Hypertension Does Not Affect Intracellular Calcium Uptake in Human Platelets

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The relationship between the Ca2+ transport of platelet endoplasmic reticulum and hypertension was analyzed in 17 untreated patients exhibiting various degrees of hypertension. Each patient underwent a 24-h recording of ambulatory blood pressure. Platelets from patients were permeabilized with saponin and the rate of ATP-driven thapsigargin-sensitive Ca2+ uptake determined using the fluorescent Ca2+ indicator fluo-3. No relationship between blood pressure (systolic, diastolic, day, night) and the rate of Ca2+ uptake into the saccoplasmonic reticulum of platelets was found. A weak but insignificant correlation between Ca2+ uptake and the heart rate was noted. Therefore, the increase in cytosolic Ca2+ of platelets in hypertension may not be due to changes of the activity of Ca2+ uptake into the sacroplasmic reticulum. Am J Hypertens 1996;9:136-142

KEY WORDS: Platelets, calcium transport, hypertension.

Despite extensive research, the relationship of calcium and arterial hypertension remains unclear. Intracellular Ca2+ is a major determinant of basal myogenic tone of vascular muscle. Therefore, an increase of cytosolic Ca2+ in arterial smooth muscle leads to an increase in peripheral resistance characteristic for hypertension. The systemic hormonal signals associated with hypertension influence endothelial cell, vascular muscle cells and blood cells. Erne et al.1 have documented an elevation of platelet cytosolic free Ca2+ in hypertension and a close correlation of the Ca2+ levels in platelets and blood pressure in untreated hypertensive patients. Furthermore, both platelet Ca2+ and vasoconstriction were normalized by antihypertensive treatment, indicating a close relationship between platolet Ca2+ dynamics and circulating hormonal factors. Cytosolic Ca2+ overload has been described in a variety of cells in both hypertension and pressure-induced cardiac dysfunction.6

Whereas the relationship of platelet cytosolic Ca2+ and vasoconstriction has been clearly established, the molecular basis of elevated Ca2+ levels remains unclear. The rapid rise in cytosolic Ca2+ observed upon exposure of platelets to humoral factors from hypertensives indicates that systemic hormonal factors influence platelet cytosolic Ca2+ and that the changes in platelet cytosolic Ca2+ are effected by a rapid mechanism, i.e., Ca2+ influx across the plasma membrane or intracellular Ca2+ mobilization. The reversal of Ca2+ levels by means of Ca2+ antagonists has led to the hypothesis that Ca2+ influx is responsible for elevated platelet Ca2+. However, the enhanced hormone-induced elevation of platelet cytosolic Ca2+ is not corrected by antihypertensive therapy, demonstrating that the fundamental defect in Ca2+ dynamics has not been restored. Moreover, voltage-operated Ca2+ channels do not mediate agonist-evoked Ca2+ entry in platelets. Besides voltage-operated Ca2+ channels, a variety of other mechanisms contribute to the Ca2+ dynamics of platelets; agonist-mediated Ca2+ in-
fluoride; ligand-gated $\text{Ca}^{2+}$ channels; $\text{Ca}^{2+}$-ATPase pumps present in both the plasma membrane and membranes of endoplasmic reticulum; $\text{Na}^+$/Ca$^{2+}$ exchanger; inositol 1,4,5-trisphosphate-induced $\text{Ca}^{2+}$ release from intracellular $\text{Ca}^{2+}$ stores; and a series of cytosolic and endoplasmic reticulum $\text{Ca}^{2+}$ binding proteins. Resink et al. have documented a reduction of the calmodulin-stimulated Ca$^{2+}$-ATPase activity and an increase in basal Ca$^{2+}$-ATPase activity in platelets from hypertensive compared to normal subjects, suggesting a reduction in plasma membrane $\text{Ca}^{2+}$ extrusion activity and an activation of the sarcoplasmic reticulum $\text{Ca}^{2+}$ pump in hypertension. Other studies also have described a decrease in $\text{Ca}^{2+}$-ATPase activity in hypertension. In the present study we have determined the rate of $\text{Ca}^{2+}$ uptake in permeabilized platelets from 17 untreated subjects with various degrees of hypertension in order to test if alteration of the sarcoplasmic reticulum $\text{Ca}^{2+}$-ATPase pump activity contributes to elevated platelet cytosolic $\text{Ca}^{2+}$ in hypertension.

