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Identification and characterization of a ouabain-like compound from human plasma

(sodium pump/digitalis/endogenous/hypertension)

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ABSTRACT **The plasma membrane sodium–potassium pumps that regulate intracellular sodium in most animal cells have specific, high-affinity receptors for the digitalis glycosides and their aglycones. This has fostered speculation that there is an endogenous ligand. We have purified and structurally identified by mass spectroscopy an endogenous substance from human plasma that binds with high affinity to this receptor and that is indistinguishable from the cardenolide ouabain. This human ouabain-like compound (OLC) displaces [^{3}H]ouabain from its receptor, inhibits Na,K-ATPase and ouabain-sensitive $^{86}Rb^+$ uptake, and has cardiotonic actions quantitatively similar to commercial ouabain. Immunoreactive OLC was detected in the plasma of many mammals, and high concentrations were found in the adrenals. The circulating OLC may modulate intracellular Na^+ and affect numerous Na^+ gradient-dependent processes including intracellular Ca^{2+} and pH homeostasis in many tissues. Furthermore, altered circulating levels of OLC may be associated with the pathogenesis of certain forms of hypertension.**

Cardiotonic steroids such as the bufodieneolides and digitalis glycosides have proven useful in the treatment of heart failure (1, 2). Synthesis of these compounds by specific plants is well known, while bufodieneolides are also endogenous to certain amphibians (3). The presence in amphibians of bufodieneolides and the conservation of a specific receptor for cardiotonic steroids on the surface of most mammalian cells (4, 5) have fueled efforts to detect and identify endogenous counterparts in mammals. Indeed, numerous Na,K-ATPase inhibitors have been detected in mammalian preparations (6, 7), although many are nonspecific, of low affinity, and may not be physiological regulators of the sodium pump (7–10). Recently, we found an endogenous ouabain-like compound (OLC)¶ in human plasma that interacted with the cardenolide receptor on the Na^+ pump and whose mechanism of inhibition was strikingly similar to that of the digitalis glycosides (11, 12). Here, we describe the identification of human OLC and its quantitation in plasma and tissue extracts.

METHODS

Purification of OLC from Human Plasma. Approximately 85 liters of human plasma, acquired from saline expanded individuals undergoing routine plasmapheresis, was dialyzed as described (11, 12). No individuals had received cardiotonic steroids. The dialyzate was applied to an Amberlite XAD-2 column (bed volume, 3.5 liters), and adsorbed materials were eluted with methanol (1 vol) and dried under vacuum at 50°C. The solids were reconstituted in water and fractionated by using a Waters Prep LC500A HPLC on a preparative-scale Vydac C-18 reverse-phase column (Millipore) and eluted with a two-step linear acetonitrile (CH_3CN) gradient (12).

Active fractions, in all purification steps, were detected by inhibition of ouabain-sensitive ^{86}Rb uptake by washed human erythrocytes using modifications of previous methods (13). Inhibitory material eluting from the column (84–88 min; ≈20% CH_3CN) was subjected to affinity extraction with partially purified lamb kidney Na,K-ATPase (14). Combined materials were incubated for 3 hr at 37°C in buffer containing 200 mM Tris·HCl (pH 7.2), 5 mM $MgCl_2$, and 5 mM NaH_2PO_4/Na_2HPO_4 to which an ≈2-fold molar excess of Na,K-ATPase was added. Under these conditions, cardenolides bind with high affinity to Na,K-ATPase (15). After incubation, enzyme–inhibitor complexes were separated by centrifugation (150,000 × *g*; 2 hr; 4°C). The pellet was resuspended in buffer containing 2 mM Tris·HCl (pH 7.2) and 5 mM Tris EDTA and was incubated for 6 hr at 37°C to induce dissociation of enzyme–inhibitor complexes. The enzyme was centrifuged as described above and the supernatant was lyophilized, reconstituted, and subjected to two sequential HPLC steps (12).

Structural Analyses. Purified OLC was analyzed by fast atom bombardment mass spectrometry (FAB MS) using a VG 70 SE double focusing mass spectrometer with a Cs gun (VG Instruments, Manchester, U.K.). FAB spectra were acquired from *m/z* 100 to *m/z* 1500. For acetylation, samples were derivatized with 50 μl of acetic anhydride/dimethylaminopyridine (DMAP) (15 mg/ml) in pyridine (1:1) for 2 hr at room temperature. Excess reagent was removed under vacuum and samples were dissolved in 0.5 μl of glycerol/thioglycerol (1:1).

