Increased Membrane-Bound Calcium in Platelets of Hypertensive Patients

Richard Cooper, Jeanette Lipowski, Earl Ford, Nasreen Shamshi, Harold Feinberg, and Guy Le Breton

The fluorescent indicator chlortetracycline was used to estimate membrane-bound calcium in mild, untreated hypertensive patients (n=39) and normotensive controls (n=42). All participants were black. After incubation with chlortetracycline, platelet-rich plasma was centrifuged into a pellet and fluorescence was measured with a microspectrofluorometer. At an interval of 45 minutes mean fluorescence values were 11% higher in the hypertensive than in the normotensive group (567±95 vs. 512±100 counts/sec, p<0.02). With both groups of participants combined, a correlation of borderline statistical significance was noted between diastolic blood pressure and chlortetracycline fluorescence (r=0.213, p=0.056). In parallel experiments, sodium and potassium concentrations were measured in red blood cells. Intracellular sodium was also significantly higher in the hypertensive group (p<0.01). These data indicate that the total cell burden of calcium is increased in the platelets of hypertensive individuals, possibly a result of abnormal cell metabolism of calcium, and further suggest that circulating platelets in hypertensive individuals may be in a hyperaggregable state. (Hypertension 1989;13:139-144)

Several lines of research have suggested that abnormalities in the storage and transport of electrolytes on the cellular level may play a role in the pathogenesis of hypertension. An increase in the content of sodium has been the most reproducible finding and is supported by the largest set of data. An increase in free intracellular calcium (Ca$_2^+$) has also been reported in some but not all cell lines studied among humans. A considerable body of evidence suggests that the levels of intracellular sodium (Na$_+$) and Ca$_2^+$ are linked through the Na-Ca exchange, although the contribution of this exchange mechanism may vary in different cell lines and has not been conclusively shown to be important under normal physiological conditions. Given the key role of Ca$_2^+$ in determining vascular smooth muscle tone, and therefore blood pressure (BP) regulation, metabolic control of this cation has been viewed as the final common pathway for the pathogenesis of hypertension. Assuming a steady state, it could be anticipated that higher levels of Ca$_2^+$ would be associated with a greater total cell burden of Ca$_2^+$, and this would be reflected in the fraction that is membrane-bound (Ca$_{mb}$). We studied this question in mild, untreated hypertensive humans by examining the content of membrane-bound calcium in platelets.

**Subjects and Methods**

**Recruitment of Participants**

Participants for this study were recruited from among patients seeking care for minor illness in the Ambulatory Screening Clinic of Cook County Hospital. The institution primarily serves the working class and poor black population of Chicago, and 600,000 outpatient visits are recorded each year from an estimated patient base of 1.5-2.0 million persons. Since relatively few patients of ethnic origin other than black are served by this facility, only blacks were recruited to maintain homogeneity of the sample. All participants for this study were free of other major medical illnesses and did not have significant pain, elevated temperature, or other acute conditions. No patients had taken antihypertensive medication for at least 2 weeks before the date of examination. Patients with a diastolic blood pressure (BP) between 90 and 115 mm Hg at the first visit were given an appointment to return within 7 days. At the second visit, after informed consent had been obtained, a standardized medical questionnaire was administered and participants were weighed on a balance scale with shoes off; height was measured with the device attached to the scale.
After a subsequent 5-minute period during which the participant sat quietly without talking, BP was measured in the right antecubital fossa with a standard mercury manometer. An initial auscultatory reading was taken and the 30-second pulse recorded. After a 30-second pause, a third and fourth BP measurement was made and a second pulse measured between readings; all reported data are based on a mean of these second two readings. Blood was sampled at the second visit in glass tubes containing acid-citrate-dextrose, transported to the laboratory, and processed within 2 hours.

Normotensive participants were recruited in the same manner from among patients with minor complaints (e.g., skin rash, backache, etc.) to serve as controls. Patients with any major medical illness and those who had taken prescription medications in the last 2 weeks were excluded.

Laboratory Methods

Blood samples were centrifuged at 160g for 10 minutes to produce platelet-rich plasma. The plasma was incubated with chlorotetracycline (CTC) (50 

\( \mu \text{M} \)), and fluorescence determinations were performed in triplicate at 30 and 45 minutes after addition of CTC. To correct for potential instrument drift during the time course of the experiment, the fluorescence of a uranium glass standard was also measured at the 30- and 45-minute time points.