**MATERIALS AND METHODS**

**Reagents** Heparin, pentosan polysulfate, saponin, and hexokinase were obtained from Sigma (St. Louis, MO). Fluoro-3 pentapotassium salt was from Molecular Probes (Eugene, OR). Fluoro-3 was stored in 50 $\mu$L aliquots at a concentration of 1 mmol/L in dimethylsulfoxide at $-70^\circ$C. Ionomycin was from Calbiochem (San Diego, CA), thapsigargin from LC Services Corporation. Aminoethyl Biogel P-2 was purchased from Bio-Rad (Richmond, CA), and Sepharose CL-2B from Pharmacia (Uppsala, Sweden).

**Buffer Solutions** All experiments were performed in 20 mmol/L HEPES buffer containing 155 mmol/L KCl, 5 mmol/L NaCl, 2 mmol/L MgCl$_2$, 2 mmol/L 1,4-dithioerythritol, and 0.3 mmol/L phenylmethanesulfonyl fluoride at pH 7.2, which was denoted buffer A. All solutions were depleted of $\text{Ca}^{2+}$ by means of chromatography on EDTA-polyacrylamide as described previously. Residual $\text{Ca}^{2+}$ concentrations of solutions following treatment with EDTA-polyacrylamide were measured as described.

**Patients** We analyzed platelets of 17 newly diagnosed hypertensive patients who were referred to the Division of Cardiology of the Kantonsspital Lucerne. None of the patients had received antihypertensive drugs before. Patients underwent a 12-lead electrocardiogram, a bicycle exercise test, and a 24-h recording of ambulatory blood pressure (HCR Blood Pressure, Disetronic, Burgdorf, Switzerland). Table 1 summarizes the clinical data and results of ambulatory 24-h blood pressure measurements.

**Preparation of Platelets** Platelets were prepared from platelet-rich plasma by gel filtration using Sepharose CL-2B (Pharmacia) and adjusted to 108 cells/mL. Platelets were permeabilized by addition of 20 $\mu$g saponin/108 cells. Selective permeabilization was confirmed by measuring the release of fluoro-3 from cells loaded with fluoro-3 acetoxymethyl ester, and by monitoring the release of $\text{Ca}^{2+}$ from internal membranes in permeabilized cells following addition of ionomycin. Platelets were used within 6 h of blood withdrawal.

**Measurement of $\text{Ca}^{2+}$** $\text{Ca}^{2+}$ concentrations were measured by means of the fluorescent $\text{Ca}^{2+}$ indicator fluoro-3. The indicator was added at a final concentration of 1 mmol/L to 1 mL of a suspension of platelets (108 cells/mL) placed into a 1-cm quartz cuvette. The suspension was continuously stirred using a magnetic stirrer. Fluorescence was measured by means of a Perkin-Elmer (Oakbrook, IL) 650-10s fluorimeter, interfaced via an analog/digital converter (PC-28, Instrument AG, Zurich, Switzerland) to a personal computer. Fluorescence of fluoro-3 was excited at 505 nm and observed at 530 nm. ATP (1 mmol/L) and saponin (20 $\mu$g) were added to the cell suspension to activate $\text{Ca}^{2+}$ uptake of intracellular $\text{Ca}^{2+}$ stores. ATP at 1 mmol/L was found to saturate thapsigargin-sensitive $\text{Ca}^{2+}$ uptake in platelets from many subjects of widely different degrees of hypertension (not shown).

