-ATPase of platelets from hypertensive subjects is altered with respect to both degree of activation by calmodulin and enzyme capacity. A defective calmodulin-stimulated Ca^{2+}-ATPase membrane calcium extrusion pump may contribute to the observed correlation between free calcium concentration in platelets and blood pressure level[1] and may render platelets (and perhaps vascular smooth muscle cells) more sensitive to hormone activation[3, 41, 42] in persons with essential hypertension. Enhanced enzyme capacity, on the other hand, may be secondary to the development of essential hypertension and represents a potentiated negative feedback mechanism that dampens the participation of platelets in hemostatic and thrombotic processes.

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