Urinary Sodium Pump Inhibitor Raises Cytosolic Free Calcium Concentration in Rat Aorta

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We were able to purify two distinct sodium pump inhibitors to homogeneity from human urine based on \(^{3}\)H]ouabain-displacing activity from intact human erythrocytes. The polar and less polar compounds were eluted off the C18 reverse-phase column with 18% and 31% acetonitrile, respectively. The polar compound cross-reacted very weakly with specific antidigoxin antibody and lacked a characteristic ultraviolet absorption peak between 190 and 300 nm. The less polar compound showed a prominent digoxinlike immunoreactivity and had an ultraviolet spectrum similar to that of digoxin. We examined the effects of these compounds on cytosolic free calcium concentration in cultured rat vascular smooth muscle cells (A10 cells) using the fluorescent calcium chelator fura-2. Only the polar ouabain-displacing compound caused a significant increase, from 108±7 to 162±8 nM (n=6, p<0.01), in cytosolic free calcium concentration in A10 cells. The rise in cytosolic free calcium concentration induced by the polar ouabain-displacing compound tended to be slower in onset and more sustained than that induced by arginine vasopressin. In contrast, ouabain and bufalin had no appreciable effects on cytosolic free calcium concentration in A10 cells. These results suggest that the polar ouabain-displacing compound we isolated from human urine may possess a vasoactive property and may play an important role in the modulation of vascular tone. (Hypertension 1989;13:916–921)

There is much interest in the possibility that the cardiac glycoside receptor on the sodium pump may be designed to bind an endogenous ligand present in the mammalian body. Such a physiological endogenous counterpart to cardiac glycoside may serve as a specific regulator of the sodium pump and may be implicated in the regulation of sodium excretion and the pathogenesis of human essential hypertension.1–8 Despite several attempts to clarify the precise nature of this endogenous digitalislike factor, it has eluded purification and definite characterization.

We have recently purified a polar digitalislike factor to a high degree from human urine and dog plasma using the inhibitory effect on \(^{3}\)H]ouabain binding to intact human erythrocytes.9,10 This compound, a reversible and competitive inhibitor of \(^{3}\)H]ouabain binding, was capable of regulating sodium pump activity in cultured vascular smooth muscle cells (VSMC).9 In the current study, we attempted to purify two distinct ouabain-displacing compounds (ODCs) to homogeneity from human urine. Since endogenous digitalislike factor has been postulated to act as a vasoactive substance, we investigated the effects of these compounds on intracellular free calcium concentration in cultured VSMC.

Materials and Methods

Purification of Ouabain-Displacing Compounds

Human urine contains four discrete peaks that inhibit \(^{3}\)H]ouabain binding to human erythrocytes when fractionated by high-performance liquid chromatography (HPLC) on an R-ODS-5 column (0.46×25 cm), as reported previously.9 Four peaks of activity were eluted off, in order of polarity, with 1%, 18%, 31%, and 45% acetonitrile in water, respectively. In the previous study, we partially purified the second (most potent) peak of activity.9 In this study, we extended our purification and attempted to purify the major (second and third) peaks of activity.
The ODC-1 was further analyzed by gel filtration and lyophilized. The active material was fractionated with 20% acetonitrile and lyophilized. The residue was suspended in distilled water and applied to the reverse-phase HPLC on a preparative C18 column (70 μm, 2.0×25 cm). The ODCs were eluted with 20% acetonitrile and lyophilized. The active material was fractionated by the reverse-phase HPLC on a D-ODS-5 column (5 μm; 2.0×25 cm) with a gradient of acetonitrile (0–40%) over 80 minutes at a flow rate of 10 ml/min. The activity from the previous step was resolved into two activity peaks, which were designated ODC-1 (retention time of 42–43 minutes) and ODC-2 (retention time of 66–67 minutes) (Figure 1). The first minor peak of activity mentioned above was not evident at this point, probably because it passed through the previous column when applied and washed with water. The ODC-1 and ODC-2 were eluted off at 18% and 31% acetonitrile, respectively; they correspond to the second and third peaks of activity mentioned above.

The polar ODC-1 was again chromatographed on a D-ODS-5 column with 8% acetonitrile at 10 ml/min and eluted at a retention time of 17 minutes. The ODC-1 emerged at 17 minutes and 36 minutes, respectively. The purification of ODC-1 was accomplished by the rechromatography on an A-402 phenyl column under conditions identical to those just described.

