Ouabain
A Stimulator of Atrial Natriuretic Peptide Secretion
and Its Mechanism of Action

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Ouabain increases atrial natriuretic peptide (ANP) secretion. When isolated superfused rat left atria were paced at 2 Hz, ouabain at concentrations of 50, 100, and 200 μM increased ANP secretion by 2.0±0.3-, 3.2±0.5-, and 4.2±0.5-fold, respectively. In this study, we examine the mechanism of ouabain-stimulated ANP secretion using the dose of 100 μM. To determine whether calcium played a role, atria were superfused with the calcium antagonist lanthanum. Superfusion with 2 mM LaCl₃, completely inhibited ouabain-stimulated secretion, suggesting that calcium influx and/or sarcoplasmic reticulum (SR) calcium release provide essential sources of calcium for the stimulatory pathway. To determine the contribution of calcium from the SR, atria were superfused with ryanodine, an agent that depletes the SR of calcium. Superfusion with 1 μM ryanodine inhibited ouabain-stimulated secretion by 47%. Inhibition of Na⁺,K⁺-ATPase allows sodium to accumulate in the cell. A rise in intracellular sodium alters Na⁺-Ca²⁺ exchange, leading to an increase in cytosolic calcium. To determine the mechanism of sodium entry, atria were superfused with 5-(N,N-hexamethylene)amiloride (HMA), an inhibitor of Na⁺-H⁺ exchange, or with bumetanide, an inhibitor of Na⁺-K⁺-Cl⁻ cotransport. Superfusion with 25 μM HMA inhibited ouabain-stimulated secretion by 71%; however, 100 μM bumetanide had no significant effect on secretion. Ouabain failed to stimulate ANP secretion by nonpaced (nonbeating) atria. Likewise, superfusion with the combination of ryanodine (1 μM) and the calcium channel antagonist isradipine (10 μM) totally blocked ouabain-stimulated ANP secretion. We conclude as follows: 1) Calcium influx and SR calcium release contribute equally to the secretory response. 2) Calcium influx through the Na⁺-Ca²⁺ exchanger is inadequate to stimulate secretion when SR calcium release and calcium channels are blocked. 3) The primary mechanism of sodium entry required for stimulation is through Na⁺-H⁺ exchange, with Na⁺-K⁺-Cl⁻ cotransport playing little to no role. 4) By inference, sodium entry by means of the fast sodium channel also plays a role in ouabain-stimulated secretion. These results lend further support to the idea that calcium is an important second messenger in regulated secretion of ANP. (Circulation Research 1993;72:1035-1043)

KEY WORDS • ouabain • atrial natriuretic peptide secretion • calcium • sarcoplasmic reticulum • Na⁻⁻H⁺ exchange • Na⁻⁻Ca²⁺ exchange • ryanodine • isradipine • lanthanum • Na⁺⁻K⁺⁻Cl⁻ cotransport • bumetanide • 5-(N,N-hexamethylene)amiloride

Inhibition of Na⁺,K⁺-ATPase activity with ouabain increases atrial natriuretic peptide (ANP) secretion.1-4 This may have physiological implications. Recently, a potent inhibitor of Na⁺,K⁺-ATPase has been isolated from human plasma5 and found to be indistinguishable from ouabain.6 This endogenous ouabain-like compound sensitizes vascular smooth muscle to vasoconstrictors.7 As a result, it may have a tendency to increase blood pressure. However, ouabain increases ANP secretion, which may counter the hypertensive effects of ouabain, since ANP is a potent hypotensive hormone. Thus, these observations may reflect a new loop in blood pressure regulation.