Biological and Immunological Characterization. The purified OLC was tested in three assay systems: the ouabain-sensitive uptake of ^{86}Rb into washed human erythrocytes, [^{3}H]ouabain binding to purified canine kidney Na,K-ATPase, and Na,K-ATPase activity by methods described elsewhere (12).

For immunological experiments, polyclonal antisera with titers >100,000 were generated against ouabain in rabbits (16). The antiserum was incorporated into an ELISA, which has an EC_{50} for ouabain of ≈0.08 pmol per well (16).

Plasma OLC was determined after extraction by octadecylsilane (C-18) columns (Analytichem International, Harbor City, CA). OLC was eluted with 25% CH_3CN in water, dried, and reconstituted in ELISA buffer. For tissue measurements, fat-free organs were obtained from rats and homogenized in

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Abbreviations: OLC, ouabain-like compound; DMAP, dimethylaminopyridine; DOCA, deoxycorticosterone acetate; FAB, fast atom bombardment.

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¶We have previously termed this material endogenous digitalis-like factor (EDLF).

methanol. The supernatants were flash evaporated, extracted, and assayed as for plasma. In some studies, hypertension was induced in uninephrectomized 300-g Sprague–Dawley rats drinking 0.9% NaCl (Unix salt) by silastic implants containing deoxycorticosterone acetate (DOCA salt) (100 mg/kg). Measurements of plasma OLC and mean arterial blood pressure (tail cuff) were determined 2 weeks later.

Inotropic properties of OLC were determined in guinea pig (250–300 g) left atria mounted in baths (1.3 ml) between a platinum stimulating electrode and a force transducer. Atria were incubated at 37°C in a salt solution (pH 7.3–7.4) containing 130 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 0.4 mM NaH_2PO_4, 19 mM $NaHCO_3$, and 5.4 mM glucose, and gassed with 95% O_2/5% CO_2. Tissues were stimulated at 1 Hz with twice threshold square pulses (0.8–1.0 V) of 0.7–1.2 msec duration.

Chromatography and Detection of OLC in Bovine Adrenal Glands and Secretion from Cultured Adrenal Cells. Bovine adrenals (1.5 kg) from an abbatoir were defatted and homogenized in 6 liters of 10 mM NH_4OAc/3 mM EDTA, pH 6.8. The supernatant (14,000 × *g*; 60 min) was lyophilized and reconstituted in 1.4 liters of H_2O, centrifuged (8000 × *g*; 10 min), and applied to an Amberlite XAD-2 column (bed vol, 2.8 liters) preequilibrated with H_2O. Bound materials were eluted with 2.5 liters of methanol and vacuum dried. The residue was reconstituted in 50 ml of H_2O containing 0.1% trifluoroacetic acid and was pumped onto a Beckman semipreparative C-18 high-performance column (1 × 25 cm). Bound materials were sequentially eluted in 5-ml fractions by a CH_3CN gradient (11). The fractions were dried under vacuum, reconstituted, and tested for their inhibitory effect on: (*i*) ouabain-sensitive uptake of ^{86}Rb by human erythrocytes, (*ii*) binding of [^{3}H]ouabain to purified dog kidney Na, K-ATPase, and (*iii*) binding of ouabain to a ouabain-specific antiserum using an ELISA.

For secretion studies, bovine adrenocortical cells from the American Type Culture Collection were grown in Dulbecco's minimal essential medium (DMEM) supplemented with 10% fetal calf serum in 95% air/5% CO_2. Culture flasks at 80% confluence were washed with serum-free DMEM. Each flask was incubated for 30 min at 37°C with DMEM (control), or with DMEM supplemented with 40 mM KCl or 40 mM NaCl as indicated. Subsequently, the medium was harvested, extracted, and assayed by ELISA. The cell protein content of each flask was determined by the BCA technique (Pierce).

RESULTS

Purification and Structural Analyses. Fig. 1 shows the results of the final step of OLC purification. A single symmetrical UV peak containing 12 μg of OLC eluted at 32 min and contained >95% of the inhibitory activity purified ≈10^{10}-fold with respect to starting plasma (dry wt/dry wt). The minimum calculated plasma concentration of OLC in the donor plasma was 321 pM after correction for losses in dialysis (25%). Chromatographic losses were at least 50%. Thus, the calculated concentration of OLC in the native plasma was >650 pM.

