Ouabain-Binding Protein(s) From Human Plasma

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Abstract—Conservation of the binding site on mammalian Na⁺,K⁺-ATPase for cardiac glycosides and the importance of the Na⁺ pump in mammalian cellular physiology has stimulated the search for a mammalian analog of these plant compounds. One candidate, isolated from brain and blood, appears to be ouabain itself or a closely related isomer, the ouabain-like compound. Little is known about the circulating form. Because human steroid hormones circulate with carrier proteins, we produced a ouabain-specific monoclonal antibody (mAb 1-10) and used it to probe normal human plasma for ouabain-protein carrier complex. Ouabain-like biological activity was isolated in association with protein bands of 80, 50, and 25 kDa. These proteins appear to be human immunoglobulins or immunoglobulin-like because they are recognized by anti-human immunoglobulin antibodies, but not by anti-mouse immunoglobulin antibodies. The protein-containing fractions inhibit the binding of mAb 1-10 to immobilized ouabain, and with further purification on protein A, the immunoglobulin-like protein binds radioactive ouabain with an IC₅₀ of 200 to 600 nmol/L, but binds digoxin with 100-fold less affinity, suggesting specificity for ouabain or its isomer. Active protein fractions after purification on C₁₈ inhibit Na⁺ pump activity in human erythrocytes (IC₅₀,4 nmol/L, ouabain equivalents), and this chromatography appears to dissociate the ouabain-like compound from the immunoglobulin protein(s). These immunoglobulin-like molecules may represent a subset of immunoglobulins (≈0.5% of total protein A immunoglobulin) that function as a reservoir and delivery system for ouabain-like compounds in the modulation of human Na⁺,K⁺-ATPase in vivo. (Hypertension. 2002;40:220-228.)

Key Words: ouabain ■ digitalis-like factor ■ immunoglobulins ■ sodium pump

Digitalis glycosides have been widely used in the clinical treatment of congestive heart failure and certain cardiac arrhythmias. The specific high-affinity binding of these compounds to the plasma membrane Na⁺,K⁺-ATPase results in the inhibition of transmembrane Na⁺ and K⁺ transport. Because it regulates the concentration of Na⁺ and K⁺ ions within mammalian cells, the Na⁺,K⁺-ATPase (Na⁺ pump) has fundamental importance in mammalian cellular physiology, being responsible for the setting of transmembrane potentials, the regulation of cell volume, the vectorial movement of solutes and water across transporting epithelia, and in cardiac muscle, the regulation of cardiac contractility.¹

Because the cardiac glycoside binding site of the Na⁺,K⁺-ATPase has been evolutionarily conserved and because the only known specific regulators of the enzyme in man are the plant kingdom cardiac glycosides such as ouabain (Oua), the notion arose that mammalian analogs to the cardiac glycosides might exist.² Over the past 2 decades, a number of investigators have reported extraction of Na⁺ pump inhibitory activity from body fluid and tissue sources, and this inhibitor has been linked to the pathogenesis of experimental volume-expanded and human essential hypertension.²,³ Structural analysis has indicated that one of these compounds, isolated from mammalian brain and human plasma, appears to be Oua itself or a closely related isomer.⁴ The source of the ouabain-like compound (OLC) is debated and not settled. The principal question is whether it is endogenous, thus, that a biosynthetic pathway exists in mammalian tissues to produce a steroid derivative closely related to the plant kingdom cardiac glycosides, or whether human tissues can accumulate small amounts of Oua/OLC from dietary sources.

Vertebrates—certain frogs and toads—synthesize digitalis-like substances, bufadienolides, which are potent inhibitors of the Na⁺ pump. Structurally, these latter compounds lack a sugar moiety at carbon 3 and have a 6-membered ring, rather than the cardenolide 5-membered lactone ring attached to carbon 17 of the steroid backbone, and thus are different evolutionarily from Oua. How humans have evolved with regard to digitalis-like substances is unknown, but this is a question fundamental to this field of study. Recent work indicates, nevertheless, that mammalian (rat) adrenal tissue can synthesize digitalis-like bioactivity using mammalian steroid pathway precursors;⁵ and both physiological and pharmacologic stimuli have been shown to influence the release or synthesis of OLC from midbrain and adrenal tissues.⁶–⁸
Although there are many reports demonstrating detection in mammalian sera, including human, of circulating Na⁺,K⁺-ATPase inhibitory activity, little is known about the form in which OLC might circulate. Human steroid hormones are often transported as complexes with protein carriers. These transport glycoproteins are made in the liver and provide a reservoir for the hormone and protection from metabolism and renal clearance. Feedback mechanisms operate to affect the degree of dissociation of the hormone from carrier, allowing titration of delivery of the active principle to tissues. A few reports have provided evidence that plasma proteins from vertebrates and mammals can bind cardiac glycosides and thus might function as carrier and delivery systems participating in the physiological regulation of Na⁺,K⁺-ATPase by OLC.