The mechanism of ouabain-stimulated secretion is not known. In this study, we examine the role that calcium may play in ouabain stimulation, since the positive inotropic effects of ouabain are associated with a rise in cytosolic calcium.8,9 Likewise, calcium has been found to play an important role in isoproterenol, phenylephrine, potassium, and endothelin-stimulated ANP secretion.10-13

Inhibition of Na⁺,K⁺-ATPase activity leads to an accumulation of sodium. In cardiac tissue, a rise in intracellular sodium alters the activity of Na⁺-Ca²⁺ exchange. This exchanger may move calcium into or out of the cell.14-17 The direction of movement is primarily dependent on the intracellular sodium concentration and on membrane potential. Under normal circumstances, the exchanger is poised to remove calcium from the cell, i.e., to remove the quantity of calcium that has entered the cell primarily through calcium channels during membrane depolarization.18 However, a rise in intracellular sodium leads to an increase in intracellular calcium by either decreasing calcium efflux and/or increasing calcium influx.14-17 Either mechanism leads to
an increase in intracellular calcium. In this study, we examined the role of calcium influx on ouabain-stimulated ANP secretion by blocking its transmembrane movement with lanthanum.

Ouabain has been found to increase diastolic calcium. A rise in diastolic calcium increases calcium uptake into the sarcoplasmic reticulum (SR), thereby expanding the SR calcium storage pool. A larger pool of stored calcium increases the amount of calcium released from the SR with each action potential. To determine whether enhanced calcium release from the SR is playing a role in ouabain-stimulated ANP secretion, ryanodine was used to deplete the SR of calcium.

The second goal of this study was to determine the mechanism of intracellular sodium accumulation. Two mechanisms of sodium influx were examined, Na+-K+-Cl- cotransport and Na+-H+ exchange. Ouabain has been found to activate Na+-K+-Cl- cotransport in 3T3 cells. To determine whether this was a significant mechanism of sodium influx, bumetanide was used to block Na+-K+-Cl- cotransport. Inhibition of Na+-H+ antiporter activity has been shown to block sodium accumulation induced by ouabain. This may be due in part to the fact that ouabain lowers pH. In this study, 5-(N,N-hexamethylene)amiloride (HMA) was used to block the Na+-H+ antiporter.

Materials and Methods

Materials

Materials were purchased from the following sources: ouabain, propranolol, atropine, and LaCl3 from Sigma Chemical Co., St. Louis, Mo.; ryanodine from Progress Agri-Systems, Wind Gap, Pa.; medium 199 from Gibco, Grand Island, N.Y.; and rat αANP and ANP antibodies from Peninsula Laboratories, Belmont, Calif. HMA was synthesized for this study as previously described. Isradipine, bumetanide, and phenolamine were gifts from Sandoz Pharmaceuticals Corp., East Hanover, N.J., from Roche Laboratories, Nutley, N.J., and from CIBA-GEIGY, Suffern, N.Y., respectively.

Experimental Design

Atrial superfusion experiments were performed as previously described with isolated rat left atria superfused with modified medium 199 containing Earle’s salts with 20 mM NaHCO3 and 4 mM KCl. All studies were performed in the presence of 1 μM propranolol, 10 μM phenolamine, and 10 μM atropine to negate possible neurotransmitter release during superfusion with ouabain. Low superfusate calcium experiments were performed by lowering the calcium concentration from 1.8 to 0.2 mM 20 minutes before the addition of ouabain. This was achieved by superfusion with modified medium 199 containing 0.2 mM CaCl2. Isradipine, ryanodine, bumetanide, and LaCl3 were added to the superfusate 55 minutes before beginning the sample collection and were continued until the termination of the experiment. Isradipine, ryanodine, and bumetanide were added in ethanol with a final concentration of 0.1%. HMA was added in dimethyl sulfoxide (final concentration, 0.1%) and was added to the superfusate 10 minutes before the addition of ouabain. All control atria were superfused with the same ethanol or dimethyl sulfoxide concentrations as the atria receiving the inhibitor. Lanthanum experiments were performed with modified medium 199 containing 1 mM CaCl2 and no phosphate, bicarbonate, or sulfate anions, which form a precipitate with lanthanum. Medium 199 used for these experiments was buffered with 10 mM HEPES and gassed with 100% oxygen. Tension measurements were obtained from a strip-chart recording of atrial performance using a Gould six-channel rectilinear oscillographic recorder.

ANP Radioimmunoassay

ANP measurements were performed on timed fractions of the superfusate by radioimmunoassay as previously described.

Data Analysis

Statistical analyses were performed by paired or unpaired t test or by one-way analysis of variance. Results are expressed as mean±SEM.