FAB spectra of the active fraction and the two adjacent fractions from the purification step (Fig. 1) were acquired over *m/z* 100–2500 Da. A unique protonated molecular ion at *m/z* 585 was observed in the active fraction. An accurate mass of 585.295 Da for OLC was determined in subsequent FAB MS by peak matching with glycerol cluster ions (data not shown) and an elemental composition of $C_{29}H_{45}O_{12}$ was predicted, matching that for ouabain (calculated mass, 585.291 for the protonated species). Linked-scan MS/MS was used to analyze the daughter ion fragments of the *m/z* 585 peak for OLC and ouabain. Both spectra showed a major ion

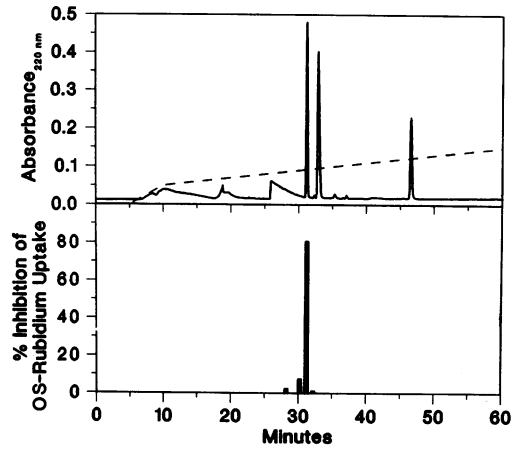

FIG. 1. Results of the final step of purification of OLC. OLC was eluted from a Beckman semipreparative column with a two-step linear gradient (10–30% at 10–60 min; dashed line) of CH_3CN containing 0.1% trifluoroacetic acid. Solid line is absorbance at 220 nm using 85 liters of plasma equivalents (PLE) and shows a baseline-resolved, symmetrical peak at 32 min (17–18% CH_3CN). Fifty milliliters of PLE from this peak inhibited ouabain-sensitive (OS) ^{86}Rb uptake by 80% with no effect on ouabain-insensitive uptake.

at *m/z* 439 corresponding to the aglycone of ouabain (data not shown), indicating that the sugar moiety of OLC, like that of ouabain, is a deoxyhexose.

Acetylated derivatives of OLC and ouabain were examined by FAB MS. The structure of ouabain is shown in Fig. 2 *Upper* (*Inset*), and the six primary and secondary OH groups are indicated. After acetylation, the protonated molecular ions at *m/z* 585 disappeared and several ions at higher masses were detected. The new peaks were interpreted as follows: *m/z* 859, [584] + 6 acetyl groups (Ac) + Na^+; *m/z* 901, [584] + 7 Ac + Na^+; *m/z* 959, [584] + 6 Ac + DMAP + H^+; *m/z* 1001, [584] + 7 Ac + DMAP + H^+; *m/z* 1043, [584] + 8 Ac + DMAP + H^+. Both ouabain and OLC generated identical spectra, indicating that human OLC, like ouabain has six primary and secondary OH groups and two hindered tertiary OH groups. The accurate mass data, identical daughter ion, and derivative spectra following acetylation show that OLC and ouabain are isomeric and probably identical compounds. As the identity of the deoxysugar and the stereochemical features of OLC remain to be established, we use the term "ouabain-like compound."

Biological and Immunological Characterization. OLC inhibited the transport and hydrolytic activities of the Na,K-ATPase and blocked the binding of [^{3}H]ouabain to the enzyme (Fig. 3). Rabbit polyclonal ouabain antibodies cross-reacted with OLC. The antiserum showed minimal cross-reactivity with common steroids: aldosterone, 0.012%; DOCA, 0.0046%; corticosterone, 0.0035%; hydrocortisone (cortisol), <0.001%; and progesterone, 0.0011%. Moreover, the slopes of the dose–response curves obtained with adrenocortical steroids in the ELISA were lower than those for ouabain or OLC (16) and the apparent half-maximal concentrations for OLC were similar to those for ouabain (see Fig. 3 legend).

