Reversal of Sodium Pump Inhibitor Induced Vascular Smooth Muscle Contraction With Digibind Stoichiometry and its Implications

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The possibility that a circulating sodium pump inhibitor contributes to the pathogenesis of volume-dependent hypertension via an action on vascular smooth muscle (VSM) is supported by multiple lines of investigation, but remains controversial. We had two goals in this study. The first was to compare the pattern of contractile response of rabbit aorta induced by two candidates, ouabain and a labile sodium pump inhibitor that we have identified in the peritoneal dialysate of volume-expanded hypertensive patients with chronic renal failure. Our second goal was to examine the ability of Digibind, a Fab fragment of antisera directed against digoxin, to reverse VSM contraction induced by both agents. Ouabain induced a concentration-dependent contraction, which was delayed in onset, was gradual, and reached a stable plateau after many hours. The labile sodium pump inhibitor induced a qualitatively similar series of responses. Digibind rapidly reversed the contractile responses to both sodium pump inhibitors, with a rate of relaxation that matched that induced by physical removal of the pump inhibitor from the bath. For ouabain, the Digibind:ouabain stoichiometry was highly predictable. When Digibind was present in a molar concentration equivalent to that of ouabain, or less, it had no effect. When the Digibind concentration was twice that of ouabain, complete relaxation occurred. Although the concentration:VSM response relationship for ouabain was steep, the concentration:effect interaction with Digibind was even more steep. The molar concentration of Digibind required to reverse the effects of the labile endogenous inhibitor from peritoneal dialysate was consistently lower than that for ouabain, which is compatible with either greater potency of the labile factor in VSM or greater affinity for Digibind. These findings are compatible with a role for one or more endogenous sodium pump inhibitors as the determinant of vascular smooth muscle tone in the volume-sensitive hypertension of renal disease. Am J Hypertens 1996;9:39-46

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ubstantial evidence suggests a role for an endogenous sodium pump inhibitor (ESPI) in salt-sensitive hypertension. Central to all hypotheses linking this ESPI to hypertension is its putative action on vascular smooth muscle (VSM). Given the central role of the VSM response in the hy-
pithetical sequence, pithetical sequence, however, there is remarkably little information regarding the influence of many proposed candidates on VSM. In part this may reflect the fact that only recently has the very gradual nature of the VSM response to sodium pump inhibition, which occurs over hours rather than minutes, been appreciated.\textsuperscript{3,10} There is an additional early response of VSM to pump inhibition, which requires a very high concentration of sodium pump inhibitor and largely reflects catecholamine release,\textsuperscript{10} most previous studies have probably been limited to this early response.

In this study we have compared the response of rabbit aorta to ouabain, a widely studied sodium pump inhibitor—suggested by some to be the major endogenous inhibitor—and another candidate, a labile endogenous sodium pump inhibitor (ESPI) isolated from the peritoneal dialysate of volume-expanded patients with chronic renal failure.\textsuperscript{11,12} This comparison, in part, was performed to determine whether the labile ESPI produced a contraction characteristic of sodium pump inhibition. Our second goal was to ascertain whether antibody Fab fragments directed against digoxin reversed ouabain or labile ESPI-induced Na pump inhibition and related VSM contraction. If the Fab fragment bound and eliminated the effects of the sodium pump inhibitor, it could serve as a "antagonist" to probe the effects of such blockade.

**MATERIAL AND METHODS**

**Preparation of the Labile ESPI**  Peritoneal dialysate (2 L) was collected in the early morning from volume-expanded hypertensive patients who were on chronic ambulatory peritoneal dialysis for renal failure. The fresh dialysate was processed rapidly through an ultrafiltration step under argon (Cortrasette, Filtron, Chicago, IL; 1000 dalton exclusion membrane) followed by a series of rapid C\textsubscript{18} HPLC chromatographic steps as described previously.\textsuperscript{11,12} In a previous clinical study employing volume expansion in these patients, only one peak of activity in the eluate of the HPLC fractionated dialysate was found to be correlated with volume expansion, blood pressure, and serum activity.\textsuperscript{12} This same material was also found to be chemically labile, and is referred to herein as the labile endogenous sodium pump inhibitor (ESPI). The solvents were removed rapidly under reduced pressure and the labile ESPI was used immediately for subsequent studies. The time from collection of specimen to its introduction into assay systems was -3.5 h, and its spontaneous disappearance typically takes 36 to 48 h.