The less polar ODC-2 was again chromatographed on a D-ODS-5 column under the same conditions mentioned above and eluted at a retention time of 66 minutes. The ODC-2 was further analyzed on a diol-60 column in 20% acetonitrile at 1 ml/min and emerged at a retention time of 17 minutes. The ODC-2 was subsequently purified by reverse-phase HPLC on a pepRPC column with a gradient of acetonitrile (0–40%) over 40 minutes at 1 ml/min.

The major activity peak, which eluted at a retention time of 31 minutes, was chromatographed on an A-402 phenyl column with a gradient of acetonitrile (0–20% and 20–40%) over 5 minutes and 40 minutes, respectively, at 1 ml/min. The ODC-2 emerged at a retention time of 33 minutes. The purification of ODC-2 was completed by reverse-phase HPLC on an R-ODS-5 column under the same conditions as on the A-402 column. The ODC-2 was eluted at a retention time of 37 minutes.

All HPLC columns except pepRPC were purchased from YMC, Kyoto, Japan. The pepRPC column was obtained from Pharmacia, Uppsala, Sweden. The HPLCs were developed by use of an LC4A (Shimadzu, Kyoto, Japan) and an M-600E system (Waters, Tokyo, Japan). Through the separation steps, an inhibitory effect on [3H]ouabain binding to intact human erythrocytes was used as a principal assay to follow the digitalislike activity. One unit of ODC was defined as that amount required to inhibit [3H]ouabain binding to human erythrocytes by 50% under the assay conditions published elsewhere.9

Characterization of Ouabain-Displacing Compounds

The ultraviolet (UV) absorption spectra of the purified ODCs were compared with those of ouabain and digoxin by use of a Hitachi Model U-3200 spectrophotometer (Hitachi, Tokyo, Japan). The ODCs were tested for ability to inhibit ouabain-sensitive 86Rb uptake into human erythrocytes and capacity to cross-react with antidigoxin antiserum according to the methods described previously.9

Cell Culture

The A10 rat aortic smooth muscle cell line was obtained from the American Type Culture Collection (Rockville, Maryland). Cells were plated in 100-mm-diameter plastic culture dishes and grown in Dulbecco's Modified Eagle Medium supplemented with 10% fetal calf serum. Cells reached confluence at approximately 4 days and were used for studies between 5 and 8 days, at which time there were approximately 2×10^6 cells per dish.

**Figure 1.** Elution profile of ouabain-displacing compounds (ODCs) by reverse-phase high-performance liquid chromatography. Active fractions from a previous step were analyzed on a D-ODS-5 column with a linear gradient of acetonitrile (0–40%) over 80 minutes at 10 ml/min. One-minute fractions were collected, freeze-dried, and assessed for their capacity to inhibit [3H]ouabain binding to human erythrocytes. Polar ODC-1 and less polar ODC-2 emerged at retention times of 42–43 minutes and 66–67 minutes, respectively.
Measurements of Cytosolic Free Calcium Concentration

Cytosolic free calcium concentration ([Ca\(^{2+}\)]\(i\)) was measured using the Ca\(^{2+}\)-sensitive fluorescent dye fura-2. A10 cells grown in 100-mm dishes were incubated with 2 \(\mu\)M fura 2-AM for 40 minutes at 37°C. The cells were then washed twice with Ca\(^{2+}\)- and Mg\(^{2+}\)-free Dulbecco’s phosphate buffered saline (PBS) and exposed to 2 ml 0.05% trypsin in Ca\(^{2+}\)- and Mg\(^{2+}\)-free PBS containing 0.02% EDTA for 10 seconds. The cells were incubated for 5 minutes at 37°C. Detached cells were dissolved in 2 ml Ca\(^{2+}\)- and Mg\(^{2+}\)-free PBS containing soybean trypsin inhibitor (0.5 mg/ml) and were placed into a cuvette. Each sample of the purified ODCs, ouabain, or bufalin was added in a volume of 20 \(\mu\)l into this cuvette, and its effect on [Ca\(^{2+}\)]\(i\) was determined. Fluorescence measurements were carried out in a Hitachi Model F-4000 spectrofluorometer equipped with magnetic stirrer and temperature control. Fura-2 fluorescence was measured at 340 and 380 nm (excitation) and 502 nm (emission).

