Evidence that a slowly acting pressor substance is present in the blood of hypertensive animals was presented as early as 1940. Subsequently, many investigators have provided evidence for the presence of a specific inhibitor of Na⁺,K⁺-ATPase in the blood of hypertensive animals, including humans. Inhibition of the vascular smooth muscle Na⁺,K⁺-ATPase (sodium pump) could cause intracellular Na⁺ to increase, which in turn could lead to an increase in intracellular Ca²⁺. Increased intracellular Ca²⁺ results in contraction of resistance vessels and a subsequent increase in blood pressure. We recently isolated and purified from human plasma a specific inhibitor of the human erythrocyte, ouabain-sensitive sodium pump. The purified substance was determined to be indistinguishable from the cardiac glycoside ouabain by mass spectrometric or biochemical means. Based on this information, we undertook an effort to prepare anti-ouabain antibodies to develop an immunologic assay with sufficient sensitivity to detect the small quantity of ouabainlike material present in the blood and tissues of living animals. In this article, we describe the preparation of polyclonal anti-ouabain antisera, the development of a highly sensitive and ouabain-specific indirect enzyme-linked immunosorbent assay (ELISA), and application of the assay to measure the ouabainlike substance in plasma samples.

Methods

Reagents

Ouabain, 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (EDAC), and Tween 20 were obtained from Aldrich Chemical Co., Milwaukee, Wis. Bovine serum albumin (BSA) (radioimmunoassay grade, fraction V), ovalbumin (OVA) (grade V), poly-d-lysine (Mₚ = 53,000), thimerosal, and all materials used in the cross-reactivity profiling (except renin) were obtained from Sigma Chemical Co., St. Louis. Human recombinant renin was a kind gift from Dr. Robert Heinrikson, Upjohn Co., Kalamazoo, Mich. Affinity purified goat anti-rabbit immunoglobulin G (IgG) (H+L) antibody coupled to horseradish peroxidase was obtained from Boehringer Mannheim Biochemicals, Indianapolis, Ind. The two-component horseradish peroxidase-H₂O₂ system was obtained from Sigma Chemical Co., St. Louis. All other reagents were of reagent grade or better.
ish peroxidase substrate system consisting of 3,3',5,5'-tetramethylbenzidine (TMB) and hydrogen peroxide solution was obtained from Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md. and was used according to the protocol accompanying the reagents. Digoxin-specific antibodies were obtained from Du Pont–New England Nuclear, Boston. All other materials used were reagent grade.

Conjugate Synthesis

Four protein–ouabain conjugates were prepared for use as either immunogens or for an ELISA developed for measuring ouabainlike immunoreactivity. The vicinal hydroxyls on the rhamnose moiety of ouabain were oxidized to the dialdehyde by dissolving 670 mg ouabain in 6.7 ml water and 4.5 ml acetone with gentle warming. The reaction was cooled to ambient temperature, and 500 mg K2HPO4 was added to raise the pH, and then 56 mg meso-erythritol was added overnight to quench the reaction (Figure 1A). Acetone was removed by rotary evaporation, and the oxidized ouabain was immediately added to the appropriate protein as described below.

Hexane diamine (1.75 g) (C6) was attached to 260 mg BSA by combining the two reagents in 10 ml water, pH 8, with 500 mg EDAC. The mixture was stirred for 20 hours at ambient temperature to effect the attachment and was exhaustively dialyzed against 30 mM K2HPO4. The same generalized protocol also was used for the linkage of hexane diamine to OVA. Succinyl dihydrazide (SDH) (1.0 g) was attached to 215 mg OVA by combining the two reagents in 14 ml water, pH 5, with 670 mg EDAC. The mixture was stirred for 20 hours to complete the attachment and was exhaustively dialyzed against 40 mM sodium acetate buffer, pH 4.5 (Figure 1B).