**Sodium Potassium, ATPase Hydrolysis Assay**  Na,K-ATPase was purified from the medullae of calf kidney. The enzyme (10 \mu g) was incubated in 120 \mu L of buffer containing (in mmol/L): 100 Na\textsuperscript{+}, 5 K\textsuperscript{+}, 3 MgCl\textsubscript{2}, 1 FGTA, and 80 Tris (pH 7.5) for 30 min at 35°C either in the presence or absence of inhibitor. The reaction was started by adding 10 \mu L of 40 mmol/L [\textsuperscript{32}P]ATP (Amersham, Arlington Heights, IL; final specific activity, 70 mCi/mol) and ended after 10 min by adding 0.87 ml of 40 g/L charcoal in 0.1 mmol/L HCl solution. After centrifugation, an aliquot of the supernate was removed and its radioactivity counted. Ouabain-sensitive activity was defined as the activity remaining in the presence of 1 mmol/L ouabain. Ouabain-insensitive inhibition was calculated as the percentage reduction of counts in the presence of ouabain or digoxin-like factor (DLF) divided by counts in the presence of buffer, after subtraction of ouabain-insensitive activity.

Ouabain was employed at a concentration (5 x 10\textsuperscript{\textendash}7 mol/L) selected to inhibit sodium potassium ATPase activity approximately 50%. The labile endogenous sodium pump inhibitor concentration could not be controlled. The labile ESPI sample reflecting the amount of ESPI extracted and purified from 2000 ml of peritoneal dialysate was split, and 50% put into each of two reaction vessels, each with a final volume of 150 \mu L. To one of each pair of vessels was added a Digibind concentration of 2 x 10\textsuperscript{\textendash}6 mol/L. To the other was added an equivalent amount of nonimmune rat IgG.

**Tissue Preparation**  Ten female New Zealand white rabbits with a body weight of 3.0 to 3.6 kg were killed by intravenous injection of 150 mg/kg pentobarbital into a superficial vein of the ear on the day that the labile ESPI was isolated from peritoneal dialysate. The thoracic aorta was quickly excised and placed in a Petri dish containing modified Krebs-Henseleit solution (in mmol/L: NaCl 118.2, KCl 4.6, CaCl\textsubscript{2} 2.5, KH\textsubscript{2}PO\textsubscript{4} 1.2, Mg\textsubscript{2}SO\textsubscript{4} 1.2, NaHCO\textsubscript{3} 24.8, dextrose 10%). Periaortic connective tissue was gently removed and the aortas spirally cut, as described in earlier reports.\textsuperscript{10} Seven strips of -1.0 to 1.2 cm length with a width of 2 to 3 mm were cut from each vessel, mounted on force displacement transducers (Grass Instruments, model FT23D, Quincy, MA), and connected to a polygraph recorder (Grass Instruments, model 7D, Quincy, MA). We employed 4.0 g tension and tissue chambers containing 18 ml (n = 6) modified Krebs-Henseleit solution. An additional chamber with a working volume of 4 ml was employed to process the tissues that would receive the peritoneal dialysate labile ESPI. The solution was maintained at 37 ± 0.5°C and continuously aerated with a gas mixture of 95% O\textsubscript{2}-5% CO\textsubscript{2} (pH 7.4). The tissues were allowed to equilibrate for at least 2 h before study. During this period, the tissue chambers were flushed with fresh buffer every 15 min.