We began to address the issue of identification of OLC in human plasma and its possible association with a protein carrier molecule by producing monoclonal antibodies (mAbs) with specificity for Oua. We employed these antibodies to purify the putative OLC-carrier complex from human plasma samples. We report here that there is a human plasma protein, Na⁺,K⁺-ATPase inhibitor complex, which is recognized by anti-Oua mAb, but not by mAbs against noncardiac glycoside moieties. This protein binds Oua with high affinity, but binds digoxin (Dig), the cardiac glycoside in prevalent clinical use, with 100-fold less affinity, suggesting specificity for OLC. This OLC-protein complex is physiologically relevant because it inhibits active Na⁺ transport as measured in human tissue.

**Methods**

**Production and Characterization of Anti-Digoxin (26-10) and Anti-Oua (1-10) mAbs**

The generation, selection, and characterization of cell lines producing Dig-specific 26-10 mAb (IgG2a, κ), Oua-specific 1-10 mAb (IgG1, κ), and p-azophenylarsonate (Ars)-specific 36-65 mAb (IgG1, κ) were previously reported. All mAbs were derived from the spleen cells of A/J mice. The mAb 1-10 was produced by immunizing A/J mice with Oua coupled to 26-10 mAb as described, allowing titration of delivery of the active principle to tissues. A few reports have provided evidence that plasma transport glycoproteins are made in the liver and provide a reservoir for the hormone and protection from metabolism and renal clearance. Feedback mechanisms operate to affect the degree of dissociation of the hormone from carrier, allowing titration of delivery of the active principle to tissues. A few reports have provided evidence that plasma proteins from vertebrates and mammals can bind cardiac glycosides and thus might function as carrier and delivery systems participating in the physiological regulation of Na⁺,K⁺-ATPase by OLC.

The binding capacity of 1-10 Sepharose was tested using 3 H-Oua. Characterization of Sepharose-Bound 1-10 mAb

Affinity Purification of Human Plasma on Sepharose-Bound 1-10 mAb and Protein A-Sepharose

Human plasma (1 to 2 weeks old, 200 mL each, stored at 4°C) was obtained from the Blood Bank (the use of plasma from blood bank donors was approved by the Institutional Review Board of the Massachusetts General Hospital). All plasma samples were filtered using 0.45 μm filters (Millipore) to remove protein aggregates that interfere with purification. One hundred or 200 mL, was passed through a column containing 1 or 2 mL of Sepharose–1-10 mAb, respectively, using a peristaltic pump at room temperature (100 to 200 μL/min). The column was washed extensively with phosphate buffered saline (PBS, 0.15 mol/L NaCl, 0.1 mol/L sodium phosphate, pH 7.2) or until the OD₂₈₀ was below 0.005. The bound material was eluted in 0.5 mL fractions (into tubes containing 1.5 mol/L Tris-Cl, pH 4.4) using 100 mmol/L Trichlyamine (TEA, pH 11.9).

Sepharose-bound protein A (PA) was used to determine the nature of immunglobulin-like proteins eluted from 1-10 Sepharose. Plasma (100 mL) was passed through Sepharose-bound PA. Bound proteins were eluted from the column by TEA.

**SDS-PAGE and Immunoblotting**

SDS-PAGE and immunoblotting were used to characterize proteins eluted from Sepharose–1-10 mAb. Purified proteins (0.5 to 2 μg) were electrophoresed on 10% SDS-PAGE and subsequently transferred onto nitrocellulose membranes. Membranes were first blocked in 10% nonfat dry milk in PBS and then detected using horseradish peroxidase (HRP)-anti-human immunoglobulin (Zymed Laboratory Inc) or HRP-anti-mouse immunoglobulin (Amersham Life Science). Proteins were detected with enhanced chemiluminescence (ECL) Western blotting detection reagents (Amersham Life Science Products). The membranes were further stained with Amidoblack (0.5% in 5% acetic acid).

