Decreased Calcium Pump Adenosine Triphosphatase in Red Blood Cells of Hypertensive Subjects

FRANK F. VINCENZI, CYNTHIA D. MORRIS, LAURA B. KINSEL, MARGARET KENNY, AND DAVID A. MCCARON

SUMMARY Several operationally defined adenosine triphosphatase (ATPase) activities were determined in vitro in red blood cell lysates of normotensive or hypertensive humans: Mg2+-ATPase, Na+,K+-ATPase, and Ca2+ pump ATPase, the latter in the calmodulin-activated and basal states. Basal Ca2+ pump ATPase was defined as the Ca2+-activated ATPase resistant to H3 trifluoperazine. Subjects were part of a double-blind study in which treatment was divided into several phases: baseline (4 weeks), placebo or calcium (1 g elemental calcium/day, 8 weeks), placebo washout (4 weeks), placebo or calcium (1 g elemental calcium/day, 8 weeks). Irrespective of the phase of treatment, the basal Ca2+ pump ATPase activity in red blood cell lysates of 36 hypertensive subjects was significantly less than that in lysates from 18 normotensive subjects. Other ATPase activities did not differ significantly, although all ATPases tended to be decreased in hypertension. The data are consistent with previous reports of altered membrane Ca2+ binding and transport in hypertension, but the precise changes are not elucidated. (Hypertension 8: 1058-1066, 1986)

KEY WORDS • calmodulin • trifluoperazine • plasma membrane

THE role of cation transport across cell membranes has been the subject of intense investigation in animal models of hypertension and in human subjects with the disorder.1-3 A principal focus has been on the identification of abnormalities of Na+ transport involving the Na+,K+-ATPase and Na+ -Li+ countertransport systems.3-5 Putative defects in one or more of the systems regulating the partition of sodium across cell membranes have been postulated to enhance vascular smooth muscle tone through alterations secondarily induced in the intracellular concentration of free Ca2+.6-7 Theoretically, an increase in intracellular Na+ would decrease the inward Na+ gradient, which may drive Ca2+ efflux through Ca2+-Na+ countertransport. Alternatively, increased intracellular Na+ could compete with Ca2+ for efflux.

The human red blood cell (RBC) contains an outwardly directed Ca2+ pump, the activity of which is coupled to phosphorylation and hydrolysis of a 150-kDa membrane-bound, Ca2+-activated, Mg2+-dependent ATPase, referred to as the (Ca2++Mg2+)-ATPase or Ca2+ pump ATPase.1-2 The fundamental observations regarding the Ca2+ pump ATPase are similar to those reported for the widely accepted, but separate and distinct (Na+ + K+ + Mg2+)-ATPase, referred to as Na+,K+-ATPase, which also exists in the RBC plasma membrane and which depends on phosphorylation and hydrolysis of a 100-kDa subunit. The ubiquitous Ca2+ binding protein calmodulin (CaM) activates both the Ca2+ pump ATPase and its associated transport.1-2 In isolated RBC membranes the Ca2+ pump ATPase exhibits two major states of activity: a "basal," or CaM-free state, and a "maximal," or CaM-loaded state.1-2 A variety of pharmacological agents antagonize the effects of CaM.1-2 The first demonstration of this effect was for psychotropic drugs,1-2 and trifluoperazine (TFP) has become a widely accepted tool that is...
used to test dependence of various phenomena on CaM. Drawbacks in the use of TFP as a specific anti-CaM agent have been noted. Nevertheless, in carefully prescribed circumstances, TFP can be used to operationally define the extent of CaM activation of a process being monitored. We have used this approach to define the extent of CaM activation of the Ca\(^{2+}\) pump ATPase in saponin lysates of RBCs in the present study.

Although a variety of genetic, environmental, and life-style factors contribute to essential hypertension, a role for disturbed calcium homeostasis recently has emerged as a possibly important factor in the initiation and maintenance of increased arterial pressure in both experimental animal models and humans. A number of reports have suggested that in both RBCs and vascular smooth muscle cells, Ca\(^{2+}\) transport is abnormal in hypertension.

Because the plasma membrane Ca\(^{2+}\) pump is one of multiple transport processes that contributes to regulation of cellular calcium content, distribution, and compartmentalization, we assessed the activity, both basal and CaM-activated, of the cation pump enzyme in plasma membranes of RBC lysates obtained from subjects with untreated essential hypertension and from subjects with normal blood pressures. The RBCs were collected in the course of a randomized, double-blind, placebo-controlled trial of 1000 mg of elemental calcium given for 8 weeks in the management of mild to moderate hypertension. Samples were collected during the baseline, placebo, and calcium phases of the intervention study. Assessment of Na\(^{+}\),K\(^{+}\)-ATPase and Mg\(^{2+}\)-ATPase activities was also performed in the same RBC membrane lysates.