Intraplatelet Ca\(^{2+} \) binding was assessed by measuring CTC fluorescence intensity,\(^ {12,13} \) which increases in proportion to Ca\(^{2+} \) associated to intracellular storage sites. In brief, 1-ml samples were layered over silicone oil (100 

\( \mu \text{l} \)) of a mixture of Dow 220:Dow 550 (14:4) in the bottom of 1.5-ml plastic conical centrifuge tubes and then centrifuged at 7,000g for 1 minute. Immediately after centrifugation, the supernatant and silicone oil were aspirated, leaving the undisturbed platelet pellets in the tube tips. The tube tips were removed and placed in acrylic holders for fluorescence determination with a photon-counting microspectrofluorometer. Control fluorescence values were normalized to 100,000 counts/sec. As previously shown, the concentration of CTC used in this study (final concentration 0.4 

\( \mu \text{M} \)) does not interfere with platelet shape change, aggregation, or secretion.\(^ {13} \)

Because the degree of fluorescence is also dependent on the amount of CTC taken up by the cells, the possibility that platelets from hypertensive subjects accumulated CTC at different rates than did platelets from normotensive controls was studied. Specifically, platelet-rich plasma was incubated with CTC (50 

\( \mu \text{M} \)) and 0.2 

\( \mu \text{Ci/ml} \) (0.4 

\( \mu \text{M} \) final concentration) of tritiated tetracycline for 45 minutes. At this time, aliquots of the platelet-rich plasma were centrifuged at 7,000g to prepare platelet pellets for fluorescence and tritium measurements.

In an attempt to establish that potential differences in platelet CTC fluorescence between the normal and hypertensive groups were associated with intraplatelet calcium stores and not calcium bound to the external membrane, EGTA pulse experiments were performed. In these cases, 3 mM EGTA was added to the platelets at 30 or 45 minutes subsequent to the addition of CTC, and aliquots of the plasma were prepared for fluorescence determination 2 minutes later.

Additional experiments were also carried out to determine whether differences existed between normal and hypertensive groups in the ability of prostaglandin (PGI\(_2\)) to stimulate intraplatelet calcium sequestration.\(^ {14} \) In these studies, PGI\(_2\) (65 nM) was added to the platelet-rich plasma after the 45-minute time point, and aliquots for fluorescence determination were collected 1 minute later.

Na, and intracellular potassium (K\(_i\)) were measured on red blood cells obtained from the same sample as the platelets.\(^ {3} \) After washing in a solution containing 112 mM magnesium chloride, cells were lysed with distilled water. Assays were performed under cold conditions (4° C) and cells were stored up to 2 hours before processing. Sodium and potassium were measured in the lysate with the flame photometer and corrected for the initial hematocrit.

To estimate the accuracy of the laboratory methods, duplicate specimens were submitted blindly for each assay. The technical error, representing intra-assay variation, was calculated with the following formula and expressed as a percentage of the sample mean:

\[
\sqrt{\frac{d^2}{2N}} + x
\]

where \( d \) is the difference between pairs of readings, \( N \) is the number of pairs of duplicates, and \( x \) is the mean of all pairs of duplicates. For the CTC measurements the technical error for the 30- and 45-minute readings were, respectively, 3.9 and 5.3%. For the stimulation with PGI\(_2\) the technical error was 18.6%, and for the uptake of tritiated tetracycline, it was 5.2%. All these estimates were based on six pairs of duplicates. Technical errors for Na and K were 3.6 and 1.8%, respectively.

Statistical Methods

Data were collected on precoded forms, entered into the computer data base system, and analyzed with programs available on Statistical Analysis Systems.\(^ {15} \) Two sample comparisons were carried out with Student's \( t \) test for continuous variables and with the \( \chi^2 \) test for categorical variables. In view of the strong prior hypothesis that Ca\(_{ib} \) would be higher in hypertensive individuals, a one-tailed statistic was used for tests involving this variable. Correlation and regression analyses were used to assess associations among the variables.

Results

Descriptive characteristics of the participants are presented in Table 1. Participants in the hypertensive group were somewhat older than those in the
TABLE 1. Descriptive Characteristics of Participants