The action of OLC on guinea pig left atrium is shown in Fig. 4. OLC (85 nM) reversibly increased peak force, reaching a plateau in 34 min. Developed force increased further with 170 nM OLC to a value 313% ± 32% (n = 3) above control and the half-time for washout of the inotropic response to OLC was ≈4 min. For comparison, 160 nM ouabain evoked a similar inotropic response (310% ± 31%; n = 4) and showed similar washout kinetics (data not shown). In each case, the augmented contractility was due to an increase in the rate of

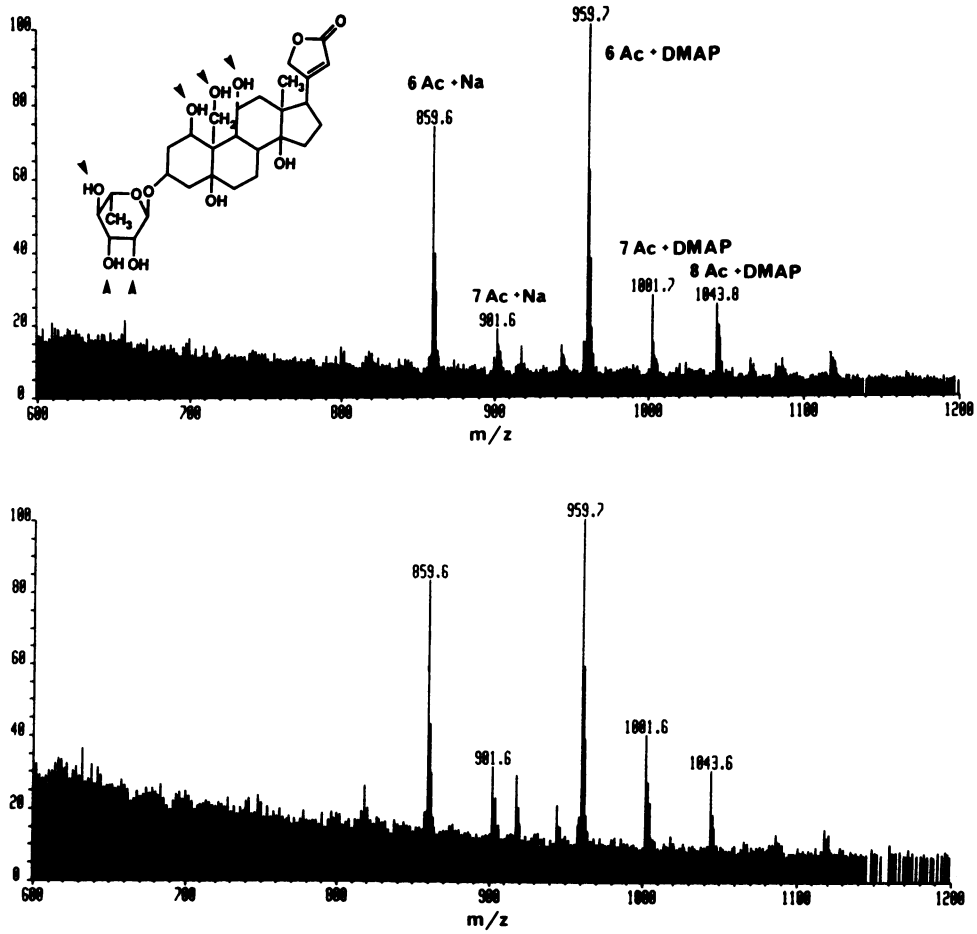

FIG. 2. MS of acetylated ouabain and OLC. Results are shown for ouabain (upper spectrum) and OLC (lower spectrum). Both samples were acetylated and analyzed as described in *Methods*.

force development with no appreciable prolongation in contractile duration (lower tracings).

An ELISA was used to estimate the levels of immunoreactive OLC in blood and tissue extracts of several mammals (Table 1). Plasma levels were similar in humans, dogs, and rats, and most tissues contained ouabain immunoreactivity. The HPLC retention time of the main peak of tissue immunoreactivity was indistinguishable from that of ouabain (data not shown) and the dose–response curves of the tissue extracts paralleled those for ouabain. The specific content of the adrenals was ≈500-fold greater than the plasma level in the rat.

Plasma immunoreactive OLC was increased (1.6-fold) by uninephrectomy of rats drinking 0.9% saline (Table 1). In hypertensive DOCA-treated uninephrectomized rats, plasma OLC levels were 9-fold higher than controls.