Results

Ouabain increased ANP secretion in a dose–response fashion. Superfusion with 50, 100, and 200 μM ouabain increased ANP secretion by 2.0±0.3-, 3.2±0.5-, and 4.2±0.5-fold, respectively (Figure 1). Ouabain increased the peak rise in developed tension (DT) by 2.1±0.1, 2.8±0.2, and 3.5±0.3-fold in response to 50, 100, and 200 μM ouabain, respectively (Table 1). The rises in DT between 50 and 100 μM ouabain (p<0.003) and 100 and 200 μM ouabain (p<0.038) were significant. Resting tension (RT) did not change at 50 μM ouabain; however, RT rose significantly, by 57±19% (p<0.022) and 79±6% (p<0.002) with 100 and 200 μM ouabain, respectively (Table 1). Figure 2 illustrates a typical functional response to 100 μM ouabain.
TABLE 1. Effect of Ouabain on Atrial Function

<table>
<thead>
<tr>
<th>Ouabain dose</th>
<th>Developed tension (g)</th>
<th>Resting tension (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50 μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>0.14±0.01</td>
<td>0.27±0.02</td>
</tr>
<tr>
<td>After</td>
<td>0.31±0.03*</td>
<td>NC</td>
</tr>
<tr>
<td>100 μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>0.16±0.04</td>
<td>0.24±0.03</td>
</tr>
<tr>
<td>After</td>
<td>0.44±0.08*</td>
<td>0.37±0.06†</td>
</tr>
<tr>
<td>200 μM</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>0.14±0.03</td>
<td>0.36±0.06</td>
</tr>
<tr>
<td>After</td>
<td>0.47±0.08*</td>
<td>0.65±0.11*</td>
</tr>
</tbody>
</table>

NC, no change. Values are mean±SEM.
*p<0.01, †p<0.03 vs. corresponding value before ouabain.

To determine whether calcium influx plays a role in the secretory response to ouabain, atria were superfused with medium 199 containing 0.2 mM calcium in lieu of the normal 1.8 mM calcium. We have previously shown this concentration of calcium to totally negate the secretory response to isoproterenol and phenylephrine.10,13 Superfusion with 0.2 mM calcium blunted the response to 100 μM ouabain by 63% (Figure 3). DT was undetectable in atria superfused with 0.2 mM calcium and remained undetectable with the addition of 100 μM ouabain (Table 2). Ouabain raised DT by 2.8±0.1-fold (p<0.001) in atria superfused with 1.8 mM calcium. Superfusion with ouabain raised DT 21±4% (p<0.001) and 30±6% (p<0.003) in atria superfused with 0.2 or 1.8 mM calcium, respectively. The increases in RT with low or normal calcium were not different (p=0.15). The persistence of the rise in RT induced by ouabain in the presence of 0.2 mM calcium suggested that significant calcium influx remained even at this low calcium concentration.

Further lowering the calcium concentration or adding a calcium chelator may deplete intracellular calcium and give misleading results. Instead, atria were superfused with the calcium antagonist lanthanum. These studies were performed with 1.0 mM calcium. The concentration of lanthanum was that which rendered DT undetectable in preliminary studies. Superfusion with 2 mM lanthanum totally negated the secretory response to 100 μM ouabain (Figure 4). DT was undetectable in atria superfused with lanthanum and remained as such with the addition of ouabain (Table 2). DT rose 4.4±0.4-fold in response to ouabain in the absence of lanthanum. RT rose minimally, 5±2% (p<0.043), in atria superfused with lanthanum alone between 15 and 45 minutes, whereas ANP secretion fell during this time (Figure 4). RT rose 9±2% (p<0.012) in atria superfused with lanthanum and ouabain. This rise in RT was not different from that seen in control atria superfused with lanthanum alone (p=0.21). Superfusion with ouabain alone raised RT by 15±5% (p<0.008 versus control value). These results suggest that calcium influx is required for ouabain-stimulated ANP secretion.