$\text{Ca}^{2+}$ concentrations were calculated from the fluorescence signal using standard titrations of fluoro-3 with $\text{Ca}^{2+}$: Binding of $\text{Ca}^{2+}$ to fluoro-3 was expressed as signal versus concentration of added $\text{Ca}^{2+}$, and evaluated as described. From the dissociation constant ($K_d$) of the $\text{Ca}^{2+}$-fluoro-3 complex, which was typically 900 nmol/L in buffer A the fluorescence signal of $\text{Ca}^{2+}$-free indicator $f_f$ and that of the $\text{Ca}^{2+}$-fluoro-3 indicator complex $f_f$ was calculated as follow:

$$\left[\text{Ca}^{2+}\right]_{\text{free}} = K_d \cdot (f_{\text{obs}} - f_f)/(f_f - f_{\text{obs}})$$

where $f_{\text{obs}}$ represents the observed fluorescence signal. $K_d$ was determined from titrations of fluoro-3 with $\text{Ca}^{2+}$ (final concentrations between 0 and 5 mmol/L; evalu-
The Ca\(^{2+}\) flux due to the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase pump, hence termed Ca\(^{2+}\) uptake, was determined from the difference of the overall Ca\(^{2+}\) flux before and after addition of thapsigargin (200 nmol/L), a specific inhibitor of the sarcoplasmic reticulum Ca\(^{2+}\)-ATPase. In the presence of thapsigargin, a spontaneous Ca\(^{2+}\) flux out of the sarcoplasmic reticulum, the so-called leak, was observed and modeled by an exponential decay toward an equilibrium value (Figure 1B):

\[
[Ca^{2+}] = a \cdot \exp(-k \cdot t) + [Ca^{2+}]_{eq}
\]

(2)

where \(a\) and \(k\) are arbitrary constants, and \([Ca^{2+}]_{eq}\) the equilibrium concentration of Ca\(^{2+}\). Thus, the rate of Ca\(^{2+}\) leak at a particular Ca\(^{2+}\) concentration is given by

\[
d[Ca^{2+}] / dt = -k \cdot a \cdot \exp(-k \cdot t) = -k \cdot ([Ca^{2+}] - [Ca^{2+}]_{eq})
\]

(3)

The rate of Ca\(^{2+}\) uptake is the difference between the slope of the Ca\(^{2+}\) transient at a particular Ca\(^{2+}\) concentration before addition of thapsigargin (overall Ca\(^{2+}\) flux, Figure 1), and the rate of Ca\(^{2+}\) leak at that Ca\(^{2+}\) concentration (equation 3).

**Data Analysis** Data evaluation and nonlinear least-squares fits were performed using standard procedures. Results are expressed as means ± SD. Individual Ca\(^{2+}\) uptake and leak rate determinations were averaged and SD values calculated if three or more individual values were measured. Linear least-squares regressions assumed uniform and unity SD values of the individual data points were used to analyze putative relationships between binding data and clinical parameters. Percentile points (P) were determined using the two-tailed t distribution.

**RESULTS**

The evaluation of fluorescence data and a typical Ca\(^{2+}\) transient used to determine Ca\(^{2+}\) uptake rates, i.e. the thapsigargin-sensitive Ca\(^{2+}\) flux, are shown in Figure 1.

