Human Adrenal Cells in Culture Produce Both Ouabain-like and Dihydroouabain-like Factors

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Background: Ouabain-like factor (OLF) and its newly discovered reduced species, dihydroouabain-like factor (Dh-OLF), are mammalian cardenolides whose structural and functional characteristics are similar to the plant-derived compounds ouabain and dihydroouabain. These endogenous compounds are believed to be produced by the adrenals and to constitute part of an hormonal axis that may regulate the catalytic activity of the α-subunit of Na⁺,K⁺-ATPase. We developed antibodies sufficiently specific to distinguish between OLF and Dh-OLF, and in this study demonstrate the selective secretion of OLF and Dh-OLF from human H295R-1 adrenocortical cells in culture.

Methods: We used reversed-phase HPLC, inhibition of Na⁺,K⁺-ATPase catalytic activity, and two enzyme immunoassays developed with antibodies specific to ouabain and dihydroouabain to purify and characterize the secretion of these two compounds by human adrenal cells in culture. Purified antisera had high titers (1×10⁶ for ouabain and 8×10⁵ for dihydroouabain) and were specific to their corresponding antigens.

Results: Human H295R-1 cells grown in serum-free medium secreted 0.18 ± 0.03 pmol of OLF and 0.39 ± 0.04 pmol of Dh-OLF per 10⁶ cells in 24 h. Both OLF and Dh-OLF inhibited the ouabain-sensitive catalytic activity of the sodium pump (0.03 μmol/L OLF inhibited 29% of the catalytic activity; 0.07 μmol/L Dh-OLF inhibited 17%). Stimulation of the cell culture by dibutyl cAMP increased the secretion of Dh-OLF 50% over control (unstimulated), whereas the secretion of OLF did not increase significantly.

Conclusions: OLF and Dh-OLF are secreted by human adrenal cells, and antibodies specific to these two compounds can be developed, using the plant-derived counterparts as antigens. The secretion of Dh-OLF is responsive to a cAMP-dependent stimulation mechanism, whereas OLF is not. Our data suggest that either the secretory or biosynthetic pathways for production of these two compounds by human adrenal cells may have different control mechanisms or that they may be linked via a precursor–product relationship.

Compounds that resemble plant-derived cardiac glycosides have been found in human plasma (1), urine (2), mammalian adrenal glands (3–6), hypothalamus (7, 8), and mammalian adrenal cells in culture (9–11). The endogenous mammalian-derived cardiac glycosides, often referred to as “mammalian cardenolides”, inhibit the catalytic activity of the α-subunit of Na⁺,K⁺-ATPase. A mechanism for direct hormonal control of the sodium pump may play an important role in understanding pathophysiologic conditions linked to control of blood volume and homeostasis (13), as well as sodium-pump-related mechanisms underlying kidney (14), cardiac (15) and neurologic disorders (16). Evidence is now mounting that suggests these mammalian-derived compounds have hormone-like properties (12, 17). Two main classes of mammalian cardenolides have been identified: the cardiac glycoside-like factors, such as digoxin-like factors (18) and ouabain-like factors (OLF)s (1, 19), and the bufodienolide-like factors, such as bufalin-like factors (20, 21) and proscillaridin-like factors (7, 22). Although the chemical structures of the mammalian-derived digoxin-like factors have not been completely elucidated, sev-

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Received March 6, 2002; accepted June 14, 2002.

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3 Nonstandard abbreviations: OLF, ouabain-like factor; Dh-OLF, dihydroouabain-like factor; EIA, enzyme immunoassay; BSA, bovine serum albumin; PBS, phosphate-buffered saline; TMB, 3,3',5,5'-tetramethylbenzidine; KLH, keyhole limpet hemocyanin; (Bu)2cAMP, dibutyl cAMP; ddH2O, distilled, deionized water; OLC, ouabain-like compound; and ACTH, adrenocorticotropic hormone.
eral reports do provide compelling evidence on the structure of OLFs (23, 24). OLFs have an oxidized lactone ring attached to the steroid nucleus (see Fig. 1) similar to their plant-derived counterpart, ouabain (12). When saturated with two protons, this lactone moiety significantly reduces the affinity for binding to the α-subunit of Na⁺,K⁺-ATPase (25, 26). Importantly, we recently isolated from bovine adrenocortical tissue a dihydroouabain-like factor, which, when saturated with two protons, is intrinsically less biologically active than OLF and is characterized by having a chemically reduced lactone ring similar to that of dihydroouabain (4). These findings underscore the importance of understanding the natural biosynthesis of these newly discovered chemically reduced forms of the mammalian cardenolides (27).

The steroid-like nature of the endogenous cardenolides (21, 28) and their increased concentrations in adrenal cortical tissues (5, 29) suggest that the steroidogenic pathway is likely involved in their biosynthesis. One approach for elucidating the biosynthetic pathway of a natural steroid-like compound is to selectively stimulate steroid synthesis in adrenal cells and subsequently characterize the secretion of the compounds (11). Hence, the availability of well-characterized cell culture model systems is important for undertaking this work. Although several animal adrenal cells have been shown to secrete OLFs (9, 10), to date no reports have documented the secretion of both OLF and Dh-OLF from any human cell line. In addition to a well-defined cell culture system, the availability of immunoassays sufficiently specific for distinguishing the two forms (unsaturated and reduced) of OLF molecules would provide a valuable tool for characterizing the biosynthesis and physiologic role of these compounds.