In a separate study, lanthanum was found to have no effect on stretch-stimulated ANP secretion. Nonbeating rat left atria were mounted with a tension of 0.5 g and superfused with or without 2 mM LaCl3. Tension was subsequently raised to 1.5 g. The peak secretory responses with or without lanthanum were 1.7±0.1- and 1.6±0.1-fold of basal ANP secretion, respectively. Thus, inhibition of stimulated ANP secretion by lanthanum is not universal. This suggests that inhibition by lanthanum of ouabain-stimulated secretion is not related to a purely nonspecific effect.
TABLE 2. Effect of Experimental Agents on Atrial Function

<table>
<thead>
<tr>
<th>Group</th>
<th>Ouabain (100 μM) Developed tension (g)</th>
<th>Resting tension (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Calcium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.10±0.02</td>
<td>0.30±0.03</td>
</tr>
<tr>
<td>0.2 mM calcium</td>
<td>After 0.28±0.04*</td>
<td>0.39±0.04*</td>
</tr>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>Lanthanum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.05±0.01</td>
<td>0.37±0.05</td>
</tr>
<tr>
<td>2.0 mM lanthanum</td>
<td>After 0.20±0.03*</td>
<td>0.42±0.06*</td>
</tr>
<tr>
<td>Ryanodine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.06±0.01</td>
<td>0.33±0.02</td>
</tr>
<tr>
<td>1 μM ryanodine</td>
<td>After 0.25±0.01*</td>
<td>0.39±0.03*</td>
</tr>
<tr>
<td>Pacing</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control (2 Hz)</td>
<td>Before 0.12±0.02</td>
<td>0.33±0.03</td>
</tr>
<tr>
<td>0 Hz</td>
<td>After 0.37±0.06*</td>
<td>0.41±0.04*</td>
</tr>
<tr>
<td>Ryanodine+isradipine</td>
<td>Before 0.08±0.02</td>
<td>0.38±0.05</td>
</tr>
<tr>
<td>Control</td>
<td>After 0.29±0.09†</td>
<td>0.55±0.06*</td>
</tr>
<tr>
<td>1 μM ryanodine+10 μM isradipine</td>
<td>Before ND</td>
<td>0.30±0.03</td>
</tr>
<tr>
<td></td>
<td>After ND</td>
<td>0.42±0.04*</td>
</tr>
<tr>
<td>Bumetanide</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.09±0.03</td>
<td>0.29±0.02</td>
</tr>
<tr>
<td>100 μM bumetanide</td>
<td>After 0.27±0.10†</td>
<td>0.38±0.04*</td>
</tr>
<tr>
<td>HMA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.11±0.01</td>
<td>0.29±0.02</td>
</tr>
<tr>
<td>25 μM HMA</td>
<td>After 0.29±0.04*</td>
<td>0.41±0.03*</td>
</tr>
</tbody>
</table>

ND, not detectable; HMA, 5-(N,N-hexamethylene)amiloride.
*p<0.01, †p<0.03 vs. corresponding value before ouabain.

Blocking calcium influx with lanthanum results in a commensurate lowering of calcium release from the SR, since SR calcium release is dependent on calcium influx. To determine the dependence of ouabain-stimulated secretion on SR calcium release, atria were superfused with ryanodine, an inhibitor of SR calcium release. Superfusion with 1 μM ryanodine delayed the onset of the secretory response to 100 μM ouabain and also blunted the peak secretory response by 47% (Figure 5). DT was undetectable in atria superfused with ryanodine and remained undetectable with the addition of ouabain. DT rose 4.6±0.6-fold with ouabain in the absence of ryanodine (Table 2). Ouabain raised RT to a similar degree in the presence or absence of ryanodine (Table 2). Thus, ouabain-stimulated ANP secretion is partially dependent on SR calcium release.

To further examine the calcium dependence of ouabain-stimulated ANP secretion, the effect of ouabain on nonbeating atria was studied. In nonbeating atria, calcium influx through voltage-dependent calcium channels does not occur to a significant degree and neither does calcium release from the SR that is due to the absence of calcium influx. Ouabain (100 μM) failed to stimulate ANP secretion in nonbeating atria (Figure 6) and did not increase RT (Table 2). DT and RT rose 3.1±0.3-fold (p<0.002) and 25±6% (p<0.009), as did ANP secretion, in atria paced at 2 Hz and superfused with ouabain. Thus, events surrounding the calcium transient that are due to membrane depolarization appear to be necessary for ouabain-stimulated ANP secretion.

