Inrecent decades, there has been increasing evidence of an abnormality of sodium transport across the cell membrane associated with elevated intracellular sodium concentration ([Na⁺]) in erythrocytes, leukocytes, and lymphocytes of patients with essential hypertension. Alternatively, abnormal calcium handling and increased intracellular free calcium concentration ([Ca²⁺]) have been reported in erythrocytes and platelets of patients with essential hypertension. The increases in [Na⁺] and [Ca²⁺] are considered, at least in part, to play an important role in the pathogenesis of essential hypertension. Although a link between sodium transport and calcium handling at the cellular level has been postulated, only limited information is available concerning the relation between abnormalities in [Na⁺] and [Ca²⁺] in patients with essential hypertension.

An abnormality of cellular cations may be demonstrated only in a subgroup of and not in all patients with essential hypertension because of the pathogenetic heterogeneity of the hypertensive populations. If elevations of [Na⁺] and [Ca²⁺] can be detected in essential hypertension, it is important to assess the factors determining such alterations in cation metabolism at the cellular level. Thus, in the present study, [Na⁺], intracellular sodium concentration ([Na⁺]), intracellular free calcium concentration ([Ca²⁺]), and plasma renin activity ([PRA]) were determined simultaneously in lymphocytes of patients with essential hypertension to elucidate the relation of [Na⁺], [K⁺], and [Ca²⁺] to each other and to several clinical indices such as age, blood pressure,
Serum electrolyte concentration, plasma renin activity, and plasma norepinephrine concentration.

Subjects and Methods
Thirty outpatients with essential hypertension and 30 normotensive controls were studied. All subjects were Japanese. No subjects had taken antihypertensive drugs or any other drugs for 4 weeks before the study. All the patients with essential hypertension had systolic blood pressure of more than 160 mm Hg or diastolic blood pressure of more than 95 mm Hg in the sitting position (or both) on at least three occasions in the outpatient clinic. All the normotensive controls had systolic blood pressure of less than 140 mm Hg and diastolic blood pressure of less than 90 mm Hg when screened. Patients with secondary causes of hypertension and with extensive organ damage were excluded on the basis of appropriate clinical, biochemical, and radiological examinations. After the nature and purpose of the study were explained, informed consent was obtained from each subject.

All of the subjects were on a diet containing 8 to 10 g of sodium chloride per day for 1 week before the day of the study. During that week, 24-hour urine samples were used to determine the excretion of sodium, potassium, calcium, and creatinine as a measure of intake.

Subjects fasted overnight, and a catheter then was inserted in the forearm vein and kept patent with 5% glucose to obtain blood samples. After the subjects remained supine for at least 30 minutes in a quiet and dark room, blood pressure was measured with a mercury sphygmomanometer every minute for 10 minutes. The average of 10 consecutive blood pressure measurements was used in the analyses. Mean blood pressure was calculated as diastolic blood pressure plus one third of pulse pressure. Subsequently, 45 ml of blood was obtained for hematocrit determination of the method of Tsien et al. 

In brief, the cells were washed twice with cold isotonic MgCl₂ solution, transferred to a polycarbonate capillary tube (Hematolon, Kayagaki Irirakokyo, Tokyo, Japan), and centrifuged at 15,000 g for 5 minutes. When the lymphocytes became packed at the bottom of the tube, the tube was cut at the boundary between the packed lymphocytes and supernatant. The percentage of extracellular fluid volume trapped within the lymphocyte pellet determined by ¹ⁱ¹I-labeled human serum albumin (Commissariat a L'Energie Atomique, Paris, France) was about 26%. The cells packed in the tube were then weighed and dried out at 80 °C for 48 hours. Nitric acid (14 N) was then added, and the cellular pellet was completely dissolved. The empty capillary tubes were then weighed. Thus, 74% of the weight loss equaled the wet weight of the lymphocytes. [Na⁺], and [K⁺], in lymphocytes were determined by flame photometry (Model 775-A, Hitachi, Tokyo, Japan) and expressed as millimoles per kilogram of wet weight. The intra-assay coefficient of variation was less than 5%, and the day-to-day intrasubject coefficient of variation was less than 7% for [Na⁺]. Corresponding coefficients of variation for [K⁺] were less than 6% and less than 9%, respectively.

[Ca²⁺], in lymphocytes was determined by a modification of the method of Tsien et al. 