When tissue isometric tension was at equilibrium, the entire amount of the labile ESPI obtained from 2 L of peritoneal dialysate dissolved in 10 \mu L of vehicle
was administered to the strip suspended in the tissue chamber having the 4 mL working volume. In parallel, the buffer solution of 4 other strips was changed by introducing ouabain at concentrations of 10^{-7} to 10^{-4} in one chamber and 1 x 10^{-5} mol/L in another, 2 strips remaining untreated. Isometric tension was measured for 16 h. Then, the untreated tissues were challenged with 1.0 x 10^{-6} mol/L serotonin (5HT). These control strips contracted rapidly and were allowed to relax spontaneously over the period of an hour. Then, the contracted tissues followed one of three sequences. They either received digoxin antibody Fab fragment (Digibind, Burroughs Wellcome Co., Durham, NC), or the tissue chambers were flushed with fresh buffer free of agonist for 30 s—to assess the effects of washout of ouabain compared to specific inactivation via its binding to the Fab fragment—or they were left undisturbed.

**Vascular 86Rb Uptake** Vascular Na⁻⁺K⁺ pump activity was assayed as 86Rb uptake, as described previously. 11 Tissues were incubated in 1.0 mL Krebs buffer at 37°C and bubbled with O₂—CO₂ for 2 h, in the same manner to assess vascular smooth muscle contraction. Uptake of 86Rb was initiated by adding 86Rb (10 mCi/mL; specific activity 1.1 to 14.0 mCi/mg; New England Nuclear, Boston, MA). After 120 min, the tissues were removed from the incubation vessel, dipped serially in buffer 3 times, blotted, weighed, and the radioactivity taken up by the tissue determined with a γ-well counter (Autogamma 5000, Packard, Downer’s Grove, IL). Total uptake was calculated in milliCi/liter/milligram wet tissue weight. In 4 to 10 experiments each, either ouabain (10^{-1} mol/L to 10^{-4} mol/L; Table 3) or the ESPI were added to the incubate in a 0.01 mL volume 60 min before exposure to 86Rb. In the case of the ESPI, the entire quantity isolated from a single 2 L peritoneal dialysis sample was employed.

**Digibind, Digoxin Antibody Fab Fragments** Digibind (Burroughs Wellcome, Triangle Research Park, NC) is made up of purified IgG Fab fragments of an antisem directed against digoxin. The Fab fragments have a molecular weight of 50,000 with one binding site per molecule. The digoxin antibody Fab fragments were obtained as a lyophilized solid, each vial containing 40 mg Fab, 75 mg sorbitol, and 28 mg NaCl. The solids were reconstituted in 8.0 mL 0.9% NaCl solution providing a working Digibind solution of 5.0 mg/mL. To assess its influence on the VSM response to the labile ESPI (10 experiments), ouabain (40 experiments), and 5HT (20 experiments), strips of VSM received sufficient Digibind stock solution to yield an initial bath concentration of 6 x 10^{-5} or 10^{-4} mol/L. The bath Digibind concentration was then increased in log-dose increments at 60-min intervals, and the effects of Digibind were monitored for at least 3 h, i.e., 2 h after receiving the final Digibind dose. The effects of washout of ouabain with buffer exchange and the time course of spontaneous relaxation of the 5HT treated strips were monitored in parallel.

**Statistics** Values are presented as the mean with the SEM as the index of dispersion. Changes as a function of time or concentration were evaluated by ANOVA. A P < .05 was considered significant.

**RESULTS**

**Sodium Potassium, ATPase Hydrolysis Assay** Ouabain at 5 x 10^{-7} mol/L achieved the target level of inhibition, which was about 50% (56.9 ± 7.7%) inhibition of soluble Na,K-ATPase (Table 1). The concentration of Digibind employed (2 x 10^{-4} mol/L) reversed inhibition completely (Table 1; P < .001). The concentration of the labile ESPI employed achieved only about 20% inhibition (19.9 ± 5.7%) (Table 1). Again, as with ouabain, Digibind reversed the inhibition completely (P < .05; Table 1).