**FIGURE 1.** Conversion of the fluorescence signal of fluo-3 (A) to Ca\(^{2+}\) concentrations (B). The arrows indicate (from left to right) the addition of saponin (120 mg/mL) and thapsigargin (200 nmol/L) to a suspension of platelets (108 cells/mL) containing ATP (1 mmol/L). The initial increase in fluorescence signal after addition of saponin is due to a decrease in turbidity upon permeabilization of the cells. The turbidity drops during the first 60 s and remains stable thereafter (not shown). The inset in A shows the correlation between fluorescence and added Ca\(^{2+}\) ([Ca\(^{2+}\)]\(_{obs}\), the total concentration of added Ca\(^{2+}\)) obtained in a separate experiment with an identical platelet suspension. The solid line represents the fitted binding curve \((K_d = 723 \text{ nmol/L}, f_p = 0.72, f_{inc} = 73.25\%\); see Eberhard M, Erne P\(^{22}\)). The Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{inc}\), in equation 1) in B was calculated from the fluorescence signal in A according to equation 1. The dotted curve in B represents a monoeponential fit of the Ca\(^{2+}\) transient according to equation 2, \(a = 0.233 \text{ nmol/L}, k = 0.0045 \text{ sec}^{-1}, [Ca^{2+}]_{inc} = 1598 \text{ nmol/L}\). Ca\(^{2+}\) concentrations are considerably underestimated at the start of the transient due to the turbidity change during permeabilization (see above).
At a given Ca\(^{2+}\) concentration similar Ca\(^{2+}\) uptake rates were obtained when thapsigargin was added at various degrees of store loading (not shown). Since Ca\(^{2+}\) concentrations of suspensions of permeabilized platelet were around and above 1 mmol/L, and since the Ca\(^{2+}\) uptake rate did not significantly depend on the concentration of Ca\(^{2+}\) in the range between 0.8 and 1.5 mmol/L (not shown), Ca\(^{2+}\) uptake rates were evaluated at 1 mmol/L Ca\(^{2+}\) in the present study. To examine if spontaneous Ca\(^{2+}\) leak of the Ca\(^{2+}\) stores is linked to hypertension, the Ca\(^{2+}\) leak (Ca\(^{2+}\) flux in the presence of thapsigargin) was also evaluated at 1 mmol/L Ca\(^{2+}\). In two out of the 17 patients examined the Ca\(^{2+}\) concentration of the platelet suspension after permeabilization was too high to allow the determination of Ca\(^{2+}\) uptake and leak rates at 1 mmol/L Ca\(^{2+}\).

Figure 2 shows that Ca\(^{2+}\) uptake into sarcoplasmic reticulum is not related to average daytime blood pressure. Similar results are obtained with other blood pressure parameters (Table 1) and with Ca\(^{2+}\) leak from sarcoplasmic reticulum (Table 2). A weak correlation between both Ca\(^{2+}\) uptake and Ca\(^{2+}\) leak and heart rate was observed (Figure 3, Table 2), although these correlations are flawed by the substantial error of the values of Ca\(^{2+}\) uptake and leak. Furthermore, no correlation was found between Ca\(^{2+}\) uptake and age (Table 2). To corroborate the relationship between Ca\(^{2+}\) fluxes and blood pressure we analyzed a group of blood donors consisting of four normotensive and ten hypertensive subjects who did not undergo 24-h blood pressure determination (Table 3). No significant correlation between flux parameters and blood pressure was detected within this group (not shown). In addition, the differences in Ca\(^{2+}\) uptake and leak between the group of normotensives and the two hypertensive groups were statistically not significant (Table 3).

**DISCUSSION**

There is a general agreement that platelet cytosolic Ca\(^{2+}\) is increased in hypertension and that this process can be accomplished by rapid Ca\(^{2+}\)-regulating processes. Both plasma membrane- and internal membrane-associated Ca\(^{2+}\) fluxes contribute to the observed changes in platelet cytosolic Ca\(^{2+}\). Since the Ca\(^{2+}\)-ATPase of sarcoplasmic reticulum is believed to represent the major contribution to Ca\(^{2+}\) removal from the cytoplasm, we have analyzed ATP-dependent