<table>
<thead>
<tr>
<th>Variable</th>
<th>Normotensive group (n=42)</th>
<th>Hypertensive group (n=39)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (years)</td>
<td>44.2±8.9</td>
<td>50.0±11.12*</td>
</tr>
<tr>
<td>Sex (men: women)</td>
<td>17:22</td>
<td>23:19</td>
</tr>
<tr>
<td>Family history</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>77%</td>
<td>71%</td>
</tr>
<tr>
<td>Stroke</td>
<td>48%</td>
<td>22%*</td>
</tr>
<tr>
<td>Current drinker</td>
<td>40%</td>
<td>56%</td>
</tr>
<tr>
<td>Drinks per week</td>
<td>7.8±19.1</td>
<td>6.9±16.2</td>
</tr>
<tr>
<td>Current smoker</td>
<td>76%</td>
<td>56%</td>
</tr>
<tr>
<td>Years of education</td>
<td>10.4±2.9</td>
<td>9.8±3.1</td>
</tr>
<tr>
<td>Systolic BP (mm Hg)</td>
<td>115±10</td>
<td>151±16*</td>
</tr>
<tr>
<td>Diastolic BP</td>
<td>77±7</td>
<td>102±8*</td>
</tr>
<tr>
<td>Body mass index (Ht/Wt2; Kg/M)</td>
<td>28.1±5.3</td>
<td>30.6±6.1*</td>
</tr>
<tr>
<td>Total cholesterol</td>
<td>189±55</td>
<td>204±40</td>
</tr>
<tr>
<td>HDL-cholesterol (mg/dl)</td>
<td>43±12</td>
<td>49±16</td>
</tr>
</tbody>
</table>

Values are mean±SD or percent. BP, blood pressure; HDL, high density lipoproteins.

*p<0.05.

control group (p<0.05); age was not significantly related to any of the electrolyte variables of interest, however, and was not entered as a covariate in further analysis. The high prevalence of hypertension in this population is apparent in the finding that three quarters of the participants reported hypertension in one parent; this finding invalidated analyses for inheritance patterns of the Ca²⁺ abnormalities. Serum lipids have been shown to affect membrane composition and transport, but were not different in the two groups studied here.

Platelet Ca₉ was increased in hypertensive compared with normotensive subjects at both the 30- and 45-minute readings; the difference was somewhat more significant in the latter reading (Table 2). No difference in the observed levels of radioactivity after incubation with tritiated tetracycline was noted, suggesting that higher fluorescence values among the hypertensive participants were not a result of preferential uptake of CTC. Furthermore, EGTA pulse experiments (n=19) demonstrated that platelet fluorescence levels did not change significantly on addition of EGTA, indicating that increased calcium binding in hypertensive individuals is associated with greater intraplatelet calcium stores and not calcium bound to the external membrane (data not shown).

Cell sodium content, as assayed in red blood cells, was also significantly higher among the hypertensive participants (p<0.02), while Kᵢ levels were the same.

Changes in fluorescence after stimulation with PGI₂ were not different between the two groups, with a 40% increase seen in the normotensive group (n=32) and a 39% increase in the hypertensive group (n=27) (mean increase, 651±135 vs. 691±83; p=0.187). This finding suggests a comparable ability of normal and hypertensive individuals to sequester additional calcium after stimulation with PGI₂.

With participants from both groups combined into a single sample, a borderline significant correlation between diastolic BP and Ca₉ was noted (Table 3), as well as significant correlations between Naᵢ and diastolic BP and Ca₉ and Kᵢ.

TABLE 2. Cellular Electrolytes in Hypertension

<table>
<thead>
<tr>
<th>Variable</th>
<th>Normotensive group (n=42)</th>
<th>Hypertensive group (n=39)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelet assays</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Membrane-bound Ca²⁺*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>30-minute</td>
<td>529±106</td>
<td>564±861</td>
</tr>
<tr>
<td>45-minute</td>
<td>512±99</td>
<td>558±934</td>
</tr>
<tr>
<td>Tritiated tetracycline$</td>
<td>620±142</td>
<td>618±188</td>
</tr>
<tr>
<td>(n=31)</td>
<td>(n=27)</td>
<td></td>
</tr>
<tr>
<td>Red blood cell assays</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intracellular Na (mmol)</td>
<td>6.70±1.97</td>
<td>7.88±2.24$</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intracellular K (mmol)</td>
<td>72.9±14.0</td>
<td>74.3±12.4</td>
</tr>
</tbody>
</table>

*Units reported in counts per second normalized to a uranium glass standard.

$p<0.05$.

$tp<0.02$.

$tp<0.02$.

§Units reported as counts per minute normalized to 10⁸ platelets.