**Effect of Purified Proteins on Sodium Pump Activity in Human Erythrocytes Using ⁸⁶Rubidium (⁸⁶Rb) Uptake**

Fractions eluted from 1-10 Sepharose were preprepared to remove interfering ions by adsorption on C-18 Sep-Pak cartridges (Waters Corporation) and elution with 25% acetonitrile in water. C18 columns have been used by others to purify steroids and Oua-like compounds from both plasma and tissue extracts. The samples were lyophilized and reconstituted in potassium-free buffer.

The effect on Na⁺,K⁺-ATPase activity was measured as inhibition of active K⁺ transport into human erythrocytes using ⁸⁶Rb chloride (19 Ci/mmol, New England Nuclear) as previously described. Oua-insensitive Rb⁺ uptake was subtracted and results expressed as the percentage inhibition of control uptake (absence of plasma samples or Oua). A standard curve for inhibition of ⁸⁶Rb by Oua was determined for comparison.

**Immunosassays**

Inhibition ELISA was used to determine whether protein-containing fractions eluted from 1-10–Sepharose and PA-Sepharose inhibit binding of 1-10 mAb to Oua-BGG. Oua-BGG (50 μL, 3 to 5 μg/mL in PBS) was immobilized in the wells of microtiter plates. Binding of 1-10 mAb (25 μL, concentration equivalent to 50% binding to Oua-BGG, usually 0.2 to 0.4 μg/mL) to Oua-BGG was determined in the absence or presence of different concentrations of 1-10–Sepharose purified human plasma protein (25 μL). Binding was detected using HRP-anti-mouse immunoglobulin (Sigma-Aldrich, Inc).

Direct binding radioimmunoassays were used to determine whether 1-10 Sepharose–purified human plasma proteins bind to ¹³¹I-Oua-BGG. Protein-containing fractions eluted from 1-10–
Seprahose (50 μL of 10 μg/mL PBS of protein) were immobilized in the wells of microtiter plates that were previously coated either with anti-human immunoglobulin (Zymed) or anti-mouse immunoglobulin (Sigma). Binding of 125I-Oua-BGG or 125I-Ars-BGG (control) was determined in an ELISA reader.

**Results**

**Purification of Human Plasma Components on 1-10 mAb**

Human plasma (100 to 200 mL) was passed through a column containing 1 to 2 mL 1-10 Sepharose and OD280 was monitored. In every plasma tested, several eluted fractions contained compounds that exhibited absorbance at 280 nm, suggesting that eluted fractions contain protein. There were significant variations (OD280/1000 = 0.2 to 0.8) in the amount of bound protein among individuals. The pattern of elution of human plasma proteins from 1-10 Sepharose for 2 blood bank donors (P4 and P7) is shown in Figure 1. To exclude the possibility that fractions were contaminated with 1-10 mAb that may have leaked from the 1-10 Sepharose, 200 mL buffer was passed through 2 mL unused 1-10 Sepharose, and the column was eluted with TEA. There was no detectable OD280 absorbance in fractions collected from the control 1-10 Sepharose column (Figure 1).

**Characterization of the Eluted Protein From 1-10 Sepharose**

To determine the nature of the eluted proteins, SDS-gel electrophoresis and immunoblotting were used. Stained gels of protein-containing fractions under reducing conditions indicated that all proteins eluted from 1-10 Sepharose contained a major band of ~80 kDa and two other bands of ~50 and 25 kDa. Figure 2A shows a representative experiment for plasma 4 (P4), fraction 5. All 3 bands reacted with anti-human immunoglobulin Abs (Figure 2B), but not with anti-mouse immunoglobulin Abs (Figure 2C), indicating that the immunoglobulin-like proteins eluted from the 1-10 Sepharose are of human origin and not leached from the column. The lack of reactivity of the eluted protein from 1-10 Sepharose with anti-mouse immunoglobulin probe indicates that the human protein does not cross-react with mouse immunoglobulin. The blotted proteins did not react with Abs against human serum albumin (HSA), which is a low-affinity carrier of certain human steroid hormones (immunoblots not shown).

**Eluted Proteins From 1-10 Sepharose Do Not Bind to Anti-Mouse Immunoglobulin**

Because it has been reported that human serum can contain pre-existing Abs reactive with murine immunoglobulin,23–25 and also because 1-10 mAb was raised against Oua coupled to murine A/J-derived anti-digoxin mAb 26-10,13 we tested whether 1-10 bound immunoglobulin proteins could react
with Abs against mouse immunoglobulins. Figure 3A demonstrates that the anti-mouse immunoglobulin binds to immobilized 1-10 mAb but not to 1-10–purified human proteins (P4 and P7). These latter proteins however bound specifically to anti-human probe (Figure 3B), indicating that 1-10–reactive plasma proteins are of human origin and not cross-reactive with mouse proteins.