Ouabain dialdehyde was attached to each of four carrier proteins (BSA–C6, OVA–C6, OVA-SDH, or poly-D-lysine) using the same generalized protocol. For BSA–C6, 260 mg of the derivatized carrier protein was mixed with 670 mg ouabain dialdehyde in 25 ml water, pH 6. Reduction amination was initiated after 30 minutes by the addition of 70 mg NaBH3CN. This was followed by a second portion of 170 mg NaBH3CN 90 minutes later (Figure 1C). After 17 hours, the reaction was briefly dialyzed against 25 mM Tris buffer, pH 7.9, concentrated by lyophilization, and applied to a 3.0x36 cm Sephadex (Sigma Chemical Co.) G-25 (20–50 μm) column. The protein fractions, well separated from oxidized ouabain, were collected and dialyzed against 25 mM Tris buffer, pH 7.9. The attachment of ouabain to BSA–C6 was estimated to be 15 mol/mol of protein, 20 for OVA–C6, 7 for OVA-SDH, and 20 for poly-D-lysine, as determined by UV difference spectroscopy at 220 and 278.5 nm.
Antibody Production

Three New Zealand White rabbits were immunized by intradermal injection of an emulsion consisting of 1 mg OVA-\(\text{C}_5\)-ouabain, 0.5 ml saline, and 0.5 ml Freund's complete adjuvant into 10 sites along the spinal column on the shaved back. Four weeks after the initial immunization, the animals were boosted by administration of an emulsion of 1 mg poly-D-lysine-ouabain conjugate, 0.5 ml saline, and 0.5 ml Freund's incomplete adjuvant injected as described previously. A final boost of 1 mg OVA-\(\text{SDH}-\text{C}_5\)-ouabain, 0.5 ml saline, and 0.5 ml Freund's incomplete adjuvant injected as described above was administered 4 weeks later. Animals were bled 11 days after the final boost, and serum was harvested and stored at \(-70^\circ\text{C}\).

Indirect ELISA Procedure

Half-area enzyme immunoassay plates (Costar Corp., Cambridge, Mass.) were coated with BSA-\(\text{C}_5\)-ouabain by adding to each well 50 \(\mu\text{l}\) of 1 ng/ml BSA-\(\text{C}_5\)-ouabain in coating buffer (50 mM sodium carbonate buffer, pH 9.6, containing 11.4 mg/l thimerosal). Plates were treated by storage for 1-7 days at 4\(^\circ\)C before use. Unbound BSA-\(\text{C}_5\)-ouabain was removed by washing each well three times with 250 \(\mu\text{l}\) rinse solution (0.09% NaCl containing 0.05% Tween 20). Unoccupied protein binding sites were blocked by adding 100 \(\mu\text{l}\) blocking/diluent buffer (50 mM sodium/potassium phosphate buffer, pH 7.4, containing 0.08% NaCl, 0.05% Tween 20, 11.4 mg/l thimerosal, and 10 g/l BSA) to each well for 2 hours at room temperature. Before use, plates were washed with three rinses of 250 \(\mu\text{l}\) per well of rinse solution.

The anti-ouabain antibody titer in immunized rabbit serum was determined on plates prepared as described above. Doubling dilutions of rabbit antisera, starting at 1:1,000, were added to successive wells. Plates were incubated for 60 minutes at room temperature with continuous shaking to allow anti-ouabain antibodies present in the antisera to bind to the BSA-\(\text{C}_5\)-ouabain already attached to the plate. Antisera was washed away with four rinses of 250 \(\mu\text{l}\) per well of rinse solution. Anti-ouabain antibodies remaining bound to the BSA-\(\text{C}_5\)-ouabain were reacted with 50 \(\mu\text{l}\) per well of a 1:1,000 dilution of goat anti-rabbit IgG-peroxidase conjugate by incubating the plates for an additional 60 minutes at room temperature with continuous shaking. Unbound anti-rabbit IgG-peroxidase conjugate was washed away by rinsing as described above. The presence of peroxidase enzyme remaining in each well was determined by the addition of 50 \(\mu\text{l}\) per well TMB reagent. After 15 minutes, the substrate reaction was terminated by the addition of 50 \(\mu\text{l}\) of 1 M \(\text{H}_2\text{PO}_4\). The absorbance of each well was measured at 450 nm (Vmax Microtiter Plate Reader, Molecular Devices Corp., Palo Alto, Calif.).

