SUMMARY The effect of bumetanide, a known probe of \( \text{Na}^+\text{,K}^+ \) cotransport, on \( ^{22}\text{Na}^+ \) uptake and washout was examined in serially passed cultured vascular smooth muscle cells of spontaneously hypertensive rats (SHR), Wistar-Kyoto rats (WKY), and Wistar rats. In \( \text{Ca}^{2+} \)-deficient medium, the drug exerted the greatest effect on \( ^{22}\text{Na}^+ \) washout in vascular smooth muscle cells from SHR and the least effect on cells from WKY. The respective mean values for the apparent bumetanide-sensitive \( ^{22}\text{Na}^+ \) washout rate constants \( (K_c; \times 10^{-2}/\text{min}) \) were 7.2, 4.3, and 1.7 for cells from SHR, WKY, and Wistar rats. In both 1 mM \( \text{Ca}^{2+} \) and \( \text{Ca}^{2+} \)-deficient medium, in the presence of 1 mM ouabain, vascular smooth muscle cells from SHR had the highest plateau phase of \( ^{22}\text{Na}^+ \) uptake among the three cell preparations. All cells exhibited higher \( ^{22}\text{Na}^+ \) uptake in \( \text{Ca}^{2+} \)-deficient medium than in 1 mM \( \text{Ca}^{2+} \) medium. Under this condition, bumetanide caused an additional rise in steady state \( ^{22}\text{Na}^+ \) uptake that was most pronounced in cells from SHR (21.3% versus 16.6% for Wistar rats and 4.8% for WKY). This finding indicates that a quantitatively greater inhibition of washout than of the uptake component of the bumetanide-sensitive \( ^{22}\text{Na}^+ \) transport occurs in \( \text{Ca}^{2+} \)-deficient medium. It is concluded that, in \( \text{Ca}^{2+} \)-deficient medium, the bumetanide-sensitive \( ^{22}\text{Na}^+ \) washout is higher in vascular smooth muscle cells of SHR than in those of normotensive controls and that this phenomenon reflects a higher \( \text{Na}^+ \) turnover in vascular smooth muscle cell in the hypertensive rat strain.

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**Key Words** genetic hypertension \( \cdot ^{22}\text{Na}^+ \) washout \( \cdot ^{22}\text{Na}^+ \) uptake \( \cdot \) extracellular calcium \( \cdot \) intracellular water volume

**M**ULTIPLE studies have examined the \( \text{Na}^+\text{,K}^+ \) cotransport system in erythrocytes of patients with essential hypertension and have generated conflicting data. Several groups reported reduced erythrocyte cotransport activity,\(^1\)\(^,2\) whereas others have shown unchanged\(^3\)\(^,4\) or higher\(^5\)\(^,6\) \( \text{Na}^+\text{,K}^+ \) cotransport activity in hypertensive patients. Moreover, to our knowledge, no study has compared the \( \text{Na}^+\text{,K}^+ \) cotransport system in the vascular smooth muscle of normotensive and hypertensive subjects. Yet, increased peripheral vascular resistance is the hallmark of the pathophysiology of established essential hypertension in humans and in its most commonly used experimental laboratory model, spontaneously hypertensive rats (SHR). Therefore, it is important to assess whether abnormalities in various transport systems, including the \( \text{Na}^+\text{,K}^+ \) cotransport system, exist in vascular tissues of hypertensive subjects and whether they can contribute to the increased peripheral vascular resistance associated with hypertension.

The present study was undertaken to assess whether a bumetanide-sensitive \( ^{22}\text{Na}^+ \) transport, assumed to represent \( \text{Na}^+\text{,K}^+ \) cotransport, is present in cultured rat vascular smooth muscle cells (VSMCs) and is more active in SHR as compared with normotensive controls.
Materials and Methods

The VSMCs were derived from carotid arteries of adult male SHR, Wistar-Kyoto rats (WKY), and Wistar rats (W) weighing between 250 and 300 g. Systolic blood pressure was measured in the awake animals with tail cuff plethysmography (IITC, Landing, NJ, USA). The methods used to obtain and maintain the cells have been published. All procedures were performed in parallel. Cells from passages 8 to 11 were examined.

