Prolonged Exposure to Ouabain Eliminates the Greater Norepinephrine-Dependent Calcium Sensitivity of Resistance Vessels in Spontaneously Hypertensive Rats

HOLGER NILSSON M.D., AND MICHAEL J. MULVANY PH.D.

SUMMARY The effects of 30 minutes of exposure to ouabain on calcium sensitivity have been investigated in two types of resistance vessels from 12 pairs of spontaneously hypertensive (SHR) and Wistar-Kyoto (WKY) rats. Branches of the superior mesenteric and femoral arteries, with internal diameters of about 200 \( \mu m \), were mounted as ring preparations in a myograph capable of measuring their isometric wall tension. Dose-response curves for calcium upon norepinephrine stimulation were determined under conditions where neuronal uptake was eliminated. Initially, when stimulated with norepinephrine, the SHR vessels from both locations were more sensitive to calcium and had stronger contractions than their controls. The addition of ouabain (1 mM) to the relaxed vessels immediately elicited a moderate, transient contraction in the branches of the femoral artery, whereas no response was observed in the mesenteric vessels. Although the addition of ouabain to activated vessels produced an immediate potentiation of the response, prolonged (30-minute) exposure to ouabain reduced active tension development upon norepinephrine stimulation in all vessels. The reduction was greatest in the SHR vessels, so that, under these conditions, the norepinephrine-activated calcium sensitivity of corresponding SHR and WKY vessels was similar. By contrast, responses to norepinephrine in high potassium solution were unaffected. The results suggest that under normal conditions, SHR vessels may have a specific increase in the permeability of the norepinephrine-activated calcium channels. Prolonged exposure to ouabain appears to reduce the permeability of these channels, providing an explanation for why this treatment eliminates the difference in calcium sensitivity of the SHR and WKY vessels.

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KEY WORDS • spontaneously hypertensive rat • calcium • norepinephrine • ouabain • resistance vessels

THE effects of cardio glycosides, in particular ouabain, on the cardiovascular system have been widely investigated. In the myocardium, ouabain has in general a positive inotropic effect. In the vasculature, three effects of ouabain have been described. The addition of ouabain may increase resting tone in the vessel. Immediately following ouabain addition there is an increased sensitivity to norepinephrine (NE). Following prolonged exposure to ouabain we have found a depression of the response to NE stimulation.

Many factors may be involved in causing these effects. First, ouabain causes depolarization of the smooth muscle cells. Second, inhibition of the sodium-potassium pump causes changes in the intracellular ionic composition. Third, ouabain inhibits neuronal uptake of NE, and also causes release of NE from the nerve endings.

There are a number of reports that suggest that in genetic (as opposed to volume-expanded) forms of hypertension the sodium-potassium pump activity of the vasculature is increased. An increased sodium-potassium pump activity in erythrocytes is also seen in moderate forms of human essential hypertension. Therefore, it might be expected that the effects of blocking the sodium-potassium pump with ouabain would be greater in spontaneously hypertensive rats (SHRs) compared with normotensive Wistar-Kyoto rats (WKYs). This is indeed the case. The immediate potentiating effects of ouabain on the sensitivities of perfused vascular beds to NE, vasopressin, and...
barium are all greater in SHRs compared with WKYs. At calcium concentrations above the physiological range, ouabain increases the maximal tension development of caudal artery strips; this occurs to a pronounced extent in SHRs.

We have previously shown that the NE-activated calcium permeability of mesenteric resistance vessels from SHRs is greater than that for WKYs. In this study we have investigated the effects of prolonged (30-minute) exposure to ouabain on this calcium sensitivity. To answer whether the effects are of general significance for the circulation, we have examined both splanchnic and muscular 200-µm resistance vessels. Results suggest that the long-term depressive action of ouabain results from a specific decrease in the NE-activated calcium permeability of the vascular smooth muscle cells. Thus, while the SHR vessels had a greater calcium sensitivity than the WKY vessels before ouabain treatment, after treatment the sensitivity of all vessels was reduced. The reduction was greatest in the SHR vessels, however, so that after treatment there was no difference in the calcium sensitivities of corresponding SHR and WKY vessels.