**Protein Containing Fractions Eluted From 1-10 Sepharose Bind to C18 Column and Exhibit Biological Activity Similar to That of Exogenous Oua**

We hypothesized that the protein eluted from plasma did not bind to 1-10 mAb directly but through an OLC. To determine the relevance of the purified proteins to OLC, the effect of 1-10 Sepharose–purified plasma proteins on active Na\(^+\)/H\(^+\) transport was determined using \(^{86}\)Rb uptake into human red blood cells (see Methods). The hypothesis was that if OLC is present in these fractions, it would bind to the Oua binding site of Na\(^+\).K\(^+\)-ATPase in the red blood cells, inhibiting \(^{86}\)Rb (a K\(^+\) analog) uptake by the Na\(^+\) pump. A representative experiment (P4) is shown in Figure 4A. C18-purified fraction 5 inhibited \(^{86}\)Rb uptake by 65%. This fraction contained the highest amount of protein (Figure 1). Controls (C1 and C2, 25% acetonitrile elution of the activated C18 column) inhibited the \(^{86}\)Rb uptake between 0% to 20%. The net inhibition of 45% corresponds to 4 nmol/L Oua under identical experimental conditions as determined from standard curves using free Oua (Figure 4B).

Because steroids and steroid-like compounds exhibit absorbance at 214 nm, the 280 and 214 nm absorbance of C18-purified fractions was also determined and was compared with that before adsorption. Figure 5 shows the absorbance for P4 (fraction 5). Though the adsorbed fraction exhibited absorbance at 214 nm, the 280 nm absorbance was absent following chromatography on C18 (Figure 5). This suggests that OLC eluted from the C18 may have dissociated from the plasma protein.

**1-10–Bound Plasma Proteins and C18-Purified P4 Inhibit the Binding of 1-10 mAb to Oua-BGG**

The results of the \(^{86}\)Rb assay using fraction P4–purified proteins suggest that 1-10–purified human plasma contains an inhibitor of the sodium pump. If the Na\(^+\).K\(^+\)-ATPase inhibitor is structurally similar to Oua, then binding of 1-10 mAb to immobilized Oua-BGG should be inhibited by the protein-containing fractions as well as by the 1-10–purified, C18-adsorbed fractions. Figure 6 shows that 1-10–purified P4 (fraction 5) inhibited 22% of the binding of 1-10 mAb to Oua-BGG. The inhibition proved specific because binding of the anti-Ars mAb (36-65) to Ars-BGG was not inhibited by P4. Inhibition by C18-adsorbed P4 (fraction 5) was greater (52%, 2.3-fold higher than unadsorbed samples; Figure 6) consistent with the possibility that dissociation of the OD\(_{280}\) compounds increased free OLC in the 1-10–purified fractions (see Discussion).

**Protein A–Binding Immunoglobulin Fractions Contain Proteins That Bind to Oua**

To determine further the nature of the immunoglobulin-like protein that was purified on 1-10 Sepharose, we tested this protein for binding to PA-Sepharose. Plasma 7 (P7, 200 mL) was divided into 2 aliquots of 100 mL each. Figure 7A shows the purification protocol schematically. One hundred milliliters was passed through 1-10 Sepharose and bound proteins were eluted by TEA (1-10 pure P7; Figure 7A, part a). The second aliquot was applied to a column of PA Sepharose. The fall-through from the PA column, depleted of PA-bound immunoglobulin (PA Ig depl; Figure 7A, part b), was then applied to 1-10 Sepharose. After washing with buffer, bound material was eluted with TEA (1-10 pure PA Ig depl; Figure 7A, part c). PA-binding immunoglobulin was eluted from PA-Sepharose with TEA (Crude PA Ig; Figure 7A, part d). This crude PA-binding immunoglobulin was passed through a 1-10 Sepharose column. After washing, the bound proteins were eluted as described above (1-10 pure PA Ig; Figure 7A,
part e). The fall-through from 1-10 Sepharose that was depleted of 1-10 reactive compounds was collected (1-10 depl PA Ig; Figure 7A, part f). We then determined which of the above fractions contained protein. Figure 7B shows the OD<sub>280</sub> of eluted samples from 1-10 Sepharose: 1-10 pure P7 (a), 1-10 pure PA Ig (e), and 1-10 pure PA Ig-depl (c). 1-10 pure P7 and 1-10 pure PA Ig contained protein, whereas little protein was detected in fractions of 1-10 purified plasma that was depleted of PA-binding immunoglobulin (c). This indicated that 1-10 mAb binds to PA-binding immunoglobulin. From 100 mL plasma, 435 mg PA-binding immunoglobulin (Crude PA Ig; Figure 7A, part d) was purified, all of which was applied to 1-10 Sepharose. From 1-10 Sepharose purification of crude PA Ig, approximately 2 mg protein was obtained, which is equivalent to 0.5% of the Crude PA Ig fraction (Figure 7A, part d).