OLC in Bovine Adrenal Glands and Secretion from Cultured Adrenal Cells. Large amounts of OLC were detected in adrenal extracts from various mammals including humans (17). As illustrated in Fig. 5, a single major peak, active in all three assay systems, was found in fraction 12 (solid bar). The elution characteristics of the adrenal OLC (fraction 12) were identical

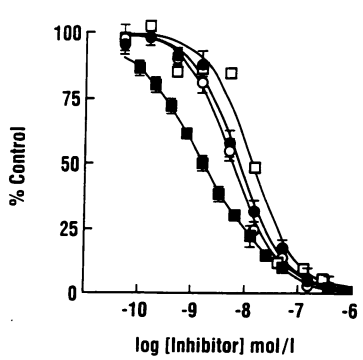

FIG. 3. Biological properties of OLC. Dose–response curves for OLC were generated for inhibition of ouabain-sensitive ^{86}Rb uptake in human erythrocytes (○), competition for [^{3}H]ouabain binding to dog kidney Na,K-ATPase (●), inhibition of dog kidney Na,K-ATPase activity (□), and competition for binding to ouabain antibodies (■). Each data set was fitted to sigmoidal functions by iterative nonlinear regression. Data points are means ± SEM of three or four determinations. Calculated apparent K_d values (and Hill coefficients) were 6.0 (−0.99), 13.9 (−1.28), 7.7 (−0.97), and 1.6 (−0.65) nM for inhibition of transport, Na,K-ATPase activity, competition for ouabain binding, and the ouabain ELISA, respectively.

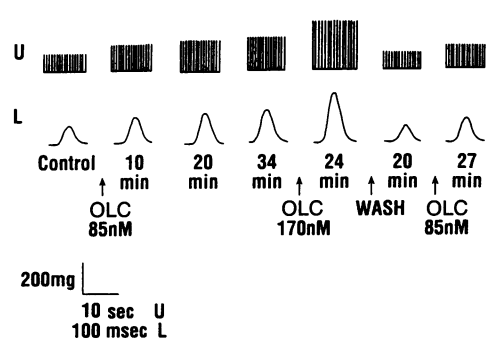

FIG. 4. Inotropic response of guinea pig atria to human OLC. The upper recordings (U) of a series of contractions and the lower recordings (L) of single contractions were obtained at the times indicated after addition or washout of OLC (arrows).

Table 1. OLC content in normal plasma and tissue and in rat plasma DOCA salt hypertension

	Immunoreactivity	Mean arterial pressure, mmHg
Species*		
Human (n = 11)	138 ± 43	
Rat (n = 11)	80 ± 18	
Dog (n = 6)	37 ± 7	
Tissue†		
Adrenal	38.6	
Pituitary	5.1	
Hypothalamus	2.5	
Atria	2.5	
Kidney	3.4	
Liver	2.1	
Model‡		
Adrenalectomy (n = 8)	36 ± 12	—
Unix salt (n = 6)	105 ± 43	113 ± 4
DOCA salt (n = 5)	975 ± 79	156 ± 12

OLC was determined in extracts of plasma or tissue by an ELISA. Plasma levels represent those in forearm venous blood in humans, trunk blood in Sprague–Dawley male rats, and femoral venous blood in beagle dogs. All data are means ± SD where appropriate.
*Plasma concentrations of OLC (pmol/liter).
†Tissue concentrations of OLC in rat (pmol/g wet wt).
‡Plasma concentrations of OLC 3 days following adrenalectomy (pmol/liter), in reduced renal mass and mineralocorticoid hypertension in the rat.

to those of ouabain. All fractions contained some assay-dependent inhibitory activity (relative to assay buffer alone) other than the main peak of OLC seen in fraction 12. This was particularly noticeable in the Na pump assays (Fig. 5*A*) in which a background level of 15–20% inhibition was common. Other small peaks of biological activity were also apparent and may be synthetic intermediates and/or metabolic products.

The secretory rate of immunoreactive OLC by cultured bovine adrenocortical cells was reduced in the presence of elevated external K^+ concentrations, which should depolarize the cells and stimulate calcium influx (Fig. 5*D*). Similar experiments with additional Na^+ indicated that the effect of K^+ was not due to hypertonicity. No OLC was found in unincubated DMEM (<0.05 pmol per 12 ml).

DISCUSSION

The data show that the endogenous digitalis-like factor in human plasma is structurally, biologically, and immunologically indistinguishable from ouabain. This is unanticipated. Ouabain-enriched extracts from plants native to East Africa and Polynesia have been used as arrow poisons (18) and the discovery of OLC in humans raises questions about the origin of this material. Six lines of evidence suggest that OLC is endogenous. First, high concentrations of OLC were detected in the adrenals of humans (17), cows (Fig. 5 *A–C*), and rats (Table 1), species with distinct diets. Second, levels of OLC in rat plasma fell after bilateral adrenalectomy (Table 1). Third, bovine adrenals contain large amounts of OLC, which inhibits the transport and receptor activity of the Na^+ pump (Fig. 5 *A–C*), and cultured adrenal cells secrete OLC (Fig. 5*D*). Fourth, plasma levels of OLC were elevated in hypertensive rats (Table 1). Fifth, plasma levels of four individuals on total parenteral nutrition for 1 week were similar to matched controls (108 ± 17 vs. 138 ± 43 pM, respectively). Sixth, oral ouabain has minimal bioavailability (19). Collectively, these observations indicate an endogenous origin.