**TABLE 2. CORRELATION OF Ca\(^{2+}\) UPTAKE AND LEAK WITH CLINICAL PARAMETERS**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Slope</th>
<th>P</th>
<th>r</th>
<th>Slope</th>
<th>P</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age</td>
<td>0.083 ± 0.055</td>
<td>&gt;.2</td>
<td>0.389</td>
<td>0.043 ± 0.029</td>
<td>&gt;.1</td>
<td>0.383</td>
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<tr>
<td>Day SBP</td>
<td>-0.025 ± 0.060</td>
<td>&gt;.5</td>
<td>-0.114</td>
<td>0.010 ± 0.031</td>
<td>&gt;.5</td>
<td>0.093</td>
</tr>
<tr>
<td>Day DBP</td>
<td>0.011 ± 0.101</td>
<td>&gt;.5</td>
<td>0.029</td>
<td>-0.003 ± 0.053</td>
<td>&gt;.5</td>
<td>-0.015</td>
</tr>
<tr>
<td>Night SBP</td>
<td>0.019 ± 0.044</td>
<td>&gt;.5</td>
<td>0.121</td>
<td>0.012 ± 0.023</td>
<td>&gt;.5</td>
<td>0.141</td>
</tr>
<tr>
<td>Night DBP</td>
<td>0.130 ± 0.086</td>
<td>&gt;.1</td>
<td>0.366</td>
<td>0.027 ± 0.048</td>
<td>&gt;.5</td>
<td>0.135</td>
</tr>
<tr>
<td>Day heart rate</td>
<td>0.141 ± 0.113</td>
<td>&gt;.2</td>
<td>0.327</td>
<td>0.105 ± 0.055</td>
<td>&gt;.05</td>
<td>0.465</td>
</tr>
<tr>
<td>Night heart rate</td>
<td>0.260 ± 0.096</td>
<td>&lt;.05</td>
<td>0.591</td>
<td>0.124 ± 0.054</td>
<td>&lt;.05</td>
<td>0.541</td>
</tr>
</tbody>
</table>

15 hypertensive patients undergoing 24-h blood pressure determination (Table 1), for which Ca\(^{2+}\) uptake and Ca\(^{2+}\) leak values were determined (see Results).

SBP, systolic blood pressure; DBP, diastolic blood pressure.
Ca\textsuperscript{2+} transport into sarcoplasmic reticulum in platelets from a series of patients exhibiting various degrees of hypertension. None of the patients received antihypertensive therapy prior to analysis. Thapsigargin, a specific inhibitor of all isoforms of internal membrane Ca\textsuperscript{2+}-ATP\textsubscript{ase}, served to evaluate Ca\textsuperscript{2+}-ATP\textsubscript{ase} transport activity and spontaneous Ca\textsuperscript{2+} leak of sarcoplasmic reticulum. No correlation of blood pressure with both Ca\textsuperscript{2+} uptake and Ca\textsuperscript{2+} leak and a weak but insignificant correlation of Ca\textsuperscript{2+} uptake with heart rate were found. Ca\textsuperscript{2+} uptake and Ca\textsuperscript{2+} leak did not significantly differ between hypertensive patients and a normotensive control group.

An earlier study has documented a substantial increase of platelet calmodulin-insensitive Ca\textsuperscript{2+}-ATP\textsubscript{ase} activity in hypertension\textsuperscript{16}, indicating a stimulation of internal membrane Ca\textsuperscript{2+}-ATP\textsubscript{ase} in hypertension. The discrepancy to our results may result from the different selection of hypertensive patients, the use of thapsigargin in the present study as opposed to calmodulin activation, and the determination of Ca\textsuperscript{2+} transport activities in permeabilized cells in the present experiments rather than Ca\textsuperscript{2+}-dependent ATP\textsubscript{ase} activity in membrane preparations used in the study of Resink et al.\textsuperscript{16} Takaya et al.\textsuperscript{17} found a decrease in maximal velocity and Michaelis constant of Ca\textsuperscript{2+}-ATP\textsubscript{ase} in platelets from hypertensive patients. Since plasma membrane and internal membrane Ca\textsuperscript{2+}-ATP\textsubscript{ase} activity was not discriminated, the respective contributions of the two Ca\textsuperscript{2+} pumps could not be evaluated in that study. In agreement with our results, no correlation of platelet sarcoplasmic reticulum Ca\textsuperscript{2+}-ATP\textsubscript{ase} with blood pressure was documented, but a decrease of platelet plasma membrane Ca\textsuperscript{2+}-ATP\textsubscript{ase} activity in hypertension was found recently.\textsuperscript{20} Consistent with the decrease in calmodulin-sensitive Ca\textsuperscript{2+}-ATP\textsubscript{ase} activity in hypertension,\textsuperscript{16} these studies indicate that removal of Ca\textsuperscript{2+} across the plasma membrane is responsible for the well established rise of cytosolic Ca\textsuperscript{2+} in hypertension.\textsuperscript{1,3}