The ion transport experiments were performed in situ in Nunc-24-well plates (Interlab, Thousand Oaks, CA, USA). Dulbecco’s modified Eagle’s medium (DMEM; 320-1885; GIBCO Laboratories, Grand Island, NY, USA) plus 2 mM L-glutamine and 10% fetal calf serum was used as the growth medium. The growth medium was removed before the transport studies were begun, and the cells in each well were washed twice with 1-ml aliquots of either DMEM containing 1 mM Ca²⁺ (for transport experiments in the presence of 1 mM Ca²⁺) or Ca²⁺-deficient DMEM (for transport studies in Ca²⁺-deficient medium). The latter medium was prepared specifically by GIBCO without calcium (formula 82-5179). Sodium pantothenate was substituted for calcium pantothenate in the DMEM. No attempt was made to prepare a Ca²⁺-free medium for use in these experiments. Bumetanide was provided by Dr. Peter Sorter, Hoffmann-LaRoche, Nutley, NJ, USA.) All transport experiments were stopped by aspiration of the media. The cells then were washed rapidly four times with 1-ml aliquots of ice-cold 0.1 M MgCl₂. The cells were extracted for 1 hour with 0.75 ml of 5% trichloroacetic acid, and their [²²Na⁺] activity was determined. Depending on the cell number in the wells, the ratio of the media to the total intracellular water volume (ICWV) in each well was between 1400 to 1900. Thus, the influence of “backflux” in these experiments was negligible.

In preliminary experiments, we examined the dose response of bumetanide on [²²Na⁺] washout. Maximal effect of bumetanide was observed at concentrations between 10⁻³ to 7.5 x 10⁻⁴ M. In further studies, we used bumetanide at concentrations of 7.5 x 10⁻⁴ M. The bumetanide was initially dissolved in ethanol. Final ethanol concentration in the medium was less than 0.1%. At these concentrations, there was no apparent ethanol effect on Na⁺ transport. In preliminary studies, we showed that in the presence of 1 mM ouabain, Na⁺ (145 mEq/L), and K⁺ (5 mEq/L) in the medium, the mean ratio of the values of the bumetanide-sensitive washout (exchange) rate constant (Kₑ) for [²²Na⁺] and the Kₑ for [⁸⁶Rb⁺] was 1.4. This value may reflect a stoichiometry of 3 Na⁺ to 2 K⁺ for the bumetanide-sensitive washout or, more likely, because of the inherent difficulty in this measurement, a stoichiometry of 1 Na⁺ to 1 K⁺.

The ICWV was measured by the equilibrium distribution of 3-α-methyl-D-glucose. Statistical analysis used linear regression and Student’s unpaired t test. The apparent Kₑ values were calculated according to the formula A = A₀ e⁻Kₑt, where A = the intracellular levels of [²²Na⁺] at a given incubation time, A₀ = the intracellular levels of the respective isotope at time 0, and t = incubation time. Data are presented as means ± SEM.

Results

The systolic blood pressure values of rats from which the VSMCs originated were as follows: 10 SHR, 190.3 ± 4.1; 7 WKY, 117.3 ± 4.4; and 8 W, 107.7 ± 4.2 mm Hg.

In 1 mM Ca²⁺ medium, the plateau phase of the Na⁺ uptake was higher for VSMCs from SHR than for VSMCs from WKY or W (p < 0.01; Figure 1). In these experiments, bumetanide had no apparent influence on the steady state (plateau phases) of Na⁺ uptake in the three cell preparations. All cell preparations showed a higher Na⁺ uptake in Ca²⁺-deficient medium than in 1 mM Ca²⁺ medium (p < 0.001; Figure 2). Under this condition, bumetanide caused a substantial increase in the steady state of Na⁺ uptake in VSMCs from SHR (21.3%; p < 0.001) and W (16.6%; p < 0.01). In contrast, cells from WKY showed only a minimal, though significant, increase in the Na⁺ uptake in response to bumetanide (4.8%; p < 0.05).

Figures 3 and 4 depict the washout of [²²Na⁺] from the VSMCs that were incubated in 1 mM Ca²⁺ or in Ca²⁺-deficient medium, respectively. The Kₑ values for the total and bumetanide-sensitive [²²Na⁺] washout are shown in Table 1. In 1 mM Ca²⁺ medium, the Kₑ values for the total Na⁺ washout were higher in cells from SHR and W than from WKY. Under this condition, the bumetanide-sensitive components of the total Kₑ values were not significantly different among the cell preparations. They constituted 18.8%, 23.0%, and 24.8% of the total Kₑ values for cells from WKY, W, and SHR, respectively. In Ca²⁺-deficient medium, however, both the total and bumetanide-sensitive Kₑ...
values of the VSMCs from SHR and W were significantly higher than those of the VSMCs from WKY. In Ca²⁺-deficient medium, the VSMCs from SHR also had a significantly higher Kᵣ value for the bumetanide-sensitive component than did the VSMCs from W. The respective contributions of the bumetanide-sensitive Kᵣ value to the total Kᵣ value in Ca²⁺-deficient medium were 8.8%, 16.6%, and 27.4% for VSMCs from WKY, W, and SHR. The effect of Ca²⁺, ouabain, and bumetanide on the ICWC is depicted in Figure 5. In the presence of 1 mM Ca²⁺, ouabain had no effect on ICWV. The addition of bumetanide to the 1 mM Ca²⁺ medium also did not exert any apparent influence on this parameter. Cells incubated in Ca²⁺-deficient medium in the presence of ouabain showed a significant increase in ICWV. The addition of bumetanide resulted in further increases in ICWV for the three cell preparations, which were significantly higher (SHR: 25.8%; p < 0.001; W: 23.3%; p < 0.001; WKY: 12.4%; p < 0.01) than the respective rises in ICWV of cells incubated in Ca²⁺-deficient medium with ouabain but without bumetanide.