Methods

Animals

Vessels were taken from 14-week-old spontaneously hypertensive rats (SHR) and normotensive Wistar-Kyoto rats (WKY); 12 rats of each strain were obtained from Mållegaards Avlslaboratorium, LI. Skensved, Denmark. After anesthetization with ether or, in some cases, ketamine (Ketalar, Parke-Davis), the blood pressure (BP) was measured via a tail artery catheter. The mean blood pressure (MAP) was 127 ± 4.3 mm Hg in SHRs and 97 ± 3.5 mm Hg in WKYs. The ratio of heart ventricle to body weight was 3.20 ± 0.14 in SHRs and 3.52 ± 0.11 in WKYs (WKY). All these differences were significant (p < 0.01).

Preparation and Apparatus

Vessels used were either 150–200 µm branches of the superior mesenteric artery from the most proximal arcade feeding the jejunum (mesenteric vessels), or the second-generation muscular branch of the femoral artery passing over the m. adductor magnus into the gracilis muscle (femoral vessels). Segments of these vessels (1–2 mm long) were dissected free from surrounding tissue and mounted as ring preparations on a myograph capable of measuring their isometric wall tension. The myograph was further developed on a myograph capable of measuring their isometric wall tension. The myograph was further developed on a myograph capable of measuring their isometric wall tension. The myograph was further developed on a myograph capable of measuring their isometric wall tension. The myograph was further developed on a myograph capable of measuring their isometric wall tension. The myograph was further developed on a myograph capable of measuring their isometric wall tension. The myograph was further developed on a myograph capable of measuring their isometric wall tension.

Solutions

The physiological salt solution (PSS) used had the following composition (mM): NaCl, 119; NaHCO₃, 25; KCl, 4.7; KH₂PO₄, 1.18; MgSO₄, 1.17; CaCl₂, 2.5; ethylenediaminetetraacetic acid (EDTA), 0.026; glucose, 5.5. In the calcium dose-response experiments, the CaCl₂ concentration was varied as required. Ethyleneglycol-bis-(β-aminoethylether)-N,N-tetraacetic acid (EGTA) was added as described below. Norepinephrine-potassium physiological salt solution (NE-K-PSS) was PSS where the NaCl was replaced by an equimolar amount of KCl, and 10 µM NE was added.

Solutions were adjusted to pH 7.4 and bubbled with 5% CO₂ in O₂ at 37°C. Drugs used were 1-NE-HCl (Sigma), cocaine chloride (May and Baker), and ouabain (Merck).

Protocol

After vessels had been equilibrated in PSS for about 1 hour and set to the internal circumference of L₁, they were stimulated for 2 minutes for 3 times with NE-K-PSS. Between stimulations, vessels were washed in PSS for 5 minutes. The last of these responses was taken as a measure of the vessel contractility (table 1, line 1). Calcium dose-response determinations were then performed as follows. First, the vessels were depleted of calcium by exposing them for 10 seconds to 5 mM EGTA in calcium-free PSS, followed by repeated

### Table 1. Active Wall Tension Responses of Mesenteric and Femoral Vessels in SHR and WKY

<table>
<thead>
<tr>
<th>Stimulation</th>
<th>Mesenteric vessel</th>
<th>Femoral vessel</th>
<th>P₁strain</th>
<th>P₁vessel</th>
<th>P₉</th>
<th>ns</th>
<th>ns</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 SHR</td>
<td>3.5 ± 0.29</td>
<td>3.7 ± 0.43</td>
<td>&lt; 0.01</td>
<td>na</td>
<td>na</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2 SHR</td>
<td>3.6 ± 0.33</td>
<td>4.0 ± 0.37</td>
<td>&lt; 0.001</td>
<td>na</td>
<td>na</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 SHR</td>
<td>3.6 ± 0.33</td>
<td>3.9 ± 0.54</td>
<td>&lt; 0.001</td>
<td>na</td>
<td>na</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Active wall tension responses in mN/mm (force developed divided by twice segment length) upon stimulation with NE-K-PSS (10 µM norepinephrine in high potassium solution) at start of the experiment (line 1), immediately following the first calcium dose-response determination (line 2), and immediately following the calcium dose-response determination in ouabain (line 3). Data compared for each line by analysis of variance, as described in Methods. Values given are from 12 mesenteric and nine femoral vessels except in Experiment 3 where they are from 11 and 8 respectively. p₉ = interaction term; ns = not significant (p > 0.05).
stimulation with norepinephrine in calcium-free PSS containing 0.1 mM EGTA. Then, the calcium concentration was increased successively from 0 to 2.5 mM at 4-minute intervals. During the second half of these intervals the vessels were stimulated with 10 µM NE in the presence of 3 µm cocaine (to inhibit the neural uptake of NE). These solutions did not contain EGTA. The vessels were then soaked in 1 mM ouabain for 30 minutes to inhibit the Na-K-ATPase, whereafter the experiment was repeated in the presence of ouabain. In control experiments on 10 pairs of vessels, the NE dose used (10 µM) was found to produce a maximal response both in the absence and presence of ouabain. One minute after each dose-response determination, the vessels were stimulated with NE-K-PSS to check the viability of the preparations (table 1, lines 2 and 3). Throughout, the tension values reported are mean values after 2 minutes of stimulation at the dose concerned. For each dose-response determination, the negative logarithm of the dose of agonist required to give half maximal response (pD₂) was calculated.