Possible other mechanisms accounting for altered cytosolic Ca\textsuperscript{2+} in hypertension include increased agonist-induced Ca\textsuperscript{2+} influx,\textsuperscript{10} activation of voltage-operated Ca\textsuperscript{2+} channels,\textsuperscript{1} altered inositol 1,4,5-trisphosphate-induced Ca\textsuperscript{2+} efflux from internal Ca\textsuperscript{2+} stores,\textsuperscript{26} and a decreased activity of Na\textsuperscript{+}/Ca\textsuperscript{2+} exchangers present in the platelet plasma membrane.\textsuperscript{15,27} Whereas the density of voltage-gated Ca\textsuperscript{2+} channels in platelets is very low and plays only a minor role in the Ca\textsuperscript{2+} balance of platelets,\textsuperscript{11} enhanced agonist-induced Ca\textsuperscript{2+} influx in platelets has been documented in patients with ischemic heart disease.\textsuperscript{28}

**TABLE 3. COMPARISON OF Ca\textsuperscript{2+} UPTAKE AND LEAK BETWEEN HYPERTENSIVE PATIENTS AND NORMOTENSIVE SUBJECTS**

<table>
<thead>
<tr>
<th></th>
<th>Normotensive (n = 4)</th>
<th>Hypertensive (n = 10)</th>
<th>Hypertensive* (n = 15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SBP (mm Hg)</td>
<td>116 ± 8</td>
<td>137 ± 8</td>
<td>146 ± 14</td>
</tr>
<tr>
<td>DBP (mm Hg)</td>
<td>75 ± 6</td>
<td>90 ± 6</td>
<td>95 ± 8</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+} uptake (nmol/L/sec)</td>
<td>8.02 ± 2.59</td>
<td>8.86 ± 2.55</td>
<td>9.85 ± 2.95</td>
</tr>
<tr>
<td>Ca\textsuperscript{2+} leak (nmol/L/sec)</td>
<td>2.89 ± 0.65</td>
<td>3.65 ± 1.24</td>
<td>3.38 ± 1.54</td>
</tr>
</tbody>
</table>

Normotensive defined as SBP (systolic blood pressure) ≤ 120 mm Hg, DBP (diastolic blood pressure) ≤ 80 mm Hg.

*This group comprises the 15 hypertensive patients undergoing 24-h blood pressure determination (Table 1), for which Ca\textsuperscript{2+} uptake and Ca\textsuperscript{2+} leak values were determined (see Results).
decrease of platelet Na+/K+-ATPase activity in mild gestational hypertension has been suggested to contribute to increased cytosolic Ca\(^{2+}\) through the Na+/Ca\(^{2+}\) exchanger.\(^9\) However, Takaya et al.\(^{17}\) did not find altered platelet Na+/K+-ATPase activity in hypertension. Therefore, a decreased activity of the plasma membrane Ca\(^{2+}\)-ATPase and increased agonist-induced Ca\(^{2+}\) influx are likely to contribute to the increase in platelet cytosolic Ca\(^{2+}\) in hypertension, whereas ATP-driven Ca\(^{2+}\) uptake into internal Ca\(^{2+}\) stores remains rather unchanged.

In the present study a weak relationship between Ca\(^{2+}\) uptake into internal membranes and heart rate was noted (Figure 3). Clearly, more patients and a wider range of heart rates will be needed to decide if this correlation is significant. A link between heart rate, which is controlled by the sympathetic and parasympathetic nervous system,\(^{30,31}\) and Ca\(^{2+}\) dynamics of the platelet sarcoplasmic reticulum could be β-adrenegic receptors present in the heart and on platelets.\(^{30,31}\) Whereas hypertension is usually associated with an increase in sympathetic activity,\(^{32}\) its role in the Ca\(^{2+}\) balance in platelets remains obscure, although β-adrenoreceptors may affect Ca\(^{2+}\) pumping activity via phospholamban phosphorylation.\(^{33}\)

REFERENCES
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