Subjects and Methods

Specimens

Subjects who provided the RBCs for analysis of membrane-bound ATPase activities were participants in a hypertension intervention trial, results of which have recently been reported. Procedures followed were in accordance with institutional guidelines. Individuals between the ages of 21 and 70 years were drawn from the outpatient clinic population at the Oregon Health Sciences University and from the community. All subjects gave informed consent. Subjects who met any of the following criteria were excluded: secondary hypertension, morbid obesity (\(> 50\%\) over average body weight for height), pregnancy, mental incapacitation, alcohol or drug dependence, congestive heart failure, myocardial infarction within 1 year, angina pectoris, kidney stone within 10 years, marked renal disease by history (or blood urea nitrogen \(> 35\) mg/dl or serum creatinine \(> 1.5\) mg/dl), or a history of cerebrovascular accident. In addition, the presence of any disease known to affect calcium balance (inflammatory bowel disease, diagnosed osteoporosis, active ulcer disease, parathyroid dysfunction, or recent bone fracture) or any medication known to alter calcium balance (thiazide diuretics, steroids, cimetidine, antibiotics, antimicrobials, calcium channel blockers, phenytion, or isoniazid) prohibited a subject from entering the trial. Withdrawal from any antihypertensive agents or from vitamin and mineral supplements was required at least 1 month before entry into the baseline phase of the trial.

During the baseline evaluation period, blood pressure was measured for 4 consecutive weeks. In addition, a medical history, physical examination, and laboratory examination were performed. Subjects were considered hypertensive if their mean arterial pressure (MAP) was consistently greater than 105 mm Hg during the four visits, if their systolic pressure was less than 200 mm Hg, and if their diastolic pressure was less than 115 mm Hg. Normotensive subjects had MAPs consistently less than 105 mm Hg.

Protocol

The trial consisted of two tablets of calcium carbonate (Biocal, Elkhart, IN, USA) taken at bedtime. Because no differences in biochemical or blood pressure responses occurred between Phase 1 and Phase 2, results were grouped over both calcium supplements. Placebo tablets were composed of microcrystalline cellulose and starch and were identical in taste and appearance to the calcium carbonate tablets. Medication was dispensed every 2 weeks. Subjects and all members of the investigative staff were blinded to the subjects' regimen throughout the trial. The ATPase data were obtained from approximately half of the total subjects in the intervention study.

Analytical Methods

Blood pressure, heart rate, and weight were measured every 2 weeks by a nurse-practitioner in a research clinic. A Hawksley random zero sphygmomanometer (Lancing, Sussex, England) was used for measurement of blood pressure after the patient had been supine for 5 minutes and after standing for 2 minutes. Electrocardiograms and routine laboratory analyses — chemistries, complete blood count, serum, multiphasic, urinalysis, automated chemistry, lipid fractions (high density, low density, and very low density lipoproteins, cholesterol), and ATPase analyses of...
RBC lysates — were done at entry and at the end of Phase 1 (Week 8) and Phase 2 (Week 20). At entry and monthly thereafter, timed overnight urine collections for electrolytes, as well as sampling for serum ionized calcium, serum total calcium, and magnesium concentrations were performed. Subjects were requested to fast after midnight on days that routine laboratory analyses were performed and lipid fractions were drawn.

**Specimen Handling for Red Blood Cell-ATPase Analysis**

Blood was drawn into heparinized tubes and stored on wet ice for up to 4 hours before processing. The specimens were centrifuged at 4°C to remove the plasma and Buffy coat. The remaining RBCs were resuspended in an approximately equal volume of holding medium composed of 140 mM KC1, 15 mM NaCl, 10 mM tris(hydroxymethyl)aminomethane (Tris), 10 mM morpholino propane sulfonic acid (MOPS), pH 7.4 at 4°C, and 10 mM glucose. The specimens were transported on wet ice from Portland, Oregon, to Seattle, Washington, generally within 24 hours. The cells were "rejuvenated" with a 1-hour incubation at 37°C in five volumes of recovery medium (5 mM KC1, 70 mM MgCl2, 85 mM sucrose, 10 mM glucose, 10 mM Tris MOPS, pH 7.4 at 37°C). The cells were subsequently washed three times at 4°C with a cold MgCl2-sucrose wash solution (75 mM MgCl2, 85 mM sucrose, 10 mM Tris MOPS buffer, pH 7.4 at 4°C). On the day of the ATPase assay, the cells were re-washed three times at room temperature in 10 volumes of unbuffered isotonic NaCl (0.9%). Blood samples from normotensive and hypertensive subjects were obtained at the same times and were transported, stored, and assayed side by side. Samples were coded, and the blood pressure status of donors was unknown to the laboratory personnel.