Briefly, the lymphocytes (1–3 × 10⁷/ml) were incubated at 37 °C for 40 minutes with 50 μM of the fluorescent dye, quin 2 tetraacetoxymethylene (quin 2 AM, Dojindo Laboratories, Kumamoto, Japan). After loading with quin 2 AM, the cells were washed, transferred to fresh RPMI-1640, and left at room temperature for 60 minutes to allow hydrolysis of the ester. The evidence that loaded lymphocytes maintain a high eosin exclusion (>95%) weighed against gross toxicity of the quin 2 concentration in the present study. The lymphocytes were centrifuged at 400 g for 10 minutes and resuspended (3–5 × 10⁸ cells/ml) in physiological saline containing (mM) NaCl, 145; KCl, 5; HEPES, 10; Na₂HPO₄, 1; CaCl₂, 1; MgSO₄, 0.5; and glucose, 5, at pH 7.4 and 37 °C. Fluorescence was measured at 37 °C with a Hitachi spectrofluorimeter (Model 204 S, Tokyo, Japan). Excitation and emission wavelengths were 339 and 492 nm with 4- and 10-nm bandwidth, respectively. Resting levels of [Ca²⁺], in lymphocytes were calculated by the equation [Ca²⁺] = 115 nM x (F – Fₘᵢₙ)/(Fₘₐₓ – F), where 115 nM is the effective dissociation constant (Kₐ) of the calcium-quin 2 complex and F is the fluorescence of the intact cells.

Isolation of lymphocytes was performed with a modification of Boyum’s method. 

It consists of diluting heparinized blood 1:2 with medium RPMI-1640 (Gibco Laboratories, Grand Island, New York, USA), layering on a Ficoll-metrizoate mixture (Lymphoprep, Nyegaard, Oslo, Norway) with a density of 1077 g/L, and centrifuging at 400 g for 35 minutes. The isolated lymphocytes were washed twice in RPMI-1640 with centrifugation at 100 g for 10 minutes and prepared for measurements of [Na⁺], [K⁺], and [Ca²⁺].
Table 1. Clinical Characteristics of Hypertensive Patients and Normotensive Controls

<table>
<thead>
<tr>
<th>Variable</th>
<th>Normotensive</th>
<th>Hypertensive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (M/F)</td>
<td>16:14</td>
<td>14:16</td>
</tr>
<tr>
<td>Age (yr)</td>
<td>52.5 ± 15.3</td>
<td>52.5 ± 10.8</td>
</tr>
<tr>
<td>Mean blood pressure (mm Hg)</td>
<td>85.2 ± 10.4</td>
<td>114.1 ± 10.5*</td>
</tr>
<tr>
<td>Serum Na (mmol/L)</td>
<td>142.0 ± 3.6</td>
<td>114.2 ± 2.2</td>
</tr>
<tr>
<td>Serum K (mmol/L)</td>
<td>2.32 ± 0.09</td>
<td>2.31 ± 0.06</td>
</tr>
<tr>
<td>Serum Ca (mmol/L)</td>
<td>0.96 ± 0.09</td>
<td>0.97 ± 0.14</td>
</tr>
<tr>
<td>Plasma Renin activity (ng Ang I/ml/hour)</td>
<td>2.26 ± 1.28</td>
<td>2.10 ± 1.67</td>
</tr>
<tr>
<td>Plasma Norepinephrine (pg/ml)</td>
<td>154 ± 64</td>
<td>138 ± 74</td>
</tr>
<tr>
<td>Urine volume (ml/day)</td>
<td>1238 ± 189</td>
<td>1212 ± 265</td>
</tr>
<tr>
<td>Urinary sodium (mmol/day)</td>
<td>156.0 ± 10.9</td>
<td>151.4 ± 10.2</td>
</tr>
<tr>
<td>Urinary potassium (mmol/day)</td>
<td>50.6 ± 8.4</td>
<td>48.8 ± 9.8</td>
</tr>
<tr>
<td>Urinary calcium (mmol/day)</td>
<td>3.45 ± 0.07</td>
<td>3.20 ± 0.08</td>
</tr>
<tr>
<td>Urinary creatinine (g/day)</td>
<td>1.12 ± 0.11</td>
<td>1.22 ± 0.13</td>
</tr>
</tbody>
</table>

Values are means ± SD. Ang I = angiotensin I.

*p < 0.05, compared with values in normotensive controls.