Statistics
Where indicated, the data have been statistically tested by analysis of variance. Elsewhere, differences were tested using the two-tail t test. For the dose-response curves presented in figure 1, analysis of variance was used to test for possible difference in the SHR and WKY characteristics by examining the effects of the different doses in the two strains. Here if the interaction term was not significant, the characteristics could be directly compared. If the interaction term was significant (p₂ < 0.05), this indicated that for at least one dose there was a significant difference in response. In this case, the responses for each dose were then compared using the t test. Values are given as means ± se.

Results
Mechanical Properties
The mechanical responses of the vessels at the start of the experiments are shown in table 1 (line 1). Following maximal activation with NE-K-PSS, the SHR vessels contracted more strongly than the WKY vessels. There was no difference in the responses of corresponding mesenteric and femoral vessels. These responses were essentially the same following each of the calcium dose-response determinations described below (table 1, lines 2 and 3).

Calcium Dose-Response Characteristics
As seen in figure 1, SHR mesenteric vessels had a greater NE-activated calcium sensitivity than the corresponding WKY vessels (Ca-pD₂ was

![Figure 1. Calcium dose-response characteristics of 12 pairs of mesenteric resistance vessels (left graph) and nine pairs of femoral resistance vessels (right graph) from spontaneously hypertensive (SHR, closed symbols) and Wistar-Kyoto rats (WKY, open symbols). Vessels were activated with 10 µM norepinephrine (in the presence of 3 µM cocaine, following the protocol indicated in fig. 3). Responses normalized with respect to responses with 1.6 mM calcium. The absolute values of the responses at 1.6 mM calcium were (left graph) 2.8 ± 0.24 mN/mm (SHR), 2.0 ± 0.16 mN/mm (WKY); (right graph) 2.9 ± 0.26 mN/mm (SHR), 2.2 ± 0.21 mN/mm (WKY). Dose-response curves were compared by analysis of variance (see Methods). In the left graph, the interaction term (p₂) was significant (p₂ < 0.001), indicating a difference in the shape of the curves; therefore, Student's t test has been used for each dose. *** = p < 0.001. In the right graph, p₂ < 0.001; p₂train < 0.01; and p₂ is not significant (p₂ > 0.05). Bars indicate SE.](http://hyper.ahajournals.org/)}
4.32 ± 0.04 (SHR, 12 vessels), 3.86 ± 0.06 (WKY, 12 vessels). The characteristic for the SHR femoral vessels also differed (p < 0.01) from its control, although the Ca-pD₄ for the femoral vessels was not significantly different (Ca-pD₄ was 4.07 ± 0.08 (SHR, 9 vessels), 3.91 ± 0.06 (WKY, 9 vessels)).

Effects of Ouabain

Figure 2 shows the effect of 1 mM ouabain on vessels held relaxed in PSS. With the addition of ouabain, six of nine SHR and seven of nine WKY femoral vessels contracted rapidly (within 1 minute) to about 10% of the NE-K-PSS response. This contraction then slowly declined and disappeared after 15 to 20 minutes. A small, short-lasting (5-minute) response of this kind was seen in one SHR mesenteric vessel. No other mesenteric vessel responded to ouabain alone. In control experiments we found that the responses of the femoral vessels could not be inhibited by phentolamine (1 μM).

When the calcium dose-response determination was repeated after vessels had been held in ouabain for 30 minutes, the form of the responses became drastically different (fig. 3). The initial fast phase of the responses was not affected (fig. 3, small arrows). However, the subsequent part of the response was generally depressed by the ouabain treatment. These subsequent parts of the responses (mean 2-minute values) are compared in figure 4. The responses were reduced at all calcium concentrations above 0.05 mM, and, more important, the difference between the rat strains as noted previously was abolished throughout. The characteristics for the mesenteric and femoral vessels were similar up to a calcium concentration of about 1 mM. At higher calcium concentrations, the responses of the femoral vessels were not as depressed as the mesenteric ones.