**Preparation of Red Blood Cell Lysates**

One volume of washed packed RBCs was allowed to lyse for 15 minutes in nine volumes of isotonic saponin solution (0.1 mg/ml saponin in unbuffered isotonic saline).2 Hematocrits and cell counts (using a Model Z2 Coulter Counter, Coulter Electronics, Hialeah, FL, USA) were determined for the washed packed RBCs to quantify the volume and number of cells used in the saponin lysis.

**Assay of Membrane-Bound ATPase Activities**

Four different operationally defined ATPase activities were determined in duplicate for each blood sample (Table 1). The method of Raess and Vincenzi32 was used with certain modifications, as noted. The incubation media contained the following components in a final volume of 0.5 ml: invariable components, 50 μl of RBC lysate; 18 mM histidine/18 mM imidazole buffer (pH 7.0, 37°C); 3 mM MgCl2; 80 mM NaCl; 15 mM KCl; 0.1 mM ethylene glycol bis(β-aminoethyl ether)-N,N',N'-tetraacetic acid; and variable components, 3 mM adenosine triphosphate (Na2ATP), pH 7.0; 0.2 mM CaCl2; 0.1 mM ouabain; and 0.1 mM TFP. At concentrations greater than 10−4 M, TFP significantly inhibited non-CaM-dependent ATPases in isolated membranes,32 as well as in pilot experiments with RBC lysates (data not shown). Although 10−4 M TFP inhibits the Mg2+ ATPase activity of isolated membranes only slightly,32 we chose to run a TFP-Mg2+ tube. The basal Ca2+ pump ATPase activity as operationally defined in this study was the result of the difference between the amounts of inorganic phosphate (P3) in tubes containing all other ingredients, including TFP, with and without added Ca2+, respectively. Table 1 specifies the contents of each assay tube. Prepared assay tubes (containing all components except lysate and ATP) were stored at −20°C before use.

All assay tubes were incubated at 37°C for 120 minutes in a shaking water bath. The addition of ATP in the appropriate tubes initiated the reaction. The reaction was terminated by the addition of 0.25 ml of 5% (wt/vol) sodium dodecyl sulfate (SDS) solution. The samples were mixed immediately after each addition, transferred to autoanalyzer sampling cups, and immediately analyzed for P3.

**Determination of Inorganic Phosphate**

The P3 determination was performed as previously described32 using a Technicon (Tarrytown, NY, USA) Autoanalyzer Pump I with a 16-channel manifold and a Technicon Sampler II fitted with a 50-1/2 cam. Output of the autoanalyzer was connected to a flow-through cell in a Gilford spectrophotometer (Oberlin, OH, USA). Absorbance at a wavelength of 820 nm (provided by a Beckman Model DU monochromator, Fullerton, CA, USA) was recorded on a Gilford Model 6050 recorder.

Autoanalyzer reagents included 1) acid molybdate solution (130 ml concentrated H2SO4, 25 g ammonium molybdate [NH4]MoO4·4H2O, 2 L to prepare 2 L); 2) SDS, 1% (wt/vol); 3) SDS, 1.667% (wt/vol); 4) ascorbic acid, 9% (wt/vol). All reagents were stored at room temperature.

A phosphate standard curve was determined at the beginning of each day’s determinations. Standard concentrations of P3 ranging from 0 to 400 nmol/ml were employed: 0, 25, 50, 100, 200, 400 nmol/ml. As with analyte for reaction tubes and blanks, the standards contained a final concentration of 1.667% SDS.