The fluorescence intensity ratio was calibrated for each experiment using 0.1% Triton X-100 (maximum fluorescence), followed by addition of 10 mM MnCl\(_2\) (minimum fluorescence) to give [Ca\(^{2+}\)]\(i\), according to the method of Grynkiewicz et al.\(^{11}\)

Chemicals

All reagents were of the highest grade commercially available. Fura-2-acetoxymethyl ester was obtained from Doujin, Kumamoto, Japan. Synthetic [arginine\(^8\)]-vasopressin was from Peptide Institute, Osaka, Japan. Bufalin, ouabain, digoxin, and other reagents were from Sigma Chemical Co., St. Louis, Missouri.

Statistics

Results were expressed as mean±SEM. The differences were analyzed by paired or nonpaired \(t\) test. A \(p<0.05\) was considered significant.

Results

Figure 2 illustrates the final purification of ODCs. The polar ODC-1 was eluted as a single peak at a retention time of 36 minutes on an A-402 phenyl column (Figure 2A). The less polar ODC-2 emerged at a retention time of 37 minutes under the single UV absorption peak on an R-ODS-5 column (Figure 2B).

The UV absorption spectra of the purified substances are given in Figure 3 in comparison with those of ouabain and digoxin. The ODC-1 lacked a characteristic spectral peak between 190 and 300 nm. The UV spectrum of ODC-2 showed a peak at 220 nm and was quite similar to that of digoxin. The purified compounds were capable of inhibiting sodium pump activity in intact human erythrocytes; 1.5 units of ODC-1 and ODC-2, which inhibit [\(^3\)H]ouabain binding to human erythrocytes by 60%, inhibited ouabain-sensitive \(^{86}\)Rb uptake into human erythrocytes by 25% and 18%, respectively. There was a pronounced difference in the digoxinlike immunoreactivity between the two substances; 1.5 units of ODC-1 and ODC-2 inhibited the binding of [\(^3\)H]digoxin to specific rabbit antidigoxin antibody by 8% and 98%, respectively.

Figure 4 represents the effects of ODC-1 and ODC-2 on [Ca\(^{2+}\)]\(i\) in A10 cells. The calcium concentrations of the preparations containing these compounds were negligible. Addition of 1 mM CaCl\(_2\) and 1 mM MgCl\(_2\) always rapidly increased [Ca\(^{2+}\)]\(i\), and the mean [Ca\(^{2+}\)]\(i\) in A10 cells in the presence of 1 mM Ca in the medium was 95±20 nM (\(n=16\)). As shown in the tracing from a typical experiment, 20 units of ODC-1 increased [Ca\(^{2+}\)]\(i\) in A10 cells from 108±7 to 162±8 nM (\(n=6\), \(p<0.01\)) (Figure 4A). The concentration dependence of the effects of ODC-1 was not examined because of the limited amount of ODC-1 available. However, 2 units of ODC-1 did not detectably affect [Ca\(^{2+}\)]\(i\) (\(n=2\)). On the other hand, 20 units of ODC-2 induced no change in [Ca\(^{2+}\)]\(i\) (\(n=3\)) (Figure 4B). Ouabain (10\(^{-3}\)–10\(^{-4}\) M) and bufalin (10\(^{-8}\)–10\(^{-5}\) M) exerted no effect on [Ca\(^{2+}\)]\(i\), in A10 cells for 60 minutes, although these substances completely inhibited [\(^3\)H]ouabain binding to human erythrocytes at this concentration.

A10 cells reportedly express well-characterized vasopressin receptors.\(^{12}\) Addition of vasopressin (10\(^{-7}\) M) caused a significant increase in [Ca\(^{2+}\)]\(i\), from 120±8 to 540±32 nM (\(n=6\), \(p<0.01\)). The increase in [Ca\(^{2+}\)]\(i\) was rapid and was followed by a prompt return toward baseline. The average time to
peak $[\text{Ca}^{2+}]_i$ was $22 \pm 6$ seconds ($n=6$). In contrast, the effect of ODC-1 on $[\text{Ca}^{2+}]_i$ was slow in onset, and the average time to peak was $45 \pm 2$ seconds ($n=6$, $p<0.01$). The rate of decline of $[\text{Ca}^{2+}]_i$ after the initial rise was greatly diminished after the addition of ODC-1. The average time for $[\text{Ca}^{2+}]_i$ to decline from the peak to half of the total increase was $21 \pm 2$ and $225 \pm 15$ seconds after addition of vasopressin and ODC-1, respectively ($p<0.001$).