To determine whether calcium influx via Na⁺-Ca²⁺ exchange occurs to a degree adequate to stimulate ANP secretion during membrane depolarization, atria were superfused with ryanodine and the calcium channel blocker isradipine. Ouabain (100 μM) failed to increase...
FIGURE 4. Graph showing the effect of lanthanum on ouabain-stimulated atrial natriuretic peptide (ANP) secretion. Rat left atria paced at 2 Hz were superfused with (○, n=6; ●, n=6) or without (△, n=6) 2 mM LaCl₃ in the presence of 1 mM CaCl₂. Atria were continuously superfused with 100 μM ouabain (●, △) from 15 to 45 minutes or superfused without ouabain (○). Results are expressed as in Figure 1. Basal ANP secretion was 94±9 (○), 121±20 (●), and 281±24 (△) pg/ml.

ANP secretion in atria superfused with 1 μM ryanodine and 10 μM isradipine (Figure 7). RT rose to a similar degree in atria superfused with ryanodine, isradipine, and ouabain as those superfused with ouabain alone (41±7% versus 43±11%, respectively) (Table 2). Again, atria superfused with ouabain alone elicited a 3.6±0.8-fold rise in DT and a 2.2±0.3-fold rise in ANP secretion.

To determine the primary mechanism of sodium entry during ouabain stimulation, atria were superfused

FIGURE 5. Graph showing the effect of ryanodine on ouabain-stimulated atrial natriuretic peptide (ANP) secretion. Rat left atria paced at 2 Hz were superfused with 1 μM ryanodine (○, n=6; ●, n=6) or with vehicle alone (△, n=6). Atria were continuously superfused with 100 μM ouabain (●, △) from 15 to 60 minutes or superfused without ouabain (○). Results are expressed as in Figure 1. Basal ANP secretion was 194±30 (○), 232±26 (●), and 205±22 (△) pg/ml.

FIGURE 6. Graph showing the effect of beating on ouabain-stimulated atrial natriuretic peptide (ANP) secretion. Rat left atria superfused with 100 μM ouabain were not paced, i.e., nonbeating (●, n=6), or were paced at 2 Hz (△, n=5). Control atria (○) not receiving ouabain were either nonpaced (n=3) or paced at 2 Hz (n=2). Results are expressed as in Figure 1. Basal ANP secretion was 179±11 (○), 178±40 (●), and 218±31 (△) pg/ml.

FIGURE 7. Graph showing the effect of the combination of isradipine and ryanodine on ouabain-stimulated atrial natriuretic peptide (ANP) secretion. Rat left atria paced at 2 Hz were superfused with 10 μM isradipine and 1 μM ryanodine (○, n=4; ●, n=6) or with vehicle alone (△, n=5). Atria were continuously superfused with 100 μM ouabain (●, △) from 15 to 60 minutes or without ouabain (○). Results are expressed as in Figure 1. Basal ANP secretion was 347±70 (○), 299±37 (●), and 358±50 (△) pg/ml.
with bumetanide to inhibit Na\(^+\)-K\(^+\)-Cl\(^-\) cotransport. Superfusion with 100 \(\mu M\) bumetanide did not significantly alter the secretory response to ouabain (Figure 8). DT rose 1.7±0.3- and 2.3±0.5-fold and RT rose 29±4% and 28±6% in the presence and absence of bumetanide, respectively (Table 2). These differences were not significant. Thus, Na\(^+\)-K\(^+\)-Cl\(^-\) cotransport does not appear to play a significant role in sodium entry with ouabain stimulation.