TABLE 3. Membrane-Bound Calcium and Hypertension: Correlation Matrix

<table>
<thead>
<tr>
<th>Variable</th>
<th>DBP</th>
<th>BMI</th>
<th>Ca₉*</th>
<th>Naᵢ</th>
<th>Kᵢ</th>
<th>Drinks/wk</th>
</tr>
</thead>
<tbody>
<tr>
<td>DBP</td>
<td>0.213</td>
<td>0.213</td>
<td>0.314</td>
<td>0.145</td>
<td>-0.099</td>
<td></td>
</tr>
<tr>
<td>BMI</td>
<td></td>
<td>0.058</td>
<td>-0.063</td>
<td>0.007</td>
<td>-0.218</td>
<td></td>
</tr>
<tr>
<td>Ca₉</td>
<td></td>
<td></td>
<td>-0.010</td>
<td>-0.317</td>
<td>0.167</td>
<td></td>
</tr>
<tr>
<td>Naᵢ</td>
<td></td>
<td></td>
<td></td>
<td>-0.216</td>
<td>-0.241</td>
<td></td>
</tr>
<tr>
<td>Kᵢ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>-0.169</td>
<td></td>
</tr>
<tr>
<td>Drinks/wk</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Numbers in parentheses are p values. DBP, diastolic blood pressure; BMI, body mass index; Ca₉, membrane-bound calcium; Naᵢ, intracellular sodium; Kᵢ, intracellular potassium.

*Forty-five-minute value.
Table 4. Cellular Electrolytes and Hypertension, Analysis by Sex

<table>
<thead>
<tr>
<th>Variable</th>
<th>Men (n=23)</th>
<th>Hypertensive (n=17)</th>
<th>Women (n=19)</th>
<th>Hypertensive (n=22)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Platelet assays</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Membrane-bound Ca(^{2+})*</td>
<td>535±98</td>
<td>573±89</td>
<td>521±116</td>
<td>558±82</td>
</tr>
<tr>
<td>30-minute</td>
<td>511±98</td>
<td>563±92t</td>
<td>513±101</td>
<td>553±94</td>
</tr>
<tr>
<td>45-minute</td>
<td>615±145</td>
<td>618±193</td>
<td>626±138</td>
<td>618±183</td>
</tr>
<tr>
<td>Tritiated tetracycline</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Red blood cell assays</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na(_i) (mmol)</td>
<td>6.34±1.35</td>
<td>7.91±1.96t</td>
<td>7.13±2.47</td>
<td>7.86±2.44</td>
</tr>
<tr>
<td>K(_i) (mmol)</td>
<td>70.9±9.4</td>
<td>72.6±8.7</td>
<td>75.3±15.6</td>
<td>75.6±18.3</td>
</tr>
</tbody>
</table>

Values are mean±SD. Na\(_i\), intracellular sodium; K\(_i\), intracellular potassium.

Regression equations were used to estimate the independent relation between Ca\(_b\) and BP. In stepwise logistic regression with case status as the dependent variable, only Na\(_i\) was statistically significant among the cellular electrolytes in a model that included sex, body mass index, Na\(_i\), K\(_i\), and Ca\(_b\). With Ca\(_b\) at 45 minutes as the dependent variable in linear regression analysis, however, diastolic BP was found to have a significant association (p=0.025) in an equation that explained 24% of the variance. It would appear, therefore, that in multivariate analysis a weak relation exists between BP with both groups combined. (Given the case-control design of this study, treating BP as a continuous variable is probably not the most sensitive method of analyzing these data and more confidence can be placed in the two-sample analyses.)

Prior evidence suggested that the relation between BP and cellular electrolytes may not be the same in the two sexes\(^7\) and we analyzed our participants by sex and case status (Table 4). The differences in Ca\(_b\) were more pronounced for men than women (10.1 vs. 7.8%) and, despite the small sample size, were significant for men at the 45-minute reading. Sodium content was also different between hypertensive and normotensive men (p=0.01) but not women. Correlation analysis demonstrated sizable and significant associations between Ca\(_b\) and Na\(_i\) and diastolic BP among men, but virtually none among women (Table 5). A strong association between Ca\(_b\) and body mass index was noted in both men and women, but of opposite direction. Thus, the association was positive for men and negative for women; this sex difference resulted in the absence of an association between these two variables in the group as a whole. Women with hypertension were more obese than those with normal blood pressure, and this may have confounded the BP-Ca\(_b\) relation in women.

Discussion

The data presented here demonstrate increased levels of Ca\(^{2+}\) bound to the membrane of platelets of hypertensive compared with normotensive individuals. Membrane-bound Ca\(^{2+}\) was also significantly, although weakly, related to BP in correlation and regression analysis, and this relation appeared to be independent of several potentially confounding factors. The possibility that increased levels of fluorescence were attributable to preferential uptake of CTC by platelets from hypertensive subjects was ruled out by experiments with radioisotope-labeled tetracycline, which demonstrated identical rates of absorption. In separate analyses performed on red blood cells from the same group of participants, sodium content was also significantly increased among patients with an elevated BP. No relation was noted in this study between Ca\(_b\), as estimated in platelets, and Na\(_i\), as assayed in red blood cells, in contrast to our previous work with Ca\(_b\). Overall these data confirm findings by our group\(^5\) and others\(^4,7\) of increased cell Ca\(^{2+}\) among hypertensive individuals and extend this observation for the first time to the membrane-bound fraction.