Several rat tissues were enriched in immunoreactive OLC (Table 1); the concentration of OLC in whole rat adrenal is ≈500-fold higher than in plasma. HPLC separation of adrenal extracts of humans, rats (data not shown), and cows (Fig. 5 *A–C*) yielded a major active fraction with the same retention time as ouabain. This fraction (*i*) inhibited the ouabain-sensitive ^{86}Rb uptake by human erythrocytes, (*ii*) inhibited [^{3}H]ouabain binding to the Na,K-ATPase, and (*iii*) showed ouabain-like immunoreactivity.

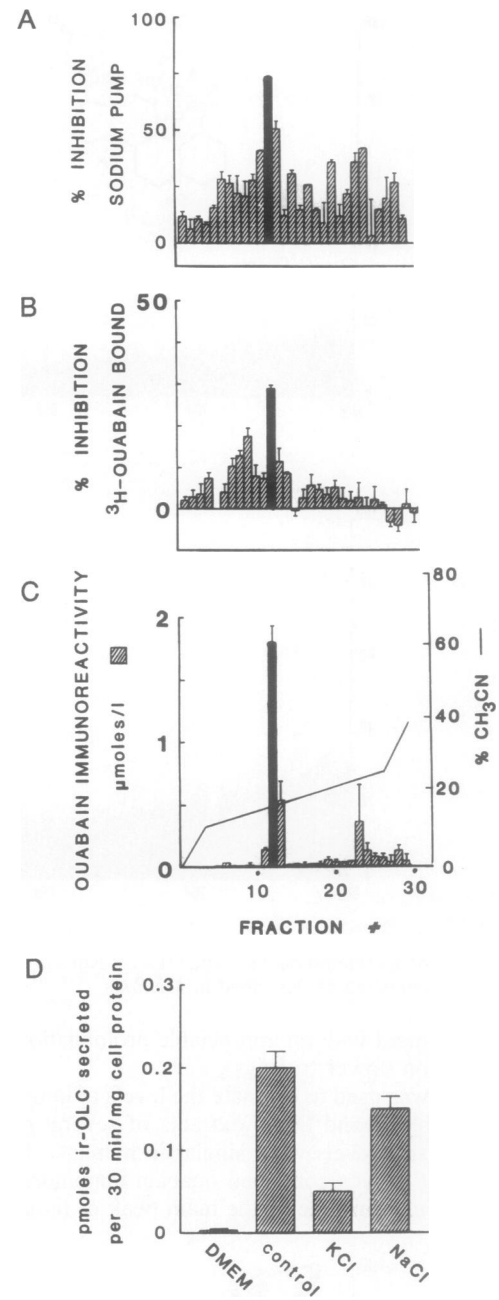

FIG. 5. Presence of OLC in bovine adrenal gland and secretion of OLC from adrenocortical cells in culture. (*A–C*) HPLC of bovine adrenal gland extract. Fractions of 5 ml were collected using the same column type, solvents, and gradient program used for purification of human OLC in Fig. 1. Fractions were assayed for inhibition of sodium pump (ouabain-sensitive ^{86}Rb uptake) (*A*), inhibition of [^{3}H]ouabain binding (*B*), and ouabain-like immunoreactivity by ELISA (*C*). Bars indicate means ± SEM of three (*C*) or four (*A* and *B*) determinations. (*D*) Secretion of immunoreactive (ir) OLC from cultured bovine adrenal cells. Cultured bovine adrenocortical cells were washed in serum-free DMEM and incubated in serum-free DMEM alone or in DMEM containing 40 mM KCl or 40 mM NaCl for 30 min at 37°C in 95% air/5% CO_2. The media were harvested and extracted by solid-phase columns as described for plasma in Table 1 legend. Data are the means ± SEM of three determinations from one set of cells; comparable results were obtained in two other sets of the same cell type.

The high OLC content in the adrenals raises the possibility that this gland may be a source of the circulating OLC. Indeed, cultured bovine adrenocortical cells secrete a polar immunoreactive OLC (Fig. 5*D*) and the secretory rate was lower in the presence of elevated levels of extracellular K^+, which may depolarize the cells and raise intracellular Ca^{2+}, a response reminiscent of that observed for secretion of renin (20) and parathyroid hormone (21). This seems an appropriate negative feedback mechanism for a humoral agent that may increase cell Na^+ and Ca^{2+}.