Intracellular cation concentrations in lymphocytes of hypertensive patients in comparison with those of normotensive controls are shown in Figure 1. [Na], in lymphocytes was significantly higher (p < 0.01) in hypertensive patients (19.8 ± 1.8 mmol/kg wet weight) than in normotensive controls (18.4 ± 1.8 mmol/kg wet weight), while [K], in lymphocytes of hypertensive patients (99.9 ± 6.0 mmol/kg wet weight) did not differ from that of normotensive controls (99.6 ± 5.8 mmol/kg wet weight). [Ca²⁺], in lymphocytes was also significantly higher (p < 0.01) in hypertensive patients (134.6 ± 13.2 nmol/L) than in normotensive controls (120.2 ± 16.4 nmol/L). The relationship between [Na], and [Ca²⁺], in lymphocytes is shown in Figure 2. A close linear correlation was present in normotensive controls (r = 0.59, p < 0.01), in hypertensive patients (r = 0.71, p < 0.001), and in the group combined (r = 0.70, p < 0.001), respectively. [K], had no relation to [Na], or [Ca²⁺], in each group.

As shown in Figure 3, plasma renin activity was negatively correlated with lymphocyte [Na], (r = -0.66, p < 0.001) and [Ca²⁺], (r = -0.60, p < 0.001) in the hypertensive patients. No correlation could be demonstrated between plasma renin activity and [Na], or [Ca²⁺], in normotensive controls. Age had a statistically significant correlation with lymphocyte [Na], and [Ca²⁺], in normotensive controls (r = 0.44, p < 0.05; r = 0.51, p < 0.01, respectively), but not in hypertensive patients (r = 0.31, NS; r = 0.25, NS, respectively). Mean blood pressure was correlated with neither [Na], nor [Ca²⁺], in normotensive controls.

Results

Age; sex; serum sodium, potassium, calcium, and creatinine concentrations; plasma renin activity; plasma norepinephrine concentration; and urinary excretion of sodium, potassium, calcium, and creatinine did not differ between normal controls and hypertensive patients (Table 1). The individual variation of the urinary excretion of sodium, potassium, and calcium was small and did not affect lymphocyte [Na], [K], or [Ca²⁺], in either group.

Autofluorescence of the cell before and after cell lysis and of added reagents such as EGTA. The intra-assay coefficient of variation was less than 7%, and the day-to-day intrasubject coefficient of variation was less than 8%.

Data are presented as means ± SD. Statistical analyses were performed using Wilcoxon rank-sum test to compare the different study groups. Results were considered significant if the p value was less than 0.05. Correlations between parameters were tested by linear regression analysis.

Results

Age; sex; serum sodium, potassium, calcium, and creatinine concentrations; plasma renin activity;
(r = 0.23, NS for [Na]; r = 0.13, NS for [Ca\(^{2+}\)]) and in hypertensive patients (r = 0.20, NS for [Na]; r = 0.11, NS for [Ca\(^{2+}\)]). There was no relation between intracellular sodium, potassium, or free calcium concentration and serum sodium, potassium, or calcium concentration or plasma norepinephrine level in each group.

Discussion

An elevation of [Na], has been reported on a number of occasions in erythrocytes,\(^1\) leukocytes,\(^2\) and lymphocytes\(^3\) of patients with essential hypertension, while [K], is considered to be unaltered in such blood cell types of this disease.\(^2\)\(^,\)\(^3\) The increased [Na], in essential hypertension has been explained by the sodium transport hypothesis and supported by multiple experimental observations, beginning with Dahl et al.\(^16\) and followed by Haddy et al.,\(^17\) de Wardener and MacGregor,\(^18\) and many others. The hypothesis is that essential hypertension is due to a rise in plasma concentration of a circulating Na\(^{+}\),K\(^{+}\)-adenosine triphosphatase (ATPase) inhibitor secreted in response to volume expansion. Na\(^{+}\), K\(^{+}\)-ATPase inhibitor increases [Na], through a reduction in sodium efflux. A Na\(^{-}\)-Ca\(^{2+}\) exchange system has been assumed to participate in the regulation of calcium handling of a number of cells. Increased [Na], would decrease the rate of extracellular Na\(^{+}\)-dependent Ca\(^{2+}\) efflux and increase the rate of intracellular Na\(^{+}\)-dependent Ca\(^{2+}\) influx and thereby raise [Ca\(^{2+}\)], and thus increase vascular reactivity. This Na\(^{-}\)-Ca\(^{2+}\) exchange has been demonstrated in vascular smooth muscle cells by Blaustein.\(^8\)