Tables 2 and 3 present results of calculations of the Na⁺ content and concentration based on the equilibrium distribution ratio of ²²Na⁺ in the uptake experiments (see Figures 1 and 2). These data are likely to represent the exchangeable Na⁺ rather than the total cellular Na⁺. In 1 mM Ca²⁺ and Ca²⁺-deficient medium, the cells from SHR showed the highest intracellular Na⁺ content and concentration. The removal of extracellular Ca²⁺ clearly resulted in a marked increase of both Na⁺ content and concentration in all cell preparations (p < 0.001). Furthermore, the addition of bumetanide to 1 mM Ca²⁺ medium exerted minimal or no effect on the cellular Na⁺ content or concentration. The addition of bumetanide to Ca²⁺-deficient medium increased the Na⁺ content in the VSMCs from SHR (21.3%; p < 0.01), W (16.6%; p < 0.01), and WKY (4.8%; p < 0.05) but not the Na⁺ concentrations.

Discussion

The Na⁺,K⁺ cotransport system has been studied extensively in red blood cells; however, this transport system exists in other cells¹²−¹⁷ and probably in porcine cultured VSMCs.¹² The observations of a bumetanide-sensitive Na⁺ and K⁺ washout in our cell preparations suggest that Na⁺,K⁺ cotransport exists in cultured rat
Table 1. $^{22}$Na$^+$ Washout Rate Constants ($\times 10^{-2}$/min) for Vascular Smooth Muscle Cells from WKY, SHR, and Wistar Rats

<table>
<thead>
<tr>
<th>VSMC source</th>
<th>Total $^{22}$Na$^+$ (mM Ca$^{2+}$ medium)</th>
<th>Bumetanide-sensitive $^{22}$Na$^+$ (mM Ca$^{2+}$ medium)</th>
<th>Total $^{22}$Na$^+$ (Ca$^{2+}$-deficient medium)</th>
<th>Bumetanide-sensitive $^{22}$Na$^+$ (Ca$^{2+}$-deficient medium)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WKY</td>
<td>20.8 ± 0.14*</td>
<td>3.9 ± 0.33</td>
<td>19.7 ± 0.32*</td>
<td>1.7 ± 0.50*</td>
</tr>
<tr>
<td>Wistar</td>
<td>24.0 ± 0.59</td>
<td>5.5 ± 0.61</td>
<td>25.8 ± 0.19</td>
<td>4.3 ± 0.31</td>
</tr>
<tr>
<td>SHR</td>
<td>23.5 ± 0.72*</td>
<td>5.8 ± 0.76</td>
<td>26.1 ± 0.21*</td>
<td>7.2 ± 0.53*</td>
</tr>
</tbody>
</table>

Values are means ± SEM. Statistical analyses are based on 4 points in each line (see Figures 3 and 4). Each point represents 10 to 12 observations.

VSMC = vascular smooth muscle cell.

*p < 0.001, *p < 0.01, compared with values for VSMCs from Wistar rats; †p < 0.01, †p < 0.001, compared with values for VSMCs from SHR.

VSMCs. Earlier findings by De Mendonca et al.\(^{18, 19}\) suggested that cotransport in erythrocytes of SHR is lower than that in WKY. However, more recent studies by Wolowyk and Slosberg\(^{20}\) and Feig et al.\(^{21}\) indicate that the cotransport system is more active in erythrocytes of SHR than in those of WKY. Similar inconsistencies have underscored studies of Na$^+$,K$^+$ cotransport in erythrocytes of patients with essential hypertension.\(^{1-7}\) The discrepancies in studies of human erythrocytes may be partially related to heterogeneity of the patient populations with respect to variables such as geographic location, racial extraction, and degree of hypertension. It is also likely that the conflicting results pertaining to erythrocyte Na$^+$,K$^+$ cotransport reflect different technical approaches to study of...
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Central to studies of erythrocyte Na⁺,K⁺ cotransport and other transport systems in genetically transmitted hypertension is the concept that abnormalities in the cellular ionic regulations are generalized in this disorder. Hence, these abnormalities are also present in the vascular smooth muscle, where they can produce vasoconstriction and predispose to hypertension. Because of the discrepancies regarding Na⁺,K⁺ cotransport in erythrocytes of hypertensive subjects, this system must be examined directly in the VSMCs of these subjects.