As indicated in Table 1, the P3 value for the blank was subtracted from the P3 value obtained from the Mg
TABLE 1. Operationally Defined ATPase Activities in Human Red Cell Lysates

<table>
<thead>
<tr>
<th>Tube</th>
<th>Buffer, Na, K, Mg, EGTA</th>
<th>Lysate</th>
<th>Ca</th>
<th>Ouabain</th>
<th>ATP</th>
<th>TFP</th>
<th>Activities</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>+</td>
<td>+</td>
<td>+/−</td>
<td>+/−</td>
<td>−</td>
<td>+/−</td>
<td>Endogenous P&lt;sub&gt;i&lt;/sub&gt;</td>
</tr>
<tr>
<td>Mg</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>Mg&lt;sup&gt;2+&lt;/sup&gt;-ATPase + blank</td>
</tr>
<tr>
<td>NaK</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>−</td>
<td>+</td>
<td>−</td>
<td>Mg&lt;sup&gt;2+&lt;/sup&gt;-ATPase + Na&lt;sup&gt;+&lt;/sup&gt;, K&lt;sup&gt;+&lt;/sup&gt;-ATPase + blank</td>
</tr>
<tr>
<td>MgTFP</td>
<td>+</td>
<td>+</td>
<td>−</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Mg&lt;sup&gt;2+&lt;/sup&gt;-ATPase (TFP) + blank</td>
</tr>
<tr>
<td>CaTFP</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Mg&lt;sup&gt;2+&lt;/sup&gt;-ATPase (TFP) + basal Ca&lt;sup&gt;2+&lt;/sup&gt;-ATPase (TFP) + blank</td>
</tr>
<tr>
<td>Ca</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>Ca&lt;sup&gt;2+&lt;/sup&gt;-ATPase + blank</td>
</tr>
</tbody>
</table>

Addition of material is indicated by +, no addition by −. See Methods for details. EGTA = ethylene glycol bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid; TFP = trifluoperazine; P<sub>i</sub> = inorganic phosphate.

The ATPase data were obtained on 91 blood samples from 54 subjects over the course of 12 months. This represented 61 samples from 36 hypertensive subjects and 30 samples from 18 normotensive subjects. Baseline samples were collected from 29 hypertensive and 11 normotensive subjects. The blood pressure and demographic profile of these two groups of subjects is summarized in Table 2. These data do not differ significantly from the profile of the study from which these subjects were drawn. Blood pressure responses were reported in that article by McCarron and Morris. Qualitatively, the ATPase results were in agreement with activities generally described for isolated RBC membrane preparations. This difference may be related to the presence of cytoplasmic enzymes in RBC lysates, which contribute to the production of P<sub>i</sub> from ATP under the Mg<sup>2+</sup>-ATPase assay conditions. The amount of P<sub>i</sub> appearing in the blank tubes (no added ATP) was minimal compared with that measured in the ATPase tubes. In any event, with the exception of the Mg<sup>2+</sup>-ATPase, all other ATPase activities are defined by subtraction, which would cancel out any such contribution of high Mg<sup>2+</sup>-ATPase.

The average value for each of the operationally defined ATPases was numerically lower in hypertensive than in normotensive samples. With the exception of basal Ca<sup>2+</sup> pump ATPase, however, no difference in ATPase activity between normotensive and hypertensive subjects achieved statistical significance (Table 3). Without regard to treatment (baseline, calcium, or placebo), the basal Ca<sup>2+</sup> pump ATPase activity was significantly less in the 61 blood samples from hypertensive subjects as compared with the 30 blood samples from the normotensive subjects (5.94 ± 1.80 vs 7.13 ± 1.86 pmol P<sub>i</sub>·min<sup>−1</sup>·10<sup>6</sup> cells<sup>−1</sup>; p<0.01). As would be predicted, the ratio of maximal to basal Ca<sup>2+</sup> pump ATPase activity also differed significantly (p<0.02). In the 61 blood samples from

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Hypertensive (n = 36)</th>
<th>Normotensive (n = 18)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Systolic pressure (mm Hg)</td>
<td>151 ± 11</td>
<td>121 ± 13</td>
</tr>
<tr>
<td>Standing</td>
<td>140 ± 11</td>
<td>111 ± 13</td>
</tr>
<tr>
<td>Diastolic pressure (mm Hg)</td>
<td>94 ± 5</td>
<td>74 ± 8</td>
</tr>
<tr>
<td>Supine</td>
<td>92 ± 7</td>
<td>74 ± 8</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>54.3 ± 9.9</td>
<td>46.3 ± 10.1</td>
</tr>
<tr>
<td>Sex (M/F)</td>
<td>23/13</td>
<td>6/12</td>
</tr>
<tr>
<td>Race (W/B)</td>
<td>35/1</td>
<td>17/1</td>
</tr>
</tbody>
</table>