Unidentified factors that cross-react with digoxin antibodies have been detected in mammalian plasma (22). The low cross-reactivity (<0.01%) of a common digoxin antibody (DuPont/NEN) to ouabain excludes OLC as the primary digoxin-immunoreactive factor in human plasma (23) and the digoxin-like factor in human urine (24).

The subnanomolar levels of OLC in plasma suggest a physiological role. The insensitivity of the rat to cardiac glycosides is documented (25), although a Na,K-ATPase isoform with high ouabain affinity is present in rat brain (26). In human arterioles, nanomolar levels of ouabain inhibit acetylcholine-induced vasodilation (27) and in volume expanded humans an inotropic action of OLC appears feasible. For example, the plasma used for purification of OLC contained calculated concentrations >0.6 nM. As the apparent affinity of the human cardiac Na,K-ATPase for ouabain is ≈2-fold greater than that of digoxin (28), plasma concentrations of ouabain (or OLC) in the range of 0.6–1.2 nM may have physiological and pathophysiological effects. Furthermore, high plasma levels of OLC may account for some idiopathic arrhythmias in otherwise normal individuals and may influence the therapeutic window for digoxin in patients with congestive heart failure. Interestingly, in a preliminary study in four patients with a rare form of untreated left ventricular cardiomyopathy (29), plasma levels of OLC were 24 ± 8 pM. The low values may indicate a propensity of some patients to develop certain forms of heart failure (30).

Increased levels of humoral Na^+ transport inhibitors have been detected during plasma volume expansion (13, 31, 32) and in hypertension (13, 23, 33–39). Our finding of elevated plasma OLC in hypertension is consistent with that in patients with primary aldosteronism and essential hypertension (38, 39).

Aside from the pathophysiological implications, the presence of OLC in animals may be of general biological significance. For example, the skin of many nonbufonidae amphibia contains polar substances that compete with [^{3}H]ouabain for binding to the Na,K-ATPase (40). In species moving between terrestrial and aquatic environments, the concentrations of these materials were higher in the dry than in the wet season (40), suggesting a role in salt and water conservation. Finally, the Na^+ pump affects several voltage and Na^+ gradient-dependent processes fundamental to growth and metabolism of the organism. A humoral agent that modulates sensitive Na^+ pumps is likely to influence numerous processes *in vivo*.