Atria were superfused with HMA to inhibit Na\(^+\)-H\(^+\) antiporter activity. Superfusion with 25 \(\mu M\) HMA was begun at 5 minutes in Figure 9. ANP secretion rose by atria superfused with HMA alone. When measurements obtained by superfusion with HMA and 100 \(\mu M\) ouabain were corrected for the rise in basal ANP secretion by HMA, inhibition by HMA was found to be 71% at 45 minutes (Figure 9). DT rose to a lesser degree with the addition of ouabain in the presence of HMA (1.8±0.1- versus 2.9±0.1-fold, \(p<0.001\)) (Table 2). Likewise, the ouabain-stimulated rise in RT was of a lesser degree with HMA (10±1% versus 31±8%, \(p<0.02\)) (Table 2). Superfusion with HMA alone did not change DT or RT. Thus, sodium entry by Na\(^+\)-H\(^+\) exchange appears to be a significant mechanism of sodium influx with ouabain stimulation.

**Discussion**

Ouabain-stimulated ANP secretion in this study was associated with a rise in DT and at higher concentrations with a rise in RT. The rise in RT reflects contraction of the atria, not an increase in stretch, since the distance between the two hooks between which the atria are mounted does not change. These functional changes suggest a rise in systolic as well as diastolic cytosolic calcium. Cytosolic calcium measurements in cardiac tissue have revealed a rise in systolic calcium with inhibitors of Na\(^+\),K\(^+\)-ATPase, and at higher inhibitor concentrations, diastolic calcium rises as well. In this study, a rise in DT was seen with all three concentrations of ouabain studied, each of which increased ANP secretion. A rise in RT was observed with the two higher concentrations at which a greater ANP secretory response was seen. Therefore, increases in systolic as well as diastolic calcium are implicated in the ouabain secretory response.

Ouabain-stimulated ANP secretion is entirely calcium dependent. Evidence for this is offered by the finding that ouabain-stimulated secretion was negated by superfusion with lanthanum or the combination of isradipine and ryanodine. Superfusion with 0.2 mM calcium only partially inhibited ouabain-stimulated secretion.
cretion, suggesting significant calcium influx even at this low concentration. In previous studies, 0.2 mM calcium was found to completely inhibit ANP-stimulated secretion by phenylephrine and isoproterenol.\textsuperscript{10,13} Rather than to lower the calcium buffer concentration further and to risk depleting cardiocytes of calcium, we chose to use the calcium antagonist lanthanum. These studies were performed in the presence of 1 mM calcium so that intracellular calcium stores were not depleted. It is possible that inhibition by lanthanum may be nonspecific; however, lanthanum does not inhibit stretch-stimulated ANP secretion.\textsuperscript{23} Although this does not prove specificity, it suggests a lower likelihood of nonspecific inhibition by lanthanum.

Inhibition of ouabain-stimulated ANP secretion by lanthanum suggests that calcium influx is a requirement for ouabain-stimulated secretion. However, the determination of the contribution of SR calcium to the secretory response required further study, since SR calcium release is mediated, at least in part, by calcium influx.\textsuperscript{30,31} Thus, blocking calcium influx also fails to release calcium from the SR during the action potential. To determine the relative contribution of SR calcium release to the secretory response, SR calcium was depleted with ryanodine.\textsuperscript{20,21}

Inhibition of ouabain-stimulated ANP secretion by ryanodine suggests enhanced calcium release from the SR by ouabain treatment. The SR calcium pool is expanded by ouabain because of a rise in diastolic calcium, leading to enhanced calcium uptake by the SR.\textsuperscript{19} This produces a greater release of calcium from the SR with each beat, which increases systolic calcium and results in a rise in DT. The following observations support this conclusion. Ryanodine does not inhibit stimulated ANP secretion by agonists that are not known to increase SR calcium stores, such as phenylephrine or endothelin,\textsuperscript{11,13} nor does it inhibit basal ANP secretion.\textsuperscript{10} In contrast, stimulated ANP secretion by secretagogues that are known to expand the SR calcium pool, such as β-adrenergic agonists and dibutryr cAMP, is nearly totally blocked by ryanodine.\textsuperscript{10} In addition, inhibition of Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity increases the magnitude of SR calcium release by caffeine, suggesting an expanded SR calcium pool.\textsuperscript{33} Thus, inhibition of ouabain-stimulated ANP secretion by ryanodine suggests a dependence on an expanded SR calcium pool size. Since superfusion with ryanodine suppresses ouabain-stimulated ANP secretion by 47\%, it appears that calcium influx and SR calcium release each account for approximately half of the calcium needed for ouabain-stimulated secretion. The dose of ryanodine used in these studies is that which lowers DT to an undetectable level and which has been shown to negate isoproterenol-stimulated ANP secretion.\textsuperscript{10} Therefore, it is unlikely that higher doses of ryanodine would further inhibit ouabain-stimulated secretion.