Blood pressure and age values are means ± SEM.
TABLE 3. ATPase Activities in Red Blood Cell Lysates: Comparison of Normotensive and Hypertensive Samples

<table>
<thead>
<tr>
<th>ATPase</th>
<th>Activity (pmol P$_{i}$ \cdot min$^{-1}$ \cdot 10$^6$ cells$^{-1}$)</th>
<th>No. of samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg$^{2+}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>13.69 ± 2.56</td>
<td>30</td>
</tr>
<tr>
<td>Hypertensive</td>
<td>13.19 ± 2.83*</td>
<td>61</td>
</tr>
<tr>
<td>Na$^+$, K$^+$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>5.47 ± 1.72</td>
<td>30</td>
</tr>
<tr>
<td>Hypertensive</td>
<td>4.90 ± 1.75†</td>
<td>61</td>
</tr>
<tr>
<td>Basal Ca$^{2+}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>7.13 ± 1.86</td>
<td>30</td>
</tr>
<tr>
<td>Hypertensive</td>
<td>5.94 ± 1.80‡</td>
<td>61</td>
</tr>
<tr>
<td>Maximal Ca$^{2+}$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>35.57 ± 5.60</td>
<td>30</td>
</tr>
<tr>
<td>Hypertensive</td>
<td>33.96 ± 6.51</td>
<td>61</td>
</tr>
</tbody>
</table>

Values are means ± SD. P$_{i}$ = inorganic phosphate. *p = 0.573, tp = 0.140, tp = 0.004, tp = 0.249, compared with normotensive values (unpaired t test).

hypertensive subjects the activation ratio was 5.96, while in the 30 normotensive samples the activation ratio was 5.18. The ATPase activities for only the baseline samples are presented in Table 4. As with the pooled data at baseline, all ATPase activities of hypertensive samples tended to be lower and basal Ca$^{2+}$ pump ATPase activity was significantly less than that in normotensive samples (p = 0.001).

Figure 1 is a plot of basal Ca$^{2+}$ pump ATPase activity as compared with baseline MAP in the same 29 hypertensive and 11 normotensive subjects presented in Table 4. Although the analysis concentrated on two groups, normotensive and hypertensive subjects, blood pressure was not skewed and represented a continuous distribution. A significant inverse relationship (r = -0.40, p = 0.005) was observed between ATPase activity and MAP in these 40 samples. Similar values were obtained when the basal Ca$^{2+}$ pump ATPase activity was correlated with both systolic (r = -0.33, p = 0.017) and diastolic (r = -0.39, p = 0.006) pressure, respectively. No significant relationship was found to exist between any of the other operationally defined ATPase activities and blood pressure whether systolic, diastolic, or MAPs were examined. Respective relationships between mean MAP and Mg$^{2+}$-ATPase (r = -0.07) and Na$^+$,K$^+$-ATPase activities (r = 0.09) were not significant.

Table 5 summarizes the effect of 8 weeks of 1000 mg of calcium given daily on the respective ATPase activities. Nineteen subjects provided samples during both the baseline and calcium treatment phases (12 hypertensive and 7 normotensive subjects). Their values served as the basis for evaluation of the possible effect of modifying calcium homeostasis on the four ATPase activities. Maximal Ca$^{2+}$ pump ATPase activity was unaffected, whereas Mg$^{2+}$-ATPase activity was increased during calcium treatment (p = 0.043), as was Na$^+$,K$^+$-ATPase activity, but not significantly (p = 0.063). By contrast, basal Ca$^{2+}$ pump ATPase activity tended to decrease with administration of calcium, though the change was not statistically significant (p = 0.096). Within hypertensive (n = 12) or normo-

Figure 1. Scatterplot of baseline blood pressure and basal Ca$^{2+}$ pump ATPase data. Basal Ca$^{2+}$ pump ATPase data (expressed as pmoles of inorganic phosphate released from ATP per minute per million cells) are plotted as a function of the standing mean arterial blood pressure (expressed in mm Hg and measured on the day blood was drawn) of 40 subjects: 29 hypertensive (■) and 11 normotensive (□) subjects. Crosses indicate the respective mean values ± SD, as indicated in Table 5. The data demonstrate a significant inverse relationship (r = -0.40, p = 0.005) between mean arterial blood pressure and the basal Ca$^{2+}$ pump ATPase activity of red blood cell lysates in these subjects. No significant relationship between blood pressure and any other of the other operationally defined ATPase activities was observed.
TABLE 5. ATPase Activities in Red Blood Cell Lysates: Effect of Calcium Treatment