1-10 Sepharose Binding PA Igs Inhibit Binding of 1-10 mAb to Oua-BGG

Inhibition ELISA was used (Figure 7C) to determine whether protein-containing fractions, 1-10 pure P7 (a) and 1-10 pure PA Ig (e), and the protein depleted fraction, 1-10 pure PA Ig depl (c), inhibit the binding of 1-10 mAb to Oua-BGG. Specific inhibition was obtained with each fraction that contained protein. Higher inhibition (25%) was obtained by 1-10 pure P7 (a) than by 1-10 pure PA Ig (e) (10%), although

![Figure 6](http://hyper.ahajournals.org/)

**Figure 6.** Inhibition of binding of 1-10 mAb to ouabain–bovine γ-globulin (Oua-BGG) by P4 (fraction 5) purified on 1-10 Sepharose pre- and post-adsorption on C18 Sep-Pak. Wells of microtiter plates were coated with Oua-BGG. Binding of 25 μL 1-10 mAb to Oua-BGG was determined in the absence or presence 25 μL of the indicated samples and detected using HRP–anti-mouse antibodies (Abs). Inhibition of binding of an anti-p-azophenylarsonate (Ars) mAb (36-65) to Ars-BGG by eluted fractions was determined for specificity control.

![Figure 7](http://hyper.ahajournals.org/)

**Figure 7.** A, Schematic presentation of purification of proteins from P7 using protein A (PA)-Sepharose as described in Results. B, Absorbance (280 nm) pattern of 1-10 purified samples from P7 as described in Results and shown in Panel A. C, Inhibition of binding of 1-10 mAb to Oua-BGG by 1-10–purified samples from P7 as described in Results and shown in Panel A. Wells of microtiter plates were coated with Oua-BGG. Binding of 25 μL 1-10 mAb to Oua-BGG was determined in the absence or presence of 25 μL of the indicated samples using the fraction containing the highest OD<sub>280</sub> (B). Binding was detected using HRP–anti-mouse Abs. Ars-BGG served as control as in Figure 5.
the latter contained more protein (Figure 7B). 1-10 pure PA Ig depl (c), with OD 280/H11005 0.09, did not inhibit binding.

SDS-gel and immunoblot analyses of 1-10 pure PA Ig fractions under reducing conditions indicated a similar pattern as those obtained previously for plasma 4 (80, 50, and 25 kDa fragments, immunoblots not shown).

1-10 Purified PA–Binding Immunoglobulin Binds to Oua In Vitro

The protein-containing fraction from P4 and different samples from P7 were immobilized in the wells of microtiter plates that were coated with either anti-human immunoglobulin or anti-mouse immunoglobulin. The direct binding of 125I-Oua-BGG to immobilized fractions was determined. Figure 8 shows that 125I-Oua-BGG binds to immobilized proteins from plasma 4 (P4; fraction 5), 1-10 pure P7 (a), and 1-10 pure PA Ig (e). PA Ig that was depleted of 1-10 reactive compounds (1-10 depl PA Ig; f), and PA-purified immunoglobulin (crude PA Ig; d) did not capture a significant amount of radiolabeled Oua-BGG. This indicates that only 1-10 Sepharose–purified human plasma proteins bind radiolabeled Oua-BGG. Immobilized fractions all showed significant binding to HRP–anti-human immunoglobulin (OD 450/H11022 2.5), but not to HRP–anti-mouse immunoglobulin, indicating that all wells were coated with plasma proteins (data not shown). 125I-Oua-BGG did not bind to anti-human immunoglobulin coated wells (Buffer), indicating that Oua binding is specific for the purified plasma proteins (Figure 8). The specificity of this binding was further confirmed using 125I-Ars-BGG (Figure 8). None of the fractions captured significant amounts of radiolabeled Ars-BGG, indicating that 1-10–purified fractions do not bind BGG. Oua binding is the property of human-derived proteins because, when anti-mouse immunoglobulin was used to immobilize the proteins from P4- and P7-derived proteins, 125I-Oua-BGG did not bind (Figure 8).