The cross-reactivity of the anti-ouabain antisera with various steroid and vasoactive compounds was determined by comparison to a ouabain standard curve, which ranged from 5 to 2,560 fmol/well. Test samples as well as standards were added to the wells in a volume of 25 \(\mu\text{l}\). Competitive binding to anti-ouabain antibody was initiated by the addition of 25 \(\mu\text{l}\) of 1:1,000,000 dilution of rabbit anti-ouabain antisera. This first incubation step was terminated after 60 minutes by rinsing the wells with 250 \(\mu\text{l}\) rinse solution. The subsequent incubations with goat anti-rabbit IgG-peroxidase conjugate and peroxidase substrate as well as measurement of UV absorption were as described above. Cross-reactivity was determined by dividing the concentration of ouabain that gave a 50% reduction in absorbance at 450 nm by the concentration of the comparison compound that also gave a 50% reduction in absorbance at 450 nm. The cross-reactivity of the anti-ouabain antibody with endogenous digitalislike factor (EDLF) was determined using EDLF that had been purified as described.\textsuperscript{11} Dilution curves for ouabain and purified EDLF were determined using 0.05-50 pmol/well
Table 1. Cross-Reactivity Profile of Anti-Ouabain Antisera

<table>
<thead>
<tr>
<th>Compound</th>
<th>% Cross-reactivity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldosterone</td>
<td>0.012</td>
</tr>
<tr>
<td>Ascorbic acid</td>
<td>n.d.</td>
</tr>
<tr>
<td>Atrial natriuretic factor</td>
<td>n.d.</td>
</tr>
<tr>
<td>Angiotensin I</td>
<td>n.d.</td>
</tr>
<tr>
<td>Angiotensin II</td>
<td>n.d.</td>
</tr>
<tr>
<td>Arginine vasopressin</td>
<td>n.d.</td>
</tr>
<tr>
<td>β-Estradiol</td>
<td>n.d.</td>
</tr>
<tr>
<td>Chlormadinone acetate</td>
<td>n.d.</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>n.d.</td>
</tr>
<tr>
<td>Citric acid</td>
<td>n.d.</td>
</tr>
<tr>
<td>Corticosterone</td>
<td>0.0035</td>
</tr>
<tr>
<td>Dihydro-ouabain</td>
<td>0.16</td>
</tr>
<tr>
<td>Deoxycorticosterone</td>
<td>0.0046</td>
</tr>
<tr>
<td>Dehydrodeoxycorticosterone</td>
<td>n.d.</td>
</tr>
<tr>
<td>Dehydrodeoxycorticosterone-3-sulfate</td>
<td>n.d.</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>n.d.</td>
</tr>
<tr>
<td>Lanatoside C</td>
<td>2.4</td>
</tr>
<tr>
<td>Ouabagenin</td>
<td>40</td>
</tr>
<tr>
<td>Progesterone</td>
<td>0.0011</td>
</tr>
<tr>
<td>Renin</td>
<td>n.d.</td>
</tr>
<tr>
<td>Strophanthin</td>
<td>66</td>
</tr>
<tr>
<td>Taurocholic acid</td>
<td>n.d.</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.0042</td>
</tr>
</tbody>
</table>

n.d., None detected

Antisera Characterization

All rabbits responded to the immunogen with titers greater than 1:800,000 (titer = dilution giving half-maximal response). The best antisera had a titer of 1:2,000,000 (Figure 2A). This antisera had a high affinity for ouabain, as evidenced by the useful assay range of 5–2,000 fmol, with 50% displacement occurring at 80 fmol/well (Figure 2B), corresponding to a concentration of 1.6 nM ouabain. None of the various steroids or vasoactive compounds tested showed any appreciable cross-reactivity with the anti-ouabain antisera, except compounds that contained an intact butenolide ring, such as digitoxin, digoxin, ouabagenin, and strophantidin. These compounds all possessed substantial cross-reactivity with the antisera (Table 1). The EDLF dilution curve was not significantly different from the ouabain standard curve (Figure 3), based on parallel line analysis on logit log curves.