<table>
<thead>
<tr>
<th>ATPase</th>
<th>No. of subjects</th>
<th>Activity (pmol Pi·min⁻¹·10⁶ cells⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mg²⁺ Baseline</td>
<td>19</td>
<td>11.75 ± 0.83</td>
</tr>
<tr>
<td>Calcium Baseline</td>
<td>19</td>
<td>13.54 ± 0.83*</td>
</tr>
<tr>
<td>Na⁺, K⁺ Baseline</td>
<td>19</td>
<td>4.69 ± 0.44</td>
</tr>
<tr>
<td>Calcium</td>
<td>19</td>
<td>5.56 ± 0.44*</td>
</tr>
<tr>
<td>Basal Ca²⁺ Baseline</td>
<td>19</td>
<td>6.94 ± 0.44</td>
</tr>
<tr>
<td>Calcium</td>
<td>19</td>
<td>6.17 ± 0.44**</td>
</tr>
<tr>
<td>Maximal Ca²⁺ Baseline</td>
<td>19</td>
<td>35.87 ± 1.60</td>
</tr>
<tr>
<td>Calcium</td>
<td>19</td>
<td>35.39 ± 1.60*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Data based on two samples from each subject (7 normotensive and 12 hypertensive subjects), one baseline and one calcium treatment value. Pi = inorganic phosphate.

*tp = 0.043, †tp = 0.063, ‡tp = 0.096, §tp = 0.765, compared with baseline values (paired t test).

motensive (n = 7) groups, none of the ATPase activities differed significantly after treatment with calcium when compared with baseline values. The small number of samples in each group may have precluded identification of differences, if such exist.

Oral calcium supplementation had no significant effect on serum Ca²⁺. Total serum calcium averaged 5.2 to 5.3 mEq/L, irrespective of treatment or blood pressure status. On the other hand, the average ionized Ca²⁺ level was higher in hypertensive subjects treated with calcium (2.14 ± 0.27 mM) than with placebo (1.97 ± 0.26 mM). In normotensive subjects ionized calcium concentration did not change significantly during calcium administration (2.14 ± 0.29 mM) compared with placebo treatment (2.06 ± 0.27 mM).

Of the 19 subjects whose ATPase activities were measured during baseline and during calcium treatment, 37% responded with at least a 10 mm Hg decrease in MAP. This is typical of the overall response of the blood pressure study from which the subjects were drawn. Baseline Na⁺, K⁺-ATPase activity (4.3 ± 0.9 pmol Pi·min⁻¹·10⁶ cells⁻¹) was significantly less (p<0.05) in those subjects whose blood pressure failed to decrease at least 10 mm Hg during the period of observation (5.5 ± 2.2 pmol Pi·min⁻¹·10⁶ cells⁻¹). No other ATPase activity was associated with a blood pressure response. In those subjects who did respond to calcium, and for whom there were measurements of ATPase activity during both the baseline and calcium phases, there were no significant changes. In nonresponders, Na⁺, K⁺-ATPase activity increased significantly (p = 0.003) after calcium treatment. There were significant changes in Na⁺, K⁺-ATPase and maximal Ca²⁺ pump ATPase activities with calcium treatment (p = 0.04 and p = 0.04, respectively); for both, activities increased overall in nonresponders but decreased in responders. The small sample size, however, limits the power of absolute conclusions of no difference between groups.

Comparison of ATPase data with subject demographic and clinical data showed that the ATPases we monitored were not significantly different according to the family history for hypertension, sex, age, weight (obese or nonobese), or possible diabetic or menopausal status of the subject. Partial correlation analysis controlling for age, sex, and/or weight did not suggest that differences were due to differences in the sex ratio of the hypertensive and normotensive groups.

Discussion

The present observations on the Ca²⁺ pump ATPase activity in RBCs of subjects with essential hypertension may be taken to suggest that the basal activity of the membrane-associated pump is altered. The implications of this finding for the pathogenesis of increased arterial pressure may be important in at least a subset of hypertensive persons. The Ca²⁺ pump ATPase is the only mechanism by which RBCs maintain low intracellular Ca²⁺ concentration and an important means by which other cells accomplish this. Maintenance of low intracellular Ca²⁺ concentration is critical for normal cell function and viability. An approximately 20% decrease in basal activity of the Ca²⁺ pump ATPase in the RBCs of hypertensive subjects may reflect a deficit in the ability of certain cells to maintain optimally low intracellular Ca²⁺.