The technique used in this study was highly reproducable and was associated with a small intra-assay variation, thus yielding a statistically significant difference with a small absolute difference and a relatively modest sample size. This method is also not subject to the problems inherent in assays of

Table 5. Cellular Electrolytes and Blood Pressure: Correlation Matrix

<table>
<thead>
<tr>
<th>Variable</th>
<th>Men (n=40)</th>
<th>Women (n=41)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ca(_b)</td>
<td>Na(_i)</td>
</tr>
<tr>
<td>Body mass index</td>
<td>0.536 (0.0001)</td>
<td>0.064 (0.028)</td>
</tr>
<tr>
<td>Diastolic BP</td>
<td>0.342 (0.031)</td>
<td>0.331 (0.045)</td>
</tr>
<tr>
<td>Na(_i)</td>
<td>0.160 (0.067)</td>
<td>-0.132 (0.013)</td>
</tr>
<tr>
<td>K(_i)</td>
<td>-0.214 (0.067)</td>
<td>0.304 (0.013)</td>
</tr>
</tbody>
</table>

Numbers in parentheses are p values. Ca\(_b\), membrane-bound calcium; BP, blood pressure; Na\(_i\), intracellular sodium; K\(_i\), intracellular potassium.
platelet Ca, with the quin/fura class of indicators with preparation in a Ca-free medium and gel filtration. The magnitude of the differences described are in the range of those previously reported for free intracellular Ca and Na. A previous report that used two fluorescent indicators simultaneously, CTC and quin-2, demonstrated a relation between Ca and Ca, in platelets, and Ca, can be taken as a measure of the total cell burden of Ca.

An entirely independent line of evidence further suggests a higher cell burden of Ca among hypertensive individuals. In both experimental animals and humans, increased aggregability of platelets has been noted in the hypertensive state. Several measures of cell function, including aggregation, shape change, release reaction, and thromboxane formation, have demonstrated the hyperaggregable state of platelets. These findings may be explained by increased stores of Ca, which can be released on exposure to a chemical agonist or other stimulation. These findings are important because of their potential implications in the pathophysiology of the thromboembolic complications of hypertension, notably stroke. Although long-standing hypertension unquestionably promotes atherosclerosis, it may also contribute to cardiovascular morbidity by increasing the acute risk of thrombosis.

Alterations in the structure and function of the cell membrane in hypertension is a topic of great current interest. Increases in Ca uptake and binding have been reported in hypertension by some investigators. A decrease in the degree to which membrane-bound Ca adenosine triphosphatase could be stimulated by calmodulin was also noted in platelets from hypertensive individuals. As in much of the research in this area, the use of different experimental models makes it difficult to compare various reports, and greater effort is needed to identify reproducible phenomena. Stimulation of platelets with PGI2 in the present study did not demonstrate evidence of enhanced Ca sequestration in response to addition of PGI2 at the concentration of 65 nM, suggesting that the cyclic adenosine monophosphate-dependent pump mechanisms are not more efficient in these hypertensive subjects.

Sodium in red blood cells was significantly higher in the hypertensive participants in this study compared with those with normal blood pressure. The relevance of this finding to the Na-Ca exchange theory is limited, however, since the two electrolytes were studied in different cell lines. Evidence for Na-Ca exchange does exist for platelets and the question of whether parallel increases in the two ions can be demonstrated in the same cell deserves further study.

Hypertension may have a single, underlying primary cause with other factors accelerating its course, or it may be the final common expression of a series of disorders. One approach to the study of this question has been to compare factors associated with an increased BP in different sex-race groups. It is possible, for example, that obesity plays a more important role among black women, while sodium overload is crucial for black men. The strength of the association between the intracellular ions and hypertension was very different in men and women in this study, and this finding has been previously reported. Similar findings exist in the relation between other risk factors for hypertension and sex, and the risk of morbid sequelae associated with hypertension appears to be different in obese and nonobese individuals. It is possible, of course, that these findings simply represent sampling variation, and they will require further verification.

The technique used in this report is highly sensitive and reproducible and does not make the assumption of normal physiological function throughout the preparation phase, as do most assays of free cytosolic Ca. If substantiated by other studies, this finding of a higher total cell burden of Ca in platelets of hypertensive humans has implications for both the pathogenesis of this disorder and the occurrence of thromboembolic sequelae.

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References


**KEY WORDS** • calcium • membranes • platelets
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