The depression of the responses did not appear to be the result of an inactivation of the contractile apparatus, for the NE-K-PSS responses immediately

![Figure 2. Effect of ouabain on femoral (upper) and mesenteric (lower) resistance vessels held relaxed on double myograph in physiological salt solution (PSS). During the time indicated by the open bar, the vessels were activated maximally with 10 μM norepinephrine (NE) in high potassium solution (NE-K-PSS) and then relaxed in PSS. Arrow indicates the solution was changed to PSS containing 1 mM ouabain. Segment length for the femoral vessel was 1.58 mm; effective lumen diameter (Lᵅ/π) was 218 μm. Segment length for the mesenteric vessel was 1.8 mm; Lᵅ/π was 178 μm.](image)

![Figure 3. Calcium dose-response records of SHR and WKY mesenteric resistance vessels obtained under control conditions (upper pair of records) and in the presence of 1 mM ouabain after soaking in 1 mM ouabain for 30 minutes (lower pair of records). Both vessels were mounted on a double myograph and exposed simultaneously to the same solutions. Vessels were initially (not shown) depleted of calcium by repeated norepinephrine (NE) stimulation in calcium-free solution. The calcium concentration was then raised successively, as indicated by the values at the top showing calcium concentration of the solutions (in mM). During the second half of each exposure period, vessels were activated with 10 μM NE (in the presence of 3 μM cocaine, as indicated by the black bars). On completion of the dose-response determination, vessels were maximally activated with 10 μM NE in high potassium solution (NE-K-PSS), as indicated by the open bar. Note widening of the trace of the SHR vessel on control stimulations, indicating rhythmic activity, most pronounced at large arrow. Small arrows indicate, as examples, initial fast phase of contractions; note that this phase is not substantially affected by the ouabain treatment. Vessel segment lengths were (SHR) 1.68 mm, (WKY) 1.77 mm; effective lumen diameters (Lᵅ/π) were (SHR) 156 μm, (WKY) 143 μm.](image)
following the dose-response determinations with ouabain (table 1, line 3) did not differ significantly from the NE-K-PSS responses obtained at the start.

Rhythmic Activity

During the calcium dose-response determinations, we noted that nine of the 12 SHR mesenteric vessels showed rhythmic contractions with a period of about 5 seconds superimposed on a stable contraction. After ouabain treatment, these rhythmic variations were not seen. None of the other vessels displayed any rhythmic activity at any time.

Discussion

The main findings of this study are that 1) the SHR resistance vessels had a greater NE-activated calcium sensitivity than the corresponding WKY vessels; 2) treatment with ouabain for 30 minutes reduced the calcium sensitivity in all vessels, but more in the SHR vessels; and 3) after ouabain treatment, the calcium sensitivity was therefore the same in the SHR and WKY vessels. Ouabain thus appeared to eliminate the difference in calcium sensitivity of SHR and WKY vessels. A secondary finding, which will not be discussed here, is that the increased contractility previously seen in SHR mesenteric resistance vessels was also seen in SHR femoral vessels (table 1).

Calcium Sensitivity

In both vessel types, a greater NE-activated calcium sensitivity was found in the SHRs. However, the difference between the rat strains was smaller in the femoral vessels, where no significant difference in CaPDr-values was found. An increased NE-activated calcium sensitivity in SHRs has been reported previously for mesenteric vessels as well as for portal veins. Calcium sensitivity was also increased in the smooth muscle of the stomach fundus of SHRs. Thus, it seems that there may be a general abnormality in calcium permeability in the SHR smooth muscle cell membrane.

Effect of Ouabain on Resting Vessels

Ouabain caused an immediate, transient increase in resting tension in both SHR and WKY femoral vessels but had no effect on mesenteric vessels. In rat tail and femoral arteries, Bonaccorsi et al. found that potassium-free solution (which also inhibited the Na-K-ATPase) induced a slow, long-lasting contraction due to NE release. However, Karaki et al. found in the rabbit aorta that, although ouabain induced a biphasic contraction, the first part of which was inhibited by a-receptor blockade with phentolamine, potassium-free solution had no effect on the tone of this vessel. These different responses may indicate that tension development is not due solely to inhibition of the pump.

As noted above, contraction of the femoral vessels in response to ouabain was immediate. This makes it unlikely that an increased intracellular sodium concentration is the cause. Furthermore, because we found that the contraction was not affected by phentolamine, it is unlikely to be caused by transmitter
release from the intramural nerves, but rather by smooth muscle cell depolarization. However, the transient nature of the contraction and the reason why it was seen only in the femoral vessels still need to be explained.