Decreased Ca²⁺ pump ATPase activity in our hypertensive subjects is consistent with a variety of other observations of RBCs of experimental animal models of hypertension and in humans with essential hypertension. These reports noted reduced binding of Ca²⁺ to membrane of RBCs in association with an increase in membrane permeability to Ca²⁺ and reduced activity of the Ca²⁺ pump ATPase. The latter previously has been identified in rat RBCs, although one group found an apparently opposite effect in rat cells. The former two abnormalities have been characterized in human and in rat RBCs. Results of our study suggest that in humans membrane-associated defects include decreased RBC Ca²⁺ pump ATPase activity.

Neither the alteration we have observed in Ca²⁺ pump ATPase activity of RBC lysates of humans with hypertension nor that which others reported in spontaneously hypertensive rats, specifically identifies the abnormality. Based on previous characterizations of this ATPase in humans and animals, several possibilities can be addressed. Our subjects exhibited an apparent defect in TFP-resistant (presumably representative of basal, or CaM-free) Ca²⁺ pump ATPase activity and a less than significantly decreased maximal or CaM-activated activity (absence of TFP). If TFP is assumed to be a relatively selective antagonist of CaM under these conditions, then any predicted decrease in out-
wardly directed Ca\(^{2+}\) transport would not be the result of a defect in CaM. This supposition is consistent with the report of normal content and distribution of CaM in the RBCs of human hypertensive subjects. As hypothesized by Postnov et al., the primary defect is related to the Ca\(^{2+}\) that activates the ATPase in the absence of CaM. An alteration in membrane binding kinetics of Ca\(^{2+}\) has been suggested, based on the reduced amount of exchangeable Ca\(^{2+}\) available from isolated membranes of animals and humans with hypertension, although total calcium of the membranes was reported to be increased. Based on the normal or near-normal CaM-activated Ca\(^{2+}\) pump ATPase activity, we suggest that there is no major deficit in the total number of ATPase sites per RBC. Further studies are necessary to address the question more directly. We are left to conclude that some subtle difference in the Ca\(^{2+}\) pump ATPase, or in its modulating environment, exists and results in decreased basal activity, but less decrease in CaM-activated activity.

The fact that other operationally defined ATPase activities were also decreased, albeit not significantly, may speak to a generalized membrane alteration.

There are potential problems in the measurement of basal Ca\(^{2+}\) pump ATPase as performed in this study. It was assumed that there is a sufficient excess of endogenous CaM in RBC lysates to maximally activate the Ca\(^{2+}\) pump ATPase. This assumption is based on the approximately micromolar concentration of CaM in normal human RBCs and the approximately 3 nM apparent \(K_d\) for CaM activation of the Ca\(^{2+}\) pump ATPase and transport. Another assumption is that 10\(^{-4}\) M TFP completely antagonized CaM in RBC lysates. Under similar conditions, 10\(^{-4}\) M TFP completely antagonized CaM added to isolated RBC membranes. It is conceivable that different amounts or effectiveness of CaM in normotensive versus hypertensive RBC lysates caused a difference in the basal Ca\(^{2+}\) pump ATPase. Postnov et al. found no difference in the CaM content or cytoplasmic and membrane distribution between normotensive and hypertensive subjects. Nevertheless, it is possible that in the present study TFP distributed differently in the complicated lysate mixture of the two types of RBCs. It is difficult to conceive that these possible complications differ in a specific fashion in hypertension. More complete TFP dose-response data are certainly needed in the future.

The difference observed we could conceivably represent artifactual effects of the assay method or an alteration in the RBC membrane induced by chronic exposure to elevated arterial pressure, for example. A nonspecific alteration in the RBC membrane might account for the general tendency toward decreased ATPase activities in RBCs from hypertensive subjects (even if not statistically significant with our sample size). Differential lysis by saponin of RBCs from hypertensive and normotensive subjects seems unlikely to account for the data. Not all of the ATPases monitored were decreased by a similar percentage, as would be expected if lysis (or resealing) of the cells differed between hypertensive and normotensive samples.

Limited discriminatory power of studies with a small sample size must always be considered with the possibility that an important difference will not be detected because of a type II, or beta, error. Using the baseline size of 29 hypertensive and 11 normotensive samples, we have calculated that a difference of one standard deviation would be detected with a power of 30%. When smaller subgroups are considered, the discriminatory power becomes less and the chance of a type II error is greater. The Mg\(^{2+}\)-ATPase and Na\(^{+}\)-K\(^{-}\)-ATPase activities were numerically similar at baseline; thus, it seems unlikely that a type II error occurred. This in no way affects the significant difference in basal Ca\(^{2+}\) pump ATPase between hypertensive and normotensive subjects.