Inhibition of ouabain-stimulated ANP secretion by the combination of isradipine and ryanodine suggests that calcium influx via Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange alone is inadequate to stimulate secretion. Alternatively, if the primary mechanism of ouabain-stimulated secretion is decreased calcium efflux, inhibition by these two drugs would also be anticipated, since calcium channel calcium influx and SR calcium release, responsible for the rise in systolic calcium, are inhibited. Thus, without a rise in systolic calcium, a reduction in calcium efflux would be of minor consequence.

Inhibition of Na\textsuperscript{+},K\textsuperscript{+}-ATPase activity induces intracellular acidification.\textsuperscript{35} Acidification may result from increased Ca\textsuperscript{2+}-H\textsuperscript{+} exchange via sarcolemma Ca\textsuperscript{2+}-ATPase, calcium displacement of hydrogen ions, or a rise in organic acid production as a result of an increase in work (energy expenditure). Intracellular acidification is calcium dependent, suggesting that one or more of the above mechanisms is involved.\textsuperscript{25} The acidification amplifies the inotropic effect of ouabain by increasing Na\textsuperscript{+}-H\textsuperscript{+} antiporter activity, which further augments intracellular sodium accumulation. The increase in intracellular sodium affects Na\textsuperscript{+}-Ca\textsuperscript{2+} exchange activity, resulting in a rise in intracellular calcium. The present study suggests that it is this rise in intracellular calcium that is responsible for ouabain-stimulated ANP secretion.

Enhanced Na\textsuperscript{+}-H\textsuperscript{+} antiporter activity plays a significant role in ouabain-stimulated ANP secretion. HMA inhibited ouabain-stimulated secretion by 71\%, suggesting that a significant mechanism of sodium entry is via Na\textsuperscript{+}-H\textsuperscript{+} exchange. A higher dose of HMA was not used in these studies, since 25 μM is 100-fold higher than the estimated K\textsubscript{i} for HMA inhibition of the antiporter\textsuperscript{26} and we have found that this dose of HMA totally inhibits frequency-stimulated ANP secretion.\textsuperscript{34} Thus, it is unlikely that higher doses would produce further inhibition.

Incomplete inhibition of ouabain-stimulated ANP secretion by HMA may be due to greater intracellular acidification, which produces displacement of calcium from protein binding sites and results in a rise in cytosolic calcium. Alternatively, incomplete inhibition of ouabain-stimulated ANP secretion may be due to an accumulation of sodium entering through fast sodium channels. Sodium entry through the sodium channel and its subsequent accumulation in the presence of ouabain is probably responsible for the initial inotropic and ANP secretory response to ouabain.\textsuperscript{35} The significance of the role of sodium entry through fast sodium channels is suggested by the failure of ouabain to stimulate ANP secretion by nonbeating atria. Thus, the primary mechanisms of sodium entry appear to be mediated by the fast sodium channel and Na\textsuperscript{+}-H\textsuperscript{+} exchanger. However, another potential mechanism of sodium entry is that mediated through Na\textsuperscript{+}-K\textsuperscript{+}-Cl\textsuperscript{−} cotransport. It has been reported that ouabain activates Na\textsuperscript{+}-K\textsuperscript{+}-Cl\textsuperscript{−} cotransport in transformed 3T3 cells.\textsuperscript{22} In the present study, bumetanide, an inhibitor of Na\textsuperscript{+}-K\textsuperscript{+}-Cl\textsuperscript{−} cotransport, failed to block ouabain-stimulated ANP secretion, suggesting that sodium entry by this mechanism does not play a significant role in ouabain-stimulated ANP secretion.