The washout component of Na⁺,K⁺ cotransport usually has been examined in media devoid of Na⁺ and Ca²⁺ and in the presence of ouabain.18-19 In the present study, however, bumetanide-sensitive Na⁺ washout was measured in medium containing physiological concentrations of Na⁺ and K⁺ and while the Na⁺,K⁺ pump was likely to be fully active.

In the presence of 1 mM Ca²⁺, bumetanide had no apparent effect on Na⁺ uptake, whereas in Ca²⁺-deficient medium the drug had a distinct influence on the plateau phase of this uptake, which was particularly pronounced in VSMCs from SHR and W. This phenomenon can be explained by the following theory. Under normal conditions, the washout and uptake components of Na⁺,K⁺ cotransport appear to be at or near equilibrium.17 Therefore, in 1 mM Ca²⁺ medium, the net inhibitory effect of bumetanide on the washout and uptake components of the cotransport results in no

![Figure 5. Effect of various experimental manipulations on intracellular water volume (ICWV). Number of observations is indicated above each bar.](image)

TABLE 2. Intracellular Na⁺ Content and Concentration of Vascular Smooth Muscle Cells from WKY, SHR, and Wistar Rats in 1 mM Ca²⁺ Medium

<table>
<thead>
<tr>
<th>VSMC source</th>
<th>No bumetanide</th>
<th>0.75 M bumetanide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Na⁺ content (μEq/10⁶ cells)</td>
<td>Na⁺ concentration (mEq/L)</td>
</tr>
<tr>
<td>WKY</td>
<td>120.5 ± 3.52 (35)*</td>
<td>53.0 ± 2.19 (35)</td>
</tr>
<tr>
<td>Wistar</td>
<td>106.4 ± 3.56 (34)</td>
<td>48.5 ± 2.25 (34)</td>
</tr>
<tr>
<td>SHR</td>
<td>137.1 ± 4.77 (35)†§</td>
<td>64.1 ± 3.07 (35)†§</td>
</tr>
</tbody>
</table>

Values are means ± SEM. Number of observations shown in parentheses. Cells were incubated for 75 minutes in the presence of 1 mM ouabain.

VSMC = vascular smooth muscle cell.

*p < 0.01, †p < 0.05, §p < 0.001, compared with values for VSMCs from Wistar rats; †p < 0.01, compared with values for VSMCs from WKY.

TABLE 3. Intracellular Na⁺ Content and Concentration of Vascular Smooth Muscle Cells from WKY, SHR, and Wistar Rats in Ca²⁺-deficient Medium

<table>
<thead>
<tr>
<th>VSMC source</th>
<th>No bumetanide</th>
<th>0.75 M bumetanide</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Na⁺ content (μEq/10⁶ cells)</td>
<td>Na⁺ concentration (mEq/L)</td>
</tr>
<tr>
<td>WKY</td>
<td>200.2 ± 5.12 (32)</td>
<td>79.2 ± 3.31 (32)</td>
</tr>
<tr>
<td>Wistar</td>
<td>224.9 ± 4.13 (30)*</td>
<td>89.8 ± 3.80 (30)</td>
</tr>
<tr>
<td>SHR</td>
<td>278.9 ± 8.45 (34)†§</td>
<td>110.6 ± 5.47 (34)†§</td>
</tr>
</tbody>
</table>

Values are means ± SEM. Number of observations shown in parentheses. Cells were incubated for 75 minutes in the presence of 1 mM ouabain.

VSMC = vascular smooth muscle cell.

*p < 0.001, †p < 0.05, compared with values for VSMCs from WKY; †p < 0.001, §p < 0.01, compared with values for VSMCs from Wistar rats.
change in the plateau phase of Na⁺ uptake. In Ca²⁺-deficient medium, however, the cells become more permeable to Na⁺, 22−24 with a marked rise in the intracellular Na⁺ content and concentration. This condition favors an increase in the washout component of Na⁺ through the cotransport system. Thus, in Ca²⁺-deficient medium, bumetanide exerts a quantitatively greater inhibitory effect on the washout than on the uptake component of Na⁺, K⁺ cotransport, with the net effect of producing an increase in steady state Na⁺ uptake.