Since the factors regulating blood pressure are complex and essential hypertension should not be regarded as a single disease entity, many investigators have separated this disease into distinct subgroups with its own characteristics. Because the distribution of plasma renin activity in patients with essential hypertension shows a continuous pattern, the division of patients into low, normal, and high renin subgroups may be arbitrary. However, many investigators have emphasized the uniqueness of patients with low renin hypertension, reporting that they have a different mechanism for their hypertension\(^19\) and an enhanced response to diuretics.\(^20\) Accordingly, it is suggested that, in these patients with low renin activity, the plasma volume is effectively expanded through a greater tendency of the kidneys to retain sodium compared with patients with normal or high renin activity.\(^21\) If this suggestion is correct, the abnormality in [Na], should be greatest in patients with low renin hypertension according to the sodium transport hypothesis that a circulating Na\(^{+}\),K\(^{+}\)-ATPase inhibitor is stimulated by volume expansion. In fact, Na\(^{+}\),K\(^{+}\)-ATPase-dependent sodium efflux from leukocytes was reported to be smallest in patients with low renin hypertension.\(^22\) In the present study, in patients with essential hypertension, both [Na] and [Ca\(^{2+}\)] were increased and negatively correlated with plasma renin activity. Our findings may support the sodium transport hypothesis; however, it is difficult to decide whether lymphocytes possess a Na\(^{-}\)-Ca\(^{2+}\) exchange mechanism. Some investigators have failed to demonstrate the existence of Na\(^{-}\)-Ca\(^{2+}\) exchange. Using quin 2, Tsien et al.\(^15\) reported that [Ca\(^{2+}\)] in lymphocytes was unchanged with changes in extracellular sodium concentration, and Shore et al.\(^23\) and Bruschi et al.\(^24\) reported that incubation with ouabain did not affect [Ca\(^{2+}\)] in lymphocytes. These observations may result from an inhibitory effect of quin 2 on Na\(^{-}\)-Ca\(^{2+}\) exchange.\(^25\) The data on Na\(^{-}\)-Ca\(^{2+}\) exchange in lymphocytes are limited by the single report of Ueda\(^9\) on Na\(^{-}\)-dependent Ca\(^{2+}\) fluxes in membrane vesicles prepared from rabbit lymphocytes. The uptake of Ca\(^{2+}\) through this Na\(^{-}\)
gradient–dependent process showed a much lower affinity for Ca\textsuperscript{2+} than that through the adenosine 5'-triphosphate–dependent process. The physiological importance of Na\textsuperscript{+}–Ca\textsuperscript{2+} exchange with low activity in lymphocytes is still a matter of dispute.

Our findings can be interpreted not only by the sodium transport hypothesis but also by another hypothesis that an abnormality in calcium metabolism is primary in the pathogenesis of essential hypertension. Resnick et al.\textsuperscript{28} reported the linkage of plasma renin activity, calcium-regulating hormones such as parathyroid hormone, calcitonin, and 1,25-dihydroxyvitamin D, and blood pressure in essential hypertension. They speculated that the lower the plasma renin activity, the larger the change in distribution of calcium from outside to inside the cell. Although we did not determine serum ionized calcium or the levels of calcium-regulating hormones, the negative correlation between [Ca\textsuperscript{2+}]\textsubscript{i}, and plasma renin activity in essential hypertension may support their suggestion. The report of Linder et al.,\textsuperscript{7} demonstrating a circulating factor that increased platelet [Ca\textsuperscript{2+}], in plasma from patients with essential hypertension, also may support the prime importance of elevated [Ca\textsuperscript{2+}]\textsubscript{i} in the pathogenesis of essential hypertension. In addition, recent studies have demonstrated that [Ca\textsuperscript{2+}]\textsubscript{i} regulates sodium fluxes across the cell membrane. Changes in [Ca\textsuperscript{2+}]\textsubscript{i}, are well known to play an important part in controlling cellular behavior, including membrane events. It is still unclear whether elevation in [Ca\textsuperscript{2+}]\textsubscript{i}, is a cause or a consequence of increased [Na\textsuperscript{+}].