**VSM Contraction With Ouabain** Aortic strips receiving ouabain showed sustained concentration-dependent contractions. Strips receiving 3 x 10^{-7} mol/L ouabain did not respond over the 16 h of monitoring (Figure 1). A concentration 5 x 10^{-7} mol/L or higher led to a slowly evolving contraction which achieved its maximum over a period of 4 to 12 h depending on the ouabain concentration. The maximal VSM contraction achieved also demonstrated significant concentration dependence to 10^{-6} mol/L which induced a maximal contraction of 2.4 ± 0.2 g. A large contractile response did not follow increasing the ouabain concentration to 10^{-5} mol/L (2.3 ± 0.2 g) or 10^{-4} mol/L (2.2 ± 0.2 g). The contractions, once maximal, were sustained thereafter until relaxation was induced either by wash or by Digibind.

**The Effect of Digibind on Responses to Ouabain** Digibind in adequate concentrations rapidly and completely reversed the VSM contractile response to ouabain (Figure 2). Relaxation occurred at the same rate as that induced by physical removal of ouabain from the

| TABLE 1. EFFECT OF DIGIBIND ON OUABAIN-INDUCED AND ESPI-INDUCED INHIBITION IN A SODIUM POTASSIUM ATPase HYDROLYSIS ASSAY |
|-------------|-------------|-------------|-------------|
| Ouabain     | Digibind    | Ouabain     | Digibind    |
| Control     | Digibind    | Control     | Digibind    |
| 56.9 ± 7.7% | 3.2 ± 1.7%  | 19.9 ± 4.7% | 0.6 ± 1.8%  |
| *P < .05*   | *P < .05*   | *P < .05*   | *P < .05*   |

*Quantum concentration was 5 x 10^{-4} mol/L.*
bath by washout (compare Figures 2A and 2B). There were also consistent stoichiometric relations. When the Digibind concentration matched that of ouabain, there was no influence on the contractile response (Figures 2B, 2C, and 3). On the other hand, when the Digibind concentration doubled the concentration of ouabain, complete relaxation followed (Figure 2B, 2C, and 3).

**VSM Contraction With Labile ESPI and Reversal by Digibind** We demonstrated previously that variable quantities of the labile ESPI were isolated from 2.0 L samples of peritoneal dialysate, as determined by their activity in a Na,K-ATPase assay. Likewise, the ESPI samples varied in their maximal action on VSM, but the pattern of the responses resembled responses to ouabain (Figures 4 and 5). The four peritoneal dialysate samples obtained from volume-contracted patients did not lead to VSM contraction. Among samples obtained from volume-expanded patients, 5 of 6 led to a contractile response. The onset of contraction was very gradual following a quiescent interval that lasted for several hours. Once a peak contractile response was achieved, the response was sustained for hours, as for ouabain. The average contractile response with labile ESPI approximated that induced by $5 \times 10^{-7}$ mol/L ouabain (compare Figures 1 and 5). The largest response (Figure 4) exceeded the average response to ouabain at $5 \times 10^{-7}$ mol/L (Figure 4).

As was the case for ouabain, in every experiment Digibind rapidly and completely reversed the contractile response induced by the labile ESPI (Figures 4 and 5). There was, however, a difference in the quantity of Digibind required. The lowest ouabain concentration resulting in a contractile response was $5 \times 10^{-7}$ mol/L. As the Digibind requirement to reverse a contractile response was twice the concentration of ouabain, the lowest effective Digibind concentration was $10^{-6}$ mol/L. In the case of the contractile response to the labile ESPI, lower concentrations of Digibind, $6 \times 10^{-7}$ mol/L, were effective (Figures 4 and 5). Digibind did not cause VSM relaxation in any of the 20 experiments in which serotonin was employed to induce contraction of the rabbit aortic strip (data not shown).

**Vascular $^{86}$Rb Uptake and Labile ESPI** Ouabain induced a concentration-dependent fall in vascular rubidium uptake (Table 2). The threshold ouabain concentration was $10^{-8}$ mol/L, which induced 17% inhibition. Ouabain insensitive $^{86}$Rb uptake was minimal at the highest ouabain concentration employed, $10^{-4}$ mol/L, which induced 89% inhibition. The ESPI induced a significant 37% inhibition of $^{86}$Rb uptake ($P < 0.01$), a level that was bracketed by ouabain at $10^{-7}$ and $10^{-8}$ mol/L (Table 2).