Inhibition of Binding of 125I-Oua-BGG to Plasma Proteins by Free Oua

The binding of 1-10 Sepharose–purified human plasma proteins to 125I-Oua-BGG could be inhibited by nanomolar concentrations of free Oua. The pattern of inhibition was compared with that of the mAb 1-10. As shown in Figure 9A,
relative apparent affinity of plasma proteins is in the range of 200 to 600 nmol/L and that for the mAb 1-10 is 20 nmol/L. The specificity of plasma proteins was tested in inhibition assays using Dig (Figure 9B). These proteins exhibited preferential binding to Oua. Approximately 3 µmol/L Dig was required for 50% inhibition of binding of ¹²⁵I-Oua-BGG to immobilized plasma proteins (100-fold more than that required for 50% inhibition by Oua). The binding specificity of 1-10–purified plasma proteins was different from that of 1-10 mAb because 1-10 mAb did not cross-react with Dig at concentrations as high as 20 µmol/L (Figure 9C).

Discussion

In an attempt to address ambiguities of specificity in the literature surrounding the detection by radioimmuno- and ELISA-based assays of Oua-like immunoreactivity detected in mammalian tissues, including in human blood, we sought to develop a monoclonal antibody to the plant cardiac glycoside Oua. We succeeded in isolating 4 clones that showed high affinity for Oua and no significant cross-reactivity to Dig, the cardiac glycoside prevalent in clinical practice. Because of continuing uncertainty as to the precise structural features of the putative endogenous ouabain-like compounds (OLC), we further sought to use one of these antibodies (mAb 1-10) as an affinity support for the isolation of OLC from the human circulation. Lack of recognition of Dig by 1-10 would allow studies of human samples, irrespective of whether or not the subject was exposed to Dig. Because a few reports have appeared suggesting that OLC can be coupled to a transporting protein in the circulation, we also considered the possibility that treatment of human plasma with a 1-10 affinity column might extract an OLC complexed with a carrier molecule.

Using 1-10 mAb-Sepharose and human plasma, a protein mixture was eluted that, under reducing conditions, exhibited bands of molecular weights (MW) 80, 50, and 25 kDa (Figure 2A). Under nonreducing conditions, only one band, with a MW of 150 to 160 kDa, was detected (immunoblots not shown). All 3 bands in the reducing gel reacted with anti-human immunoglobulin (Figure 2A) but not with anti-mouse immunoglobulin (Figure 2B). The single band (MW 150 to 160 kDa) in nonreducing gels also reacted with anti-human immunoglobulin, but not with anti-mouse immunoglobulin (data not shown), indicating that the high-MW product in the nonreducing gel (corresponding to 50 and 25 kDa fragments in reducing gels) is a human-derivated immunoglobulin. It is possible that the 80 kDa protein observed in reducing gels is the product of a larger protein, a cross-linked heterodimer of the 50 and 25 kDa bands, or residual nonreduced heavy and light chains. The latter is unlikely because control experiments using human immunoglobulin (data not shown) and mAb 1-10 (Figures 2A, 2C) indicated that the reduction of the purified proteins was complete.

We cannot say which of these fragments (human immunoglobulin or the 80 kDa protein) binds ¹²⁵I-Oua-BGG (Figure 8); the 80kDa protein is, nevertheless, the major component of the purified material from 1-10 Sepharose.

Of several human plasma samples affinity-purified on 1-10 Sepharose, P4 demonstrated the most potent biological activity in the Na⁺ pump inhibitory assay. Thus, P4 was selected for more extensive study. The data indicate that protein containing fractions of P4, which specifically inhibited 1-10 mAb binding to Oua-BGG, also contain a compound with inhibitory activity on the intact Na⁺ pump with a potency similar to that of Oua and previously reported OLC of bovine hypothalamic origin. Because human immunoglobulin itself does not bind to the Na⁺,K⁺-ATPase, we hypothesized that the OLC is bound to a transport protein in the plasma that coelutes when OLC is purified on an anti-Oua mAb Sepharose column. Further treatment of the putative OLC-binding protein complex with C18 may dissociate the two (Figures 5, 6), because OD 280 absorbance was eliminated while biological activity increased. The OLC binding protein seems to be an immunoglobulin or an immunoglobulin-like molecule.