**Effect of Ouabain on Activated Vessels**

The other effect of ouabain studied here is the reduction of the response to maximal NE stimulation following prolonged exposure to ouabain. This does not appear to be due to a general depressing action of ouabain, since the NE-K-PSS responses were unaffected in spite of the marked reduction of the responses to NE alone. We also found previously that this effect of ouabain is reversible. Furthermore, the NE dose producing maximum response is not appreciably altered by 30 minutes of ouabain treatment (see Methods). Therefore, we suggest that this effect of ouabain is due to a decrease of the calcium permeability of the cell membrane. This possibility is supported by the shape of the calcium dose response curves following ouabain treatment, which were then almost linear and did not appear to have reached their maximum value at 2.5 mM calcium.

In a model presented by Bolton, calcium is supposed to enter the cell through two kinds of "channels." One kind is a potential-sensitive channel, which is opened to calcium upon depolarization of the cell. The other channel is thought to be opened through the action of an agonist upon a receptor. Norepinephrine is thought to activate primarily the latter channels, whereas, for example, a high extracellular potassium concentration would depolarize the cell and thereby open the potential-sensitive channels. That the response to NE-K-PSS (thought to open both types of channels) is unaffected by ouabain would indicate that the potential-sensitive channels are insensitive to the ouabain treatment. We would therefore suggest that the observed depressive effects of ouabain are due to a decreased calcium permeability of the NE-activated channels.

In a preliminary study of this depressive effect of ouabain, we found similar results after 30 minutes of incubation in potassium-free medium. This suggests that the studied effect is a result of inhibiting the Na-K-ATPase. However, as the effect is first clearly visible some time after stopping the pump, it is likely to be a secondary effect.

**Intracellular Sodium Level**

The increased Na-K-ATPase activity in SHRs described in the Introduction may be secondary to an increased permeability of the cell membrane to sodium and potassium. The tendency toward an increased intracellular sodium concentration thus created stimulates the Na-K-pump to increased activity. According to Blaustein, the intracellular sodium level is of great importance in determining the excitability of the cell. Calcium is thought to be extruded from the cell by an exchange mechanism, the concentration gradient for sodium being the driving force. An increased sodium level within the cell would diminish this driving force, causing the intracellular calcium level to rise and thereby increasing the mechanical excitability of the cell. The data presented here are not easily explained by this hypothesis, however.

At a time when the intracellular sodium level almost certainly is increased, the calcium sensitivity of the cell is decreased, and even more so in the SHR vessels, which probably would have the highest sodium concentration. Of course, the results presented do not exclude the possibility of a simultaneous decrease in sodium-dependent calcium extrusion and norepinephrine-dependent calcium entry. However, the results do not support the hypothesis that vascular sodium-calcium exchange is of crucial importance in genetic hypertension. As discussed by van Breemen and his colleagues, the role of sodium-calcium exchange in vascular smooth muscle still needs to be thoroughly investigated.

**Na-K-ATPase and Genetic Hypertension**

That long-term ouabain treatment eliminates the increased NE-dependent calcium sensitivity of the SHR vessels implies that ouabain affects the SHR vessels more strongly. This is a common finding. As discussed in the Introduction, the immediate potentiating effects of ouabain are increased in the SHR vasculature. Furthermore, Hermosmeyer has shown that ouabain causes a greater than normal depolarization in the SHR caudal arteries. These findings are consistent with the evidence for an increased Na-K-ATPase activity in SHRs provided by Pamnani and colleagues, who showed an increased uptake in SHR caudal arteries. It is perhaps also significant that it was the SHR mesenteric vessels that exhibited rhythmic activity. It has been suggested that slow waves in intestinal smooth muscle are a function of spontaneous changes in Na-K-ATPase activity. If this is also the case for vascular smooth muscle, rhythmic activity could be expected to be prevalent in vessels having a high Na-K-ATPase activity.

On the basis of the model described above, the long-term effect of ouabain is compatible with that of ouabain decreasing the permeability of (and perhaps blocking) the NE-activated calcium channels. These channels, however, appear to have an increased calcium permeability in the SHR vessels. These two deductions, taken together, therefore provide a ready explanation for why the SHR and WKY vessels have the same calcium sensitivity after ouabain treatment. Blocking the NE-activated calcium channels would leave the vessels with the potential-dependent calcium channels, which appear to be similar in SHR and WKY vessels.

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