In essential hypertension, abnormal cellular calcium handling that raises [Ca\textsuperscript{2+}] \textsubscript{i}, has been reported in erythrocytes.\textsuperscript{4,5} Recently, Tsien et al.\textsuperscript{15} synthesized a fluorescent free calcium indicator, quin 2, that can be trapped in the cytoplasm of intact cells, and this technique has allowed a reliable determination of [Ca\textsuperscript{2+}]\textsubscript{i} in blood cells. Since the advent of quin 2, [Ca\textsuperscript{2+}]\textsubscript{i} has been reported to be increased in platelets of patients with essential hypertension\textsuperscript{6} and in lymphocytes of spontaneously hypertensive rats.\textsuperscript{24} However, no increase in [Ca\textsuperscript{2+}]\textsubscript{i} has been shown in lymphocytes\textsuperscript{29-30} and leukocytes\textsuperscript{31-32} of patients with essential hypertension, and these results are in contrast with our findings of increased [Ca\textsuperscript{2+}]\textsubscript{i} in lymphocytes of patients with essential hypertension. The discrepancy may be explained by the following possible causes. First, there may be differences in the characteristics of the hypertensive patients who were investigated, for example, in race or in renin profile. Second, the lack of increase in [Ca\textsuperscript{2+}]\textsubscript{i} in hypertensive patients in the previous reports could be due to free salt intake. Since the changes in salt intake alter [Ca\textsuperscript{2+}], in lymphocytes,\textsuperscript{33} if salt intake was higher in normotensive controls than in hypertensive patients, the difference in [Ca\textsuperscript{2+}]\textsubscript{i} between two groups should be diminished.

In the present study, a close positive correlation was observed between [Na\textsuperscript{+}] and [Ca\textsuperscript{2+}] in both groups. This result may indicate a considerable link between calcium handling and sodium transport across the cell membrane in patients with essential hypertension and normotensive subjects. In contrast to our result, Bing et al.\textsuperscript{32} have reported no correlation between [Ca\textsuperscript{2+}]\textsubscript{i} and sodium content in leukocytes in humans. Although [Na\textsuperscript{+}] was measured in wet weight in the present study, they measured intracellular sodium content in dry weight. If there was a marked variation in intracellular water contents, it would be difficult to detect the relation between [Na\textsuperscript{+}] and [Ca\textsuperscript{2+}]. In addition, they failed to find an elevation of [Ca\textsuperscript{2+}]\textsubscript{i}, in patients with essential hypertension.

We also observed that [Ca\textsuperscript{2+}]\textsubscript{i} in lymphocytes was higher in patients with essential hypertension than in normotensive controls and had a negative correlation with plasma renin activity. These results are in accord with the results of studies on the response to calcium antagonists. The hypotensive effects of calcium antagonists also have been demonstrated to be enhanced in patients with essential hypertension compared with normotensive subjects\textsuperscript{34-36} and negatively correlated with plasma renin activity.\textsuperscript{35} If the hypotensive effects of these drugs reflect a functional abnormality of vascular smooth muscle related to [Ca\textsuperscript{2+}], lymphocytes may be an appropriate model for calcium handling in vascular smooth muscle cells in essential hypertension.

We were unable to demonstrate a significant correlation between blood pressure and [Na\textsuperscript{+}] or [Ca\textsuperscript{2+}] in lymphocytes, and this finding contrasts to the positive correlation of blood pressure level with [Na\textsuperscript{+}] in lymphocytes reported by Ambrosioni et al.\textsuperscript{3} and with [Ca\textsuperscript{2+}] in platelets reported by Erne et al.\textsuperscript{6} In hypertensive patients with similar levels of blood pressure, the mechanism for their hypertension, such as abnormalities in cellular cation metabolism, may be different among patients with different renin profiles. Cellular cation concentration is not the only determinant regulating blood pressure. Both [Na\textsuperscript{+}] and [Ca\textsuperscript{2+}] are correlated with plasma renin activity as an index of volume expansion, but not with the level of blood pressure in essential hypertension. Thus, increased [Na\textsuperscript{+}] and [Ca\textsuperscript{2+}] may not be secondary to disturbances that are associated with high blood pressure and may be one of many factors responsible for the pathogenesis of essential hypertension.

In summary, [Na\textsuperscript{+}] and [Ca\textsuperscript{2+}] in lymphocytes were higher in hypertensive patients than in normotensive controls. There was a positive correlation between [Na\textsuperscript{+}] and [Ca\textsuperscript{2+}] in hypertensive patients and normotensive controls. Plasma renin activity was negatively correlated with [Na\textsuperscript{+}] and [Ca\textsuperscript{2+}] in hypertensive patients. We could not find a significant association of age, blood pressure, or plasma norepinephrine concentration with these alterations in cellular cation. These results suggest that the elevation in [Ca\textsuperscript{2+}] linked with increased [Na\textsuperscript{+}] is important in the genesis of volume-expanded hypertension with suppressed plasma renin activity.
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