The immunoglobulin-like OLC binding protein that coeluted with OLC from 1-10 Sepharose is contained in the total PA-bound immunoglobulin fraction (Figure 7). However, further purification on anti-Oua mAb appears to be necessary for enrichment and detection of Oua-binding immunoglobulin-like protein because 1-10 Sepharose–purified immunoglobulin captures radiolabeled Oua-BGG, whereas the total PA Ig did not (Figure 8). The Oua-binding immunoglobulin fraction constituted ≈0.5% of total PA-binding immunoglobulin. Because of the abundance of Na⁺,K⁺-ATPase in tissues, significant amounts of this carrier protein may be required in the circulation for delivery or uptake of OLC.

Based on the specific activity of ¹²⁵I-Oua-BGG (10 µCi/µg) and the assumptions that 1 to 2 molecules of Oua are coupled to 1 molecule of BGG, with 1 binding site for the Oua-binding protein, we calculated that plasma OLC concentrations for P4 and P7 could be in the range of 27 and 16 nmol/L of plasma, respectively. The concentration of Oua binding protein was calculated based on 2 mg/100 mL plasma (0.5% yield) to be in the range of 13 nmol/L of plasma. This is consistent with the amount of OLC and could indicate that 1 to 2 molecules of OLC bind to 1 molecule of the binding protein. These are estimates. Our OLC concentrations are a little higher than generally published reports (0.2 to 2 nmol/L), and this may represent differences in type and sensitivity of antibodies used in various assay techniques. It is important to mention that several plasmas from blood bank donors, which were purified under identical conditions using the same batch of 1-10 Sepharose that was used for P4 and P7, did not contain detectable Oua bioactivity, although all plasma samples showed protein by OD₂₈₀. Three plasmas that showed no inhibition in the ELISA or Rb⁺ uptake assay were nevertheless run on SDS gel, and all contained the immunoglobulin pattern seen in immuno/bioactivity–positive samples (data not shown). This indicates that either there are significant variations in the amount of the circulating OLC in individuals or there is heterogeneity among OLCs from different indi-
viduals. The former possibility is supported by findings from other investigators that levels of immunologically detectable OLC in human plasma are affected in certain pathophysiological states, including congestive heart failure and hypertension.\textsuperscript{27,28} It is important to note also that the mAb 1-10 used for coupling was precipitated from ascites by ammonium sulfate and not purified by Oua-affinity columns, thus removing the possibility of exogenous Oua contamination in our studies. Our finding of individual variation in the detection of OLC is consistent with the findings of other investigators that Oua/OLC concentrations can be less than 0.005 nmol/L in human plasma.\textsuperscript{29,30}

An additional inconvenience of the small and variably present amounts of the immunoglobulin-like Oua binding protein relates to obtaining adequate samples for necessary structural and sequencing studies. Such will be necessary to characterize not only the 1-10 Sepharose–purified product protein, but the nature and biological properties of the associated Na\textsuperscript{+} pump inhibitory activity and its dynamic relationship to the putative carrier molecule. Nevertheless, our present findings are not without precedent: Lichtstein and coworkers\textsuperscript{10} reported that 25% to 30% of digitalis-like bioactivity in toad plasma appears to be bound to plasma proteins of 48 to 53 kDa. Further structural characterization of this bufadienolide-binding protein was not reported. Antolovic and coworkers\textsuperscript{12} isolated a glycoprotein (homodimer of two 26-kDa subunits) from bovine serum, which binds the protein-reactive digoxigenin derivative HDMA (N-hydroxy succimidyl digoxigenin-3-O-methylcarbonyl-e-aminocaproate). This protein also bound Oua with 2 negatively cooperative sites with affinities in the nanomolar range.\textsuperscript{12} Consistent with our findings, Komiyama and colleagues\textsuperscript{11} reported that a plasmin-cleaved Fc fragment of human IgG, when incubated with Oua, coloeluted on HPLC, and this Fc fragment was able to reverse Oua-induced growth arrest in cultured cells.