In summary, ouabain-stimulated ANP secretion is calcium dependent. At the ouabain dose used in this study, calcium influx and SR calcium release contribute equally to the stimulatory response. The primary mechanism of sodium entry required for ouabain-stimulated secretion is through Na\textsuperscript{+}-H\textsuperscript{+} exchange. By inference, sodium entry by means of the fast sodium channel also plays a role in ouabain-stimulated ANP secretion. Sodium entry through Na\textsuperscript{+}-K\textsuperscript{+}-Cl\textsuperscript{−} cotransport does not play a significant role in the secretory response. These results lend further support to the idea that calcium is an important second messenger in regulated secretion.
of ANP. They do not support the theory that ANP secretion is negatively regulated by calcium, as proposed by Greenwald et al.30

The calcium dependence of ouabain is unique when compared with the stimuli isoproterenol, phenylephrine, and endothelin. Whereas, all four stimuli are totally dependent on calcium influx, as illustrated by superfusion with 0.2 mM calcium or lanthanum, they differ in their dependence on SR calcium release.10,11,13 Isoproterenol stimulation of ANP secretion is totally dependent on an expanded SR calcium pool.10 This is due to enhanced SR Ca2+-ATPase activity resulting from phosphorylation of phospholamban.37 In contrast, neither phenylephrine nor endothelin is dependent on an expanded SR calcium pool.11,13 This may reflect the lack of an effect of phosphoinositol pathway activation by phenylephrine and endothelin on SR function. Ouabain-stimulated ANP secretion is partially dependent on an expanded SR calcium pool. This appears to be due to an increase in SR calcium uptake resulting from a rise in diastolic calcium. This most likely is not due to an increase in Ca2+-ATPase activity, since a rise in cytosolic calcium has not been found to phosphorylate phospholamban.38 Thus, the partial dependence of ouabain on SR calcium release distinguishes it from the other three secretagogues.

The physiological significance of ouabain-stimulated ANP secretion remains to be elucidated. An endogenous digitalis-like factor (EDLF) has been isolated from human plasma and found to be identical in elemental composition to ouabain.39,40 EDLF is elevated in volume-expanded or sodium-loaded animals.41,42 Sodium loading has been observed to increase blood pressure, which may be due to the ability of EDLF to augment contractility of vascular smooth muscle in response to vasoconstrictive agents.43 Thus, EDLF may have a propensity to increase blood pressure. Indeed, elevated levels of Na+,K+-ATPase inhibitors have been found in hypertensive humans.44 However, enhanced secretion of ANP by ouabain may help blunt the pressor response to ouabain, since ANP is a potent vasodilator.45,46 Thus, these observations may reflect a new loop in blood pressure regulation. They may also explain the puzzling finding made by Dananberg et al47 that intravenous bolus administration of saline in humans induces a sustained rise in plasma ANP levels at a time when right and left atrial pressures have returned to normal. We speculate that the sustained rise in plasma ANP observed in this study may be due to an increase in EDLF. If true, this would indicate that physiological concentrations of EDLF are capable of increasing ANP secretion in vivo.

It is impossible to determine the potential physiological relevance of ouabain-stimulated ANP secretion vis-à-vis the concentrations of ouabain used in this study. Rat tissue is less sensitive to ouabain relative to other animals and humans, so comparisons are difficult to make. The concentration of EDLF in humans is estimated to be in the 0.1–1.0 nM range.39,40,48 Augmentation of norepinephrine-stimulated contraction of resistance arteries in humans is present at ouabain concentrations of 11 nM.49 The cardiac sensitivity of ouabain-stimulated ANP secretion in humans is not known. Thus, the physiological relevance of ouabain-stimulated ANP secretion in humans remains to be determined.

As a hormone-secreting tissue, however, atria may be more sensitive to ouabain than other endocrine tissues by virtue of the fact that atria are electrically very active. This hypothesis is supported by the observation that ouabain had no effect on ANP secretion by nonbeating atria in the present study. Thus, hormone-secreting cells undergoing frequent spontaneous membrane depolarization may be more prone to respond to ouabain. However, a secretory event would only occur in those cells in which a rise in cytosolic calcium was the only requisite requirement for hormone release.

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