To our knowledge, the influence of modifying calcium balance on Ca\(^{2+}\) pump ATPase activity has not been evaluated previously in tissue from laboratory animals or humans. Although it did not reach statistical significance, the tendency was for Ca\(^{2+}\) pump ATPase activity to decline with calcium treatment in both the normotensive and hypertensive subjects. In any event, calcium treatment did not appear to "correct" (i.e., increase) Ca\(^{2+}\) pump ATPase activity. Of course, if decreased basal Ca\(^{2+}\) pump ATPase were determined genetically, then it would not be expected to change, even with effective therapy. More work with larger numbers of subjects is needed. We suggest that with calcium treatment, additional (extracellular) membrane-bound calcium reduces Ca\(^{2+}\) influx and reduces the cellular dependence on the outwardly directed pump. This interpretation is consistent with reports of membrane-bound calcium acting in a variety of cell types to decrease Ca\(^{2+}\) fluxes. Inhibition by Ca\(^{2+}\) of its own flux is, in part, a CaM-dependent process according to some reports. Such an effect of Ca\(^{2+}\) is also consistent with the observation from in vitro studies of vascular tissue, which have demonstrated a membrane stabilization effect of extracellular calcium \(24-42\) of course, in cells other than the RBC other mechanisms that regulate cytosolic Ca\(^{2+}\) probably reduce their dependence on the Ca\(^{2+}\) pump ATPase.

In human subjects with essential hypertension and in spontaneously hypertensive rats, membrane-related alterations in calcium metabolism have been associated with spontaneous decreases in extracellular Ca\(^{2+}\). A defect in outwardly directed Ca\(^{2+}\) transport across the cell membrane could account for the seemingly paradoxical increase in cytosolic Ca\(^{2+}\) that theoretically underlies the increased vascular contractility in essential hypertension. In platelets, a recent report demonstrated an increase in the intracellular concentration of Ca\(^{2+}\) concurrent with a decrease in the extracellular concentration of the cation. Platelets and RBCs are not the only cells with identifiable defects in Ca\(^{2+}\) transport associated with increased arterial pressure. Active intestinal transport of Ca\(^{2+}\), a Ca\(^{2+}\) pump ATPase-dependent process, is decreased in spontaneously hypertensive rats. Renal reabsorption is also diminished, resulting in increased excretion of Ca\(^{2+}\). In humans no specific comparable defect in...
either the intestinal or renal transport of Ca\(^{2+}\) has been reported. Nevertheless, essential hypertension is associated with increased renal excretion of Ca\(^{2+}\) and its paradoxical response in the face of diminished dietary intake of the element.

Extrapolation of our findings to vascular smooth muscle must be interpreted cautiously. The presence of the Ca\(^{2+}\) pump ATPase has been demonstrated in smooth muscle, but the relationship of that pump and its regulation to the RBC is not well established. Contraction of smooth muscle is dependent on Ca\(^{2+}\) and its interaction with CaM. Decreased activity of the Ca\(^{2+}\) pump, if it existed in the vascular smooth muscle, could be one mechanism by which sustained increases in intracellular Ca\(^{2+}\) would occur with consequent increases in vascular tone, peripheral resistance, and blood pressure.

References
7. DeWardeeher HE, MacGregor GA. Dahl’s hypothesis that a saluretic substance may be responsible for a sustained rise in arterial pressure: its possible role in essential hypertension. Kidney Int 1980;18:1–9
15. Normal JA, Drummond AH, Moser P. Inhibition of calcium-dependent regulator-stimulated phosphodiesterase activity by neuroleptic drugs is unrelated to their clinical efficacy. Mol Pharmacol 1979;16:1089–1094
30. Raess BU, Vincenzi FF. Calmodulin activation of red blood cell (Ca\(^{2+}\) + Mg\(^{2+}\))-ATPase and its antagonism by phenothiazines. Mol Pharmacol 1980;18:253–258
Decreased calcium pump adenosine triphosphatase in red blood cells of hypertensive subjects.

F F Vincenzi, C D Morris, L B Kinsel, M Kenny and D A McCarron

Hypertension. 1986;8:1058-1066
doi: 10.1161/01.HYP.8.11.1058

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1986 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/8/11/1058