**DISCUSSION**

Our interest in the effects of sodium pump inhibition on VSM reflects many observations of reduced sodium pump activity accompanying volume-dependent hypertension, and parallel evidence that suggests a circulating sodium pump inhibitor is released in response to sodium retention and volume expansion. This inhibitor, according to this thesis, acts on the VSM sodium pump, reducing its activity, which results in shifts in the intracellular ionic milieu and a myogenic VSM contraction. Recently, Stewart et al demonstrated that there are two temporally distinct rabbit aorta contractions in response to ouabain. The early response is mediated by catecholamine release. A second response, labelled "myogenic", occurred much later, required lower concentrations of ouabain,
and was largely independent of catecholamine release but associated with a rise in VSM sodium, as required by the thesis.\textsuperscript{10} In this study we examined the late myogenic response to new candidates for the role of the endogenous, circulating sodium pump inhibitor, also known as the "digitalis-like factor." The first, ouabain, has been proposed to be the circulating ESI.\textsuperscript{11} The second is a stable ESI we obtained from the peritoneal dialysate of renal failure patients.\textsuperscript{11}

There were several striking similarities and one potentially important difference demonstrated in this study between ouabain and the labile ESI. Both inhibited vascular \textsuperscript{86}Rb uptake, an index of sodium pump activity. Both induced a contractile response that featured an extended delay, in which there was little or no change in tone, followed by a gradual smooth muscle contraction in vascular tone to reach a dose-related maximum tone that was stable for hours. This pattern of VSM response differs strikingly from that induced by any known agonist that acts on membrane receptors, but is a feature of maneuvers that influence the internal milieu of VSM, as induced by ouabain or other sodium pump inhibitors or by a very high extracellular concentration of potassium.\textsuperscript{11,12} In human small arteries, a gradual contraction was documented in response to ouabain, without a delay.\textsuperscript{12}

The second similar feature uncovered in this study is
the consistent reversal of the contractile response by Digibind. Digibind induced VSM relaxation occurred as quickly and progressed as rapidly as the relaxation induced by physical removal of ouabain from the bath by wash. These data indicate that the labile endoge-

TABLE 2. EFFECT OF OUABAIN AND ESPI ON VASCULAR *Rb UPTAKE

<table>
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<tr>
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<th>Uptake (mmol/L Rb/mg tissue)</th>
<th>Percent inhibition</th>
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<tbody>
<tr>
<td>Control</td>
<td>10</td>
<td>100</td>
</tr>
<tr>
<td>ESPI</td>
<td>10</td>
<td>31.5 ± 3.7*</td>
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<tr>
<td>Ouabain</td>
<td>10⁻¹</td>
<td>5.6 ± 0.9</td>
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<tr>
<td></td>
<td>10⁻²</td>
<td>6.9 ± 1.7</td>
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<tr>
<td></td>
<td>10⁻³</td>
<td>8.8 ± 1.9</td>
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<tr>
<td></td>
<td>10⁻⁴</td>
<td>20.7 ± 4.1</td>
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<tr>
<td></td>
<td>10⁻⁵</td>
<td>41.5 ± 1.3*</td>
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<tr>
<td></td>
<td>10⁻⁶</td>
<td>46.4 ± 4.8</td>
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*P < 0.01 vs control.

nous sodium pump inhibitor resembles digoxin sufficiently that it presents some, perhaps much, of the epitope to Digibind. Specificity is supported by the finding that Digibind did not influence the contractile response to serotonin. These observations confirm a number of reports of successful reversal of hypertension by immunoneutralization, although there has been controversy about this.²⁰ We have documented a blood pressure fall in a volume dependent model—DOCA salt hypertension in the rat—with the same Fab directed against digoxin.²¹

The difference between the labile endogenous sodium pump inhibitor and ouabain was evident in the stoichiometry of the interaction with Digibind. In the case of ouabain there was an intriguing stoichiometric relationship between Digibind concentration and reversal of the ouabain-induced contractile response. The Digibind concentration had to exceed the ouabain concentration by a factor of almost exactly two. It is very likely that the very steep dose-response for ouabain-induced VSM contraction—a ouabain concentration of 3 x 10⁻⁷ mol/L being subthreshold and a concentration of 10⁻⁵ mol/L achieving a maximal response—is related to the stoichiometry of reversal.