Measurement of Endogenous Digitalislike Factor

Testing by high-performance liquid chromatography (HPLC) of various fractions obtained from the EDLF isolation procedure indicated that EDLF immunoreactivity could not be separated from red blood cell rubidium-86 (\(^{86}\text{Rb}\)) uptake inhibitory activity. The initial C\(_{18}\) preparatory HPLC purification step showed only one region of anti-ouabain antibody cross-reactivity that demonstrated the presence of a single ouabainlike substance in human plasma (Figure 4A). When these same fractions were tested for cross-reactivity with antibodies to digoxin, cross-reacting material was observed to elute 35 minutes after the ouabain cross-reactivity. Further steps in the purification protocol also were unable to separate red blood cell \(^{86}\text{Rb}\) uptake inhibitory activity from EDLF immunoreactivity (Figures 4B and 4C).
Dilutions of extracted human plasma gave a curve parallel to that observed for the ouabain standard curve (Figure 5A). In addition, measurements of immunoreactivity present in human plasma spiked with ouabain, and corrected for endogenous immuno-reactivity, gave curves that were not significantly different from ouabain standard curves (Figure 5B).

The concentration of EDLF in frozen plasma samples obtained from 11 normotensive male volunteers was found to be 138±43 fmol/ml. No significant difference was observed when some of the plasma samples also were extracted before freezing (75.8±15.3 fmol/ml). Plasma obtained from four patients who received total parenteral nutrition showed levels of cross-reactive material of 108±17 fmol/ml.

Discussion

Several laboratories have tried to isolate and quantitate the endogenous inhibitor of Na⁺,K⁺-ATPase, also known as the sodium pump inhibitor, EDLF, or endoxin, by use of antibodies directed toward digoxin. We, however, elected to study these endogenous factors by using ouabain-specific antibodies, because our mass spectral results suggested that the substance we had isolated and purified could not be distinguished from ouabain. Interestingly, the presence of a circulating factor with ouabainlike immunoreactivity in patients with primary aldosteronism has been reported previously. Subsequently, these investigators demonstrated that plasma levels of ouabainlike material decreased in patients with primary aldosteronism after removal of the adenoma. They also demonstrated that levels of the immunoreactive ouabainlike substance were elevated in patients with essential hypertension and could be stimulated to increase even further with high sodium intake. Of significance is the observation that the apparent levels of material reported by these investigators are in the range of 1–3 nM. The levels in normal individuals reported in their studies appear to
be substantially higher than those reported herein. Two possibilities may explain this difference. The first pertains to the nature and selectivity of the procedure used to extract the plasma. Some researchers have used the dried supernatant from an ethanol precipitation of plasma, which probably included a wide variety of other materials, such as steroids and lipids known to nonselectively interact with antibodies raised against cardiac glycosides. The C₈ extraction used in our study provides a highly selective enrichment of the plasma EDLF and greatly minimizes the presence of other potential cross-reactive materials into the assay. Second, the antisera produced by Masugi et al was not extensively characterized and may not possess a similar cross-reactivity profile with the antisera we have produced. For example, Masugi et al used a simple BSA-ouabain conjugate as the antigen, whereas we used three different ouabain conjugates as antigens in an effort to enhance the antibody recognition to the ouabain portion of the antigen. Our antisera demonstrated a high degree of specificity for ouabain and closely related cardenolides such as strophanthidin, ouabagenin, digoxin, and digitoxin. Furthermore, the antisera cross-react poorly with dihydro-ouabain, which indicates that the butenolide ring of ouabain is an essential structural component of the epitope recognized by the antisera. Removal of the rhamnose moiety, as in ouabagenin, had little effect on cross-reactivity, as might be expected, considering that rhamnose was oxidized and used to couple ouabain to the carrier protein for the immunogen preparation. In addition, the effective affinity of our antisera is approximately 1.6 nM for ouabain and is at least 10-fold greater than that reported elsewhere. Of further importance is the fact that the antisera do not cross-react with common steroids in plasma or other tested endogenous substances known to be involved in blood pressure regulation or to be associated with hypertension. Within the error of the assay, EDLF appears to be fully cross-reactive relative to ouabain. The small differences between the dilution curves for EDLF compared with ouabain reflect the fact that the concentration of purified EDLF in the sample used for dilution was estimated based on results obtained with UV absorption and enzyme titration. The error in these procedures probably accounts for the small, although not statistically significant, differences observed. The fact that the EDLF dilution curve is parallel to the curve obtained for ouabain supports the premise that the same epitope is being recognized on both compounds and provides additional evidence for the similarity between EDLF and ouabain. Thus, the ouabainlike factor appears to be immunologically as well as mass spectrometrically and biochemically identical to ouabain.