Discussion

Much evidence has accumulated that suggests that an endogenous digitalislike factor may be involved in the control of cation transport, sodium
excretion by the kidney, and vascular reactivity in arterioles. However, the nature, source, and mode of action of this factor have remained elusive. We have recently found that an inhibitory effect on [3H]ouabain binding to intact human erythrocytes is the most sensitive and relatively specific method for determination of digitalislike activity. In this study, we were able to purified two distinct ODCs to homogeneity from human urine based on this assay method. Both compounds possessed the capacity to inhibit sodium-potassium–adenosine triphosphatase (Na⁺,K⁺-ATPase) activity in intact human erythrocytes.

A recent communication documents the importance of excluding exogenous sources of digitalislike substance. At present we do not have direct evidence that these ODCs are actually produced in the human body. However, we have already confirmed the existence of identical ODC-1 in the mammalian plasma and rat hypothalamus (unpublished observation). Furthermore, Tamura et al reported the existence of a similar compound in bovine adrenal gland. Accordingly, the ODC-1 we isolated from human urine is likely to be a substance of endogenous origin. The less polar ODC-2 strongly cross-reacted with specific antidigoxin antibody. The UV spectrum of the ODC-2 was analogous to that of digoxin. These observations clearly indicate the structural similarity between ODC-2 and digoxin. However, none of the normal male volunteers from whom we collected urine had received cardiac glycosides. When we fractionated the urine from patients with cardiac diseases on digoxin treatment, the fraction eluted with 20% acetonitrile from C18 reverse phase column contained barely detectable amounts of digoxin and its metabolites. The elution position of digoxin on an R-ODS-5 column under the same condition mentioned in Materials and Methods was 46 minutes. Taken together, the ODC-2 is different from digoxin and may be a substance of endogenous origin.

The polar ODC-1 increased [Ca²⁺] in cultured VSMC. This is the first demonstration that a sodium pump inhibitor purified from the human body is capable of increasing [Ca²⁺] in VSMC. This finding is compatible with our previous report that partially purified ODC-1 increased [3H]ouabain and decreased Ca²⁺ influx to A10 cells. These findings indicate that the ODC-1 possesses a vasoactive property. Similar vasoactive sodium transport inhibitor has been purified from rat hypothalamic cells. On the other hand, [Ca²⁺] in A10 cells did not change at all after addition of ODC-2 possessing the same [3H]ouabain-displacing activity as that of ODC-1. However, it is still possible that ODC-2 is less potent in its capacity to increase [Ca²⁺], than ODC-1 and that larger amounts of ODC-2 are required to exert an effect on VSMC.

In view of the known role of Na⁺,K⁺-ATPase in modulation of vascular contractility, sodium pump inhibition could lead to a rise in intracellular calcium and the subsequent development of increased vascular tone. Actually, Matlib et al have reported that ouabain induced increased steady-state [Ca²⁺] in cultured rat aortic smooth muscle cells, probably through Na⁺-Ca²⁺ exchange system. However, plant-derived ouabain and toad-derived bufalin, specific inhibitors of Na⁺,K⁺-ATPase, did not affect [Ca²⁺] in A10 cells. This discrepancy may be explained by the difference in the dosages or the cells used. In our previous report, 2 mM of ouabain decreased Ca²⁺ influx from A10 cells. In contrast, the final concentration of ouabain in this study was 10⁻³–10⁻⁴ M because of the technical reason (100-fold dilution of sample). This dosage of ouabain may be insufficient for the inhibition of sodium pump in rat-derived A10 cells. Although it is likely that the ODC-1 increased [Ca²⁺] in VSMC through Na⁺-Ca²⁺ exchange system, other explanations may be possible. Inhibition of sodium pump may reduce the membrane potential and increase calcium influx through a voltage-dependent calcium channel. Moreover, it has been speculated that the link between Na⁺,K⁺-ATPase-receptor function and calcium fluxes could be more direct and that endogenous digitalislike factor might cause increased vascular tone by a direct effect on calcium.

In conclusion, we purified two distinct ouabain-displacing compounds to homogeneity from human urine. The polar ODC-1 raised [Ca²⁺] in cultured VSMC. The ODC-1 may possess a vasoactive property and play an important role in the modulation of vascular tone. Chemical identification and sufficient amounts of this compound are clearly needed to elucidate its role in the pathogenesis of human essential hypertension.

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References


**KEY WORDS** • essential hypertension • sodium transport • sodium-potassium ATPase • ouabain • digoxinlike immunoreactivity
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