In the case of the as yet unidentified labile endogenous sodium pump inhibitor from peritoneal dialysate we cannot assign a molar concentration, and thus we cannot know its potency in inducing a VSM contraction, which on average resembled the VSM response to 5 x 10⁻⁷ mol/L ouabain. Nor can we provide a doubt-free interpretation of its stoichiometry with Digibind. What is clear from these experiments is that the Digibind concentration required to reverse the VSM contractile response to the labile ESPI was less than required for ouabain over an overlapping range of contraction. There are two interpretations compatible with the available data. First, the labile ESPI may be far more potent in its action on VSM than ouabain, so that Digibind was present in excess for binding the labile ESPI, though its concentration was subthreshold.
for ouabain. The second possibility is that Digibind has greater affinity for the labile endogenous sodium pump inhibitor than for ouabain. No definitive information available from our observations would allow separation of these two possibilities, as they are equally compatible with the data. A measurement of concentration of the labile ESPI would resolve this important issue and efforts are being made to define sensitive and quantitative analytical methodologies that might accomplish this.

The labile endogenous sodium pump inhibitor was effective in inhibiting sodium potassium ATPase in the chemical assay and in inducing a gradual and sustained contraction characteristic of sodium pump inhibition. It is instructive to compare the responses to ESPI in the two systems. Although there is no independent measure of the quantity of agent employed in any assay or day, they were drawn from a common source, and it is reasonable to assume that on average equivalent amounts were employed. The chemical assay involves far less dilution (130 μl) than does the smooth muscle chamber (4000 μl). The quantity of material available was reduced for the chemical assay because the sample was split with half employed for the baseline assay and half to assess the effect of Digibind. Thus, the concentration was 15-fold higher in biochemical assay than in the VSM system, but the effect was smaller. The results suggest that the endogenous agent is substantially more active in VSM than in the chemical assay perhaps because the biochemical assay employed renal Na,K-ATPase. Although indirect, these results support the suggestions from the interaction of Digibind with the endogenous ESPI that it might have greater potency in VSM.

One striking feature of the Fab-induced relaxation of the ouabain contracted tissues was the extraordinary stoichiometric requirement. When the concentration of Fab was less than or equal to that of ouabain, no effect was seen. When the ratio of Fab to ouabain was 2.0 or greater, complete relaxation was effected. In other words, there was an all-or-nothing effect. The mechanisms governing VSM contraction and relaxation differ when sodium pump inhibition is involved. Contraction was gradual, but relaxation was rapid. The slowness of contraction due to sodium pump inhibition presumably reflects the rate of rise of tissue sodium, which is due to leak which goes uncompensated with inhibition of the pump. The more rapid relaxation probably provides a measure of the capacity of the pump to remove sodium when it is activated at a very high intracellular sodium concentration, and could provide a useful index of the state of the pump under pathological conditions. Sodium pump inhibition with ouabain in VSM produces a slowly evolving, dose-related contraction, which plateaus at a well-defined maximum contractile force. The labile ESPI related from peritoneal dialysis, selected for its correlations with patient volume status, arterial blood pressure, and serum DLF activity, induced a VSM response with similar features. Either of these agents was bound to, and their effects blocked effectively by, the Fab fragment directed against digoxin. In VSM precontracted with either agent, this resulted in a rapid and complete relaxation. Digibind appeared to have no nonspecific effects.

Our results confirm the utility of Digibind as a pharmacologic probe to explore this system. Although it has been suggested that the depressor response to antiserum directed against digoxin in some hypertension models reflects a nonspecific mechanism, such as complement activation or histamine release, that is far less likely with a Fab fragment, especially when it is employed in vitro.

Our findings lend further support to the concept of a volume-dependent sodium pump inhibitor that acts on the vascular smooth muscle sodium pump, at least in the hypertension associated with volume expansion in the patient with advanced renal failure.

REFERENCES