One possible explanation for the ability of 1-10 mAb to purify Oua or a similar molecule from plasma is that this mAb binds to the ring structure of Oua,\textsuperscript{13} and the sugar residues are free to interact with the immunoglobulin-like Oua/OLC binding protein. This idea is supported by the observation that protein fractions purified by 1-10 mAb contain both Na\textsuperscript{+} pump inhibitory activity (Figure 4A) and the immunoglobulin-like proteins capable of binding specifically to radiolabeled Oua-BGG (Figure 8). In addition, as noted above, comparison of UV absorbance at 280 and 214 nm of 1-10 purified plasma proteins before and after adsorption on C18 suggests that Oua or OLC dissociates from the immunoglobulin-like plasma proteins during this chromatography (Figure 5). The inhibition of binding of 1-10 mAb to Oua-BGG by the C18-eluted product was 2.5-fold higher than that before adsorption from C18 (Figure 6), raising the possibility that the greater inhibition is due to enhanced free (dissociated) Oua or OLC. It is also possible that the chromatography resulted in increased inhibitory activity by removing other interfering compounds.

It might be considered that the immunoglobulin-like protein is an antibody to exogenous Oua or an autoantibody to endogenous OLC, rather than a storage/transport vehicle. However, we think it unlikely that once Oua were bound to human anti-Oua Abs, it could simultaneously bind to 1-10 mAb (we propose that Oua is bound to Oua-binding protein through its sugar). We know that 1-10 mAb binds to Oua and ouabagenin with the same affinity,\textsuperscript{13} indicating binding through the steroid moiety, but not the sugar. For simultaneous binding of Oua and 1-10 mAb to a reactive (or auto) Oua Ab would imply that endogenous Oua is a multimer compound.

Three lines of evidence exclude the possibility that the Oua-binding protein is either 1-10 mAb that leaks from Sepharose or human immunoglobulins that carry cross-reactive epitopes with mouse immunoglobulins and thus bind to 1-10 mAb. First, protein-containing fractions of P4 and P7 bound to Oua-BGG only when they were captured and immobilized on anti-human immunoglobulin–, but not anti-mouse immunoglobulin–coated wells (Figure 8). Second, the specificity of the 1-10 purified proteins was different from that of 1-10 mAb because, at 20 \( \mu \text{mol/L} \) Dig concentrations, the former cross-reacted with Dig, but 1-10 mAb did not (Figures 9B and 9C). Third, in direct binding assays, anti-mouse immunoglobulin did not show significant binding to 1-10–purified plasma proteins at the highest concentration tested (30 ng/well; Figure 3).

Using another approach to isolate the Oua-binding, immunoglobulin-like proteins, we passed human plasma on Oua-BGG Sepharose. Though some immunoreactive proteins could be eluted from such columns, none of the fractions contained Na\textsuperscript{+} pump inhibitory activity or bound to 125\text{I}-Oua-BGG (data not presented).

Peptidic mimics of Oua and Dig with regard to complementary epitopes for binding to anti-Dig monoclonal antibodies have been selected from phage-displayed peptide libraries, but in this case, too, the selected peptides did not inhibit activity of the Na\textsuperscript{+} pump, even though they did inhibit Dig binding to a monoclonal anti-Dig antibody.\textsuperscript{31} Such Oua-like immunoreactive compounds, therefore, are distinct from our isolates purified using mAb 1-10 because these latter contain both a Na\textsuperscript{+} pump inhibitor and Oua-binding proteins. It should be emphasized that the identity of the Na\textsuperscript{+} pump inhibitor in our current studies is unknown and may or may not be the same or related to OLC that we have previously isolated from bovine hypothalamus and characterized biochemically and physiologically in detail.\textsuperscript{32} Though speculative, the finding that the current OLC is recognized by an anti-ouabain mAb has a potency in the rubidium-uptake assay in line with our previous bioassay results, and that it is a stable molecule with the same elution profile from C18, suggest structural homology between the plasma and hypothalamus-derived inhibitors. Larger quantities of inhibitory activity will be needed to allow biochemical and physiologically profiling of the OLC activity that copurifies with the immunoglobulin-like proteins from human plasma reported in this work.

In summary, using a mAb with high specificity for the cardiac glycoside Oua, human plasma immunoglobulin-like proteins have been isolated in association with Oua-like biological activity, and these proteins bind Oua in vitro with 100-fold greater affinity than for the related cardiac glycoside Dig, suggesting specificity for Oua or OLC in vivo. Further
chromatographic purification appears to dissociate the inhibitor from the putative carrier proteins, raising the possibility that these immunoglobulin-like molecules may function as a reservoir and delivery system for OLC. Further structural and physiological characterization of both moieties will be necessary to determine the nature of their interaction and a possible role as a regulatory system in the modulation of human Na⁺,K⁺-ATPase activity in vivo.

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