In other experiments, we have shown that, in the presence of Ca²⁺ in the medium, VSMCs from SHR manifested higher Na⁺ uptake and washout rate constants than did controls. 22 Furthermore, when extracellular Ca²⁺ was removed, a greater increase in cellular Na⁺ and Na⁺, K⁺ pump activity was observed in cells from SHR than in cells from normotensive rats. These observations support the notion of higher turnover of Na⁺ in VSMCs from SHR, a phenomenon that is consistent with findings of the present investigation. Also of interest is that the removal of extracellular Ca²⁺ resulted in a greater increase in the washout component of Na⁺, K⁺ cotransport in the VSMCs from SHR than in those from W or WKY.

In both 1 mM Ca²⁺ and Ca²⁺-deficient medium, the bumetanide-sensitive K⁺ values were highest in VSMCs from SHR and lowest in cells from WKY. These differences were not significant in 1 mM Ca²⁺ medium, but they were highly significant in Ca²⁺-deficient medium. The reason for this difference is not clear. It is possible, however, that removal of extracellular Ca²⁺, besides causing a greater increase in Na⁺ uptake in the VSMCs from SHR than in those from W or WKY, also alters intrinsic properties related to Na⁺, K⁺ cotransport (i.e., the affinity of the Na⁺, K⁺ carrier to intracellular Na⁺).

The uptake studies also support the findings of higher bumetanide-sensitive Na⁺ washout in SHR compared with normotensive controls in Ca²⁺-deficient medium. In these experiments, bumetanide exerted an effect on the Na⁺ uptake that was stratified in the same manner as the K⁺ values for the bumetanide-sensitive Na⁺ washout. Chipperfield 25 showed that extracellular Ca²⁺ inhibited the washout component of human erythrocyte cotransport in a medium of 142 mM NaCl and 8 mM KCl. These results may actually reflect the effect of extracellular Ca²⁺ on Na⁺ uptake in these cells. In other words, in the presence of extracellular Ca²⁺, these cells are less permeable to Na⁺ and, thus, the magnitude of the Na⁺ washout component mediated by Na⁺, K⁺ cotransport is lower.

Finally, the impact of various experimental perturbations on ICWV has been poorly explored. Some of the changes in ionic transport may lead to changes in ICWV. Conversely, alterations in the cell membrane that lead to a change in ICWV may also change transport processes. The question of whether a change in ICWV due to an experimental perturbation is primary or secondary is not always resolved. It is clear that in our experiments removal of extracellular Ca²⁺ resulted in a disproportionate increase in Na⁺ content as compared to the rise in ICWV (11.2%, 12.5%, 18.0% rise in ICWV; 66.1%, 111.3%, 103.4% increase in cellular Na⁺ content for VSMCs of WKY, W, and SHR, respectively). This phenomenon resulted in a rise of the intracellular Na⁺ concentration. In contrast, the addition of bumetanide to 1 mM Ca²⁺ medium did not influence intracellular Na⁺ content or concentration. In Ca²⁺-deficient medium, however, the drug appeared to exert equivalent effects on Na⁺ content and ICWV, since the intracellular Na⁺ concentrations for each cell preparation were not statistically different with or without bumetanide. These findings suggest a role for Na⁺, K⁺ cotransport in cell volume regulation. 26 It should be emphasized, however, that with respect to changes in ICWV, the VSMCs from WKY were the least sensitive and those from SHR were the most sensitive to incubation in Ca²⁺-deficient medium with or without bumetanide (see Figure 5).

Taken together, it can be concluded from the measurements of ICWV and Na⁺ that 1) incubation in a Ca²⁺-deficient medium (in the presence of ouabain) results in a substantial increase in ICWV, Na⁺ content, and Na⁺ concentration and 2) when incubated in a Ca²⁺-deficient medium, VSMCs from SHR manifest the highest ICWV, Na⁺ content, and Na⁺ concentration among the three rat strains.

In conclusion, the greatest effect of bumetanide on Na⁺ uptake and washout was observed in VSMCs from SHR in a Ca²⁺-deficient medium. This finding appears to relate to the Na⁺, K⁺ cotransport–mediated inhibitory effect of bumetanide on Na⁺ washout. Removal of extracellular Ca²⁺ affected the intracellular Na⁺ concentration and ICWV in VSMCs from SHR more than in cells from WKY or W. The present study also illustrates that specific experimental conditions may substantially influence results of Na⁺, K⁺ cotransport measurement.

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