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1. Withering, W. (1795) *An Account of the Foxglove and Some of Its Medical Uses: With Practical Remarks on Dropsy and Other Diseases* (Swinney, Birmingham, U.K.), p. 207.
2. Meyer, K. & Linde, H. (1971) in *Venomous Animals and Their Venoms*, eds. Bucherl, W. & Buckley, E. E. (Academic, New York), Vol. 2, pp. 521–556.
3. Siperstein, M. D., Murray, A. W. & Titus, E. (1957) *Arch. Biochem. Biophys.* **67**, 154–160.
4. Schatzmann, H.-J. (1953) *Helv. Physiol. Acta* **11**, 346–354.
5. Skou, J. C. (1960) *Biochim. Biophys. Acta* **42**, 6–23.
6. Lichtstein, D. S., Samuelov, S., Deutsch, J., Xu, H., Lutz, R. A., Chernick, S. S. & Chernick, S. S. (1987) *Klin. Wochenschr.* **65**, Suppl. 8, 40–48.
7. Hamlyn, J. M. (1988) *ISI Atlas Sci. Pharmacol.* **2**, 339–344.
8. Hamlyn, J. M., Schenden, J. A., Zyren, J. & Baczynskyj, L. (1987) *Hypertension* **10**, Suppl. I, I-71–I-77.
9. Tamura, M., Kuwano, H., Kinoshita, T. & Inagami, T. (1985) *J. Biol. Chem.* **260**, 9672–9677.
10. Kelly, R. A., O'Hara, D. S., Mitch, W. E. & Smith, T. W. (1986) *J. Biol. Chem.* **261**, 11704–11711.
11. Hamlyn, J. M., Harris, D. W., Clark, M. A., Rogowski, A. C., White, R. J. & Ludens, J. H. (1989) *Hypertension (Dallas)* **13**, 681–689.
12. Hamlyn, J. M., Harris, D. W. & Ludens, J. H. (1989) *J. Biol. Chem.* **264**, 7395–7404.
13. Hamlyn, J. M., Ashen, M. D., Forrest, B., Rogowski, A. C. & White, R. J. (1988) *Prog. Biochem. Pharmacol.* **23**, 22–34.
14. Lane, L. K., Potter, J. D. & Collins, J. H. (1979) *Prep. Biochem.* **9**, 157–170.
15. Van Winkle, W. B., Allen, J. C. & Schwartz, A. (1972) *Arch. Biochem. Biophys.* **151**, 85–92.
16. Harris, D. W., Clark, M. A., Fisher, J. F., Hamlyn, J. M., Kolbasa, K. P., Ludens, J. H. & DuCharme, D. W. (1991) *Hypertension (Dallas)* **17**, 936–943.
17. Hamlyn, J. M., Harris, D. W., Resau, J. & Ludens, J. H. (1990) *FASEB J.* **4**, A295.
18. Fieser, L. F. & Fieser, M., eds. (1959) *Steroids* (Reinhold, New York), p. 768.
19. Tanz, R. D. (1986) in *Modern Pharmacology*, eds. Craig, C. R. & Stitzel, R. E. (Little, Brown, Boston), 2nd Ed., pp. 345–351.
20. Churchill, P. C. (1979) *J. Physiol. (London)* **294**, 123–134.
21. Shoback, D. M., Thatcher, J., Leombruno, R. & Brown, E. M. (1984) *Proc. Natl. Acad. Sci. USA* **81**, 3113–3117.
22. Graves, S. W. (1983) *Crit. Rev. Clin. Lab. Sci.* **23**, 177–200.
23. Hamlyn, J. M., Ringel, R., Schaeffer, J., Levinson, P. D., Hamilton, B. P., Kowarski, A. A. & Blaustein, M. P. (1982) *Nature (London)* **300**, 650–652.
24. Goto, A., Ishiguro, T., Yamada, K., Ishii, M., Yoshioka, M., Eguchi, C., Shimora, M. & Sugimoto, T. (1990) *Biochem. Biophys. Res. Commun.* **173**, 1093–1101.
25. Repke, K., Est, M. & Portius, H. J. (1965) *Biochem. Pharmacol.* **14**, 1785–1802.
26. Blanco, G., Berberian, G. & Beauge, L. (1990) *Biochim. Biophys. Acta* **1027**, 1–7.
27. Woolfson, R. G. & Poston, L. (1990) *J. Hypertens.* **8**, 1057–1058.
28. Brown, L. & Erdmann, E. (1984) *Arch. Int. Pharmacodyn. Ther.* **271**, 229–240.
29. Topol, E. J., Traill, T. A. & Fortuin, N. J. (1985) *N. Engl. J. Med.* **312**, 277–282.
30. Szent-Gyorgyi, A. (1953) *Chemical Physiology of Contraction in Body and Heart Muscle* (Academic, New York), pp. 86–91.
31. Buckalew, V. M., Jr., Martinez, F. J. & Green, W. E. (1970) *J. Clin. Invest.* **49**, 926–935.
32. Nutbourne, D. M., Howse, J. D., Schrier, R. W., Talner, L. B., Ventom, M. G., Verroust, P. J. & de Wardener, H. E. (1970) *Clin. Sci.* **38**, 629–648.
33. Haddy, F. J. & Pamnani, M. B. (1983) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **42**, 2673–2680.
34. Songu-Mize, E., Bealer, S. L. & Caldwell, R. W. (1987) *J. Hypertens.* **5**, 461–468.
35. Hamlyn, J. M. (1989) *J. Endocrinol.* **122**, 409–420.
36. Moreth, K., Kuske, R., Renner, D. & Schoner, W. (1988) *Klin. Wochenschr.* **64**, 239–244.
37. Pamnani, M. B., Burris, J. F., Jemionek, J. F., Huot, S. J., Price, M., Fries, E. D. & Haddy, F. J. (1989) *Am. J. Hypertens.* **2**, 524–531.
38. Masugi, F., Ogihara, T., Hasegawa, T., Tomii, A., Nagano, M., Higashimori, K., Kumahara, Y. & Terano, Y. (1986) *Biochem. Biophys. Res. Commun.* **135**, 41–45.
39. Masugi, F., Ogihara, T., Hasegawa, T. & Kumahara, Y. (1987) *Clin. Exp. Hypertens.* **A9**, 1233–1242.
40. Flier, J., Edwards, M. W., Daly, J. W. & Myers, C. W. (1980) *Science* **208**, 503–505.