Previously, we have referred to the inhibitor of the sodium pump we isolated from human plasma as EDLF. This general nomenclature was used because we found our inhibitor to possess many of the properties of the cardiac glycosides. Our current finding that this substance is indistinguishable from ouabain suggests that more specific nomenclature such as endogenous ouabainlike compound (OLC) should be used.

It is interesting to note that although our antisera have approximately 5% cross-reactivity with digoxin, the digoxin antibody we tested failed to recognize the purified ouabainlike substance. This observation may explain why others who tried to use digoxin
immunoreactivity to guide a purification effort were unable to isolate sufficient quantities of a pure substance to permit structural identification. We also attempted to use a digoxin radioimmunoassay to guide our purification efforts. However, during these studies, it became apparent that the presence of digoxin immunoreactivity was inconsistent and was not well correlated with the presence of inhibition of red blood cell sodium pump activity. Conversely, the presence of ouabain immunoreactivity was highly correlated with the HPLC elution of the major peak of sodium pump inhibitory activity at each step in the purification procedure. In addition, no other region eluted from the columns exhibited ouabain immunoreactivity. This indicates that a single substance that cross-reacts with anti-ouabain antibodies is present in plasma extracts.

We found that plasma samples could be prepared for immunassay simply by passing them over a C18 disposable Bond Elut column. Dilution of the extracted samples yielded a curve with slopes identical to the ouabain standard curve. Additionally, plasma samples that had been spiked with ouabain gave a curve superimposable on the ouabain standard curve when corrected for endogenous immunoreactive OLC. These two observations demonstrated that extracted plasma samples were free of interfering factors and that the ELISA could be used for the assay of immunoreactive OLC in plasma. Samples were obtained from 11 normotensive male volunteers who were not hypertensive and were not receiving any medications. The concentration of OLC was found to be 138±43 fmol/ml. The obvious question was whether the endogenous OLC was of nutritional origin. In an attempt to answer that question, plasma was obtained from four patients who were on total parenteral nutrition for not less than 7 days. As the average level of the OLC for those patients was 108±17 fmol/ml, it appears unlikely that the factor is of dietary origin.

Plasma OLC levels determined by the ELISA were lower than those estimated based on yield of OLC during purification. However, it should be emphasized that the ELISA measurements were conducted with plasma from normotensive individuals, whereas the purification protocol used material obtained from mildly volume-expanded patients irrespective of blood pressure status. Both volume expansion and a hypertensive status would be expected to raise OLC levels, and thus these factors may account for the higher estimates of circulating OLC reported elsewhere.

In conclusion, polyclonal antibodies to conjugates of the commercially available cardiac glycose ouabain were raised in rabbits. Antiserum obtained contained a high antibody titer and showed high cross-reactivity with an OLC purified from human plasma. The antisera were used to develop an indirect ELISA with sufficient selectivity and sensitivity to quantitate endogenous levels of the circulating OLC. As the level found in patients after at least 7 days of total parenteral nutrition was not lower than that found in normal volunteers, we suggest that the source of OLC is not the diet.

Acknowledgments

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Development of an immunoassay for endogenous digitalislike factor.
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