In this study we report the development of two specific enzyme immunoassays (EIAs) for detection and quantification of OLF and Dh-OLF. Using a combination of reversed-phase HPLC, inhibition of Na⁺,K⁺-ATPase catalytic activity, and two immunoassays developed with antibodies specific to ouabain and to dihydroouabain, we characterized the secretion of these two compounds from human adrenal cells (H295R-1) in culture. Our data indicate that although both OLF and Dh-OLF are secreted by human adrenal cells, when the cells are challenged with cAMP, the secretion of Dh-OLF is favored over that of OLF. These results suggest a cAMP-dependent pathway for production of these compounds with possible differential regulation. A preliminary account of portions of this work has been presented (24).

### Materials and Methods

**MATERIALS**

All chemicals used were reagent grade. Chromatography-grade CH₃CN was obtained from Aldrich Co.; 5-sulfosalicylic acid, CaCO₃, ouabain, dihydroouabain, porcine cerebral cortex, all reagents for catalytic inhibition of the sodium pump [ATP, ammonium methylolate, Tween 80, and bovine serum albumin (BSA)] as well as goat antirabbit horseradish peroxidase conjugate, phosphate-buffered saline (PBS)-Tween (pH 7.4), and antibodies to progesterone were purchased from Sigma. 3,3',5,5'-Tetramethylbenzidine (TMB) soluble reagent and TMB stop buffer were purchased from SKYTEK Laboratories.

Human cells in culture were disrupted by sonication in a Microson ultrasonic cell disruptor (Heat Systems Inc.). For the EIA assays, microtiter plates (Immulon 2) were purchased from Dynex Technologies, Inc. Ultraviolet absorbance was measured on an SLT Rainbow Microplate Reader, and the data were analyzed by WinSelect Data analysis Software (Microsoft Corp). For sodium pump inhibition assays, disposable nonsterile 96-well flat-bottomed polystyrene microtiter plates were purchased from Dynex Technologies, Inc.
METHODS

Ouabain and dihydroouabain conjugates. Ouabain and dihydroouabain were each conjugated to both keyhole limpet hemocyanin (KLH) and BSA by HTI Bio-products Inc. to our specifications: KLH and BSA were conjugated through the rhamnose sugar moieties of ouabain and dihydroouabain (see Fig. 1) to increase the antigenic response in rabbits. The ouabain-BSA and dihydroouabain-BSA conjugates were used to coat the microtiter plate wells in the development of both EIAs as detailed below.

Ouabain and dihydroouabain antisera. Production of ouabain and dihydroouabain antisera from rabbits was performed by HTI Bio-products according to our specifications and schedule of injection/boosting. Briefly, KLH-conjugated ouabain and dihydroouabain were each emulsified in Freund’s complete adjuvant and used in intradermal immunization of rabbits. Three weeks later, the animals were boosted for the first time, followed by a total of four boosts, each 2 weeks apart. Blood samples from the rabbits were drawn 3 weeks after the primary injection and every 2 weeks after subsequent booster injections to test antisera titers. Animals were bled 14 days after the final boost, and serum was harvested and stored at −80 °C for further use.

Immunoglobulins were further purified by ammonium sulfate precipitation as described elsewhere with some modifications (30). Briefly, serum was clarified by centrifugation at 10 000g at 4 °C. The supernatant was decanted and stirred slowly in a beaker. A saturated solution of (NH4)2SO4 was slowly added until precipitate started forming and until an equal volume of saturated (NH4)2SO4 was added. The precipitate was allowed to form for an additional 4–5 h. The contents of the beaker were then centrifuged at 10 000g at 4 °C for 30 min, and the precipitate was dissolved in PBS (one-half of the original volume of serum). The IgG concentration was measured spectrophotometrically at 280 nm. Finally, the plate was washed, and 100 μL of TMB soluble reagent was added to each well. Color development was monitored at 650 nm for a maximum of 30 min, after which the reaction was stopped with 100 μL of TMB stop buffer and the plate was read at 450 nm. The readings were blanked and adjusted for nonspecific binding. We used the plant-derived ouabain and dihydroouabain as calibrators in the respective immunoassays. Therefore, all concentrations and amounts of measured OLF and Dh-OLF refer to the respective immunoequivalences to their plant-derived counterparts.

Cell culture growth conditions and stimulation of steroidogenesis. Human adrenal cells (H295R-1) were a gift from Dr. William E. Rainey (University of Texas Southwestern Medical Center, Dallas, TX). The cells were grown and maintained in DMEM/F12 medium containing 25 mL/L ITS culture supplements, and antibiotics in 75-cm2 flasks at 37 °C under a humid atmosphere of 5% CO2–95% air, as described previously (31). Tissue culture flasks were immediately increased to neutral by the addition of 100 μL of 1 mol/L Tris-Cl, pH 7.6, and the absorbance spectra of the proteins were monitored at 280 nm.
seeded with H295R-1 cells and grown to ~80% confluent density at 37 °C in 5% CO2 (~1 × 10⁶ cells). The cells were then washed twice with PBS, and serum-free DMEM/F-12 medium was added to the cells. The incubation was continued for an additional 24 h, after which the medium was removed and stored at −80 °C for isolation of OLF and Dh-OLF. Steroidogenesis was stimulated by replacing the medium with fresh serum-free culture medium containing 1 mmol/L dibutyryl cAMP [(Bu)₂cAMP] and incubating for an additional 24 h, after which OLF, Dh-OLF, and progesterone in the cell medium were measured. Progesterone was measured directly in the cell medium by RIA as described previously (32). The data are expressed as µg progesterone/L of medium, and the mean values ± SD were determined from triplicate determinations for each sample.

To isolate OLF and Dh-OLF, the culture medium was extracted by solid-phase extraction followed by HPLC purification, and the final purified substance was reconstituted in plasma before measurement by EIA. The amount of each compound measured after 24 h (OLF, Dh-OLF, and progesterone) in serum-free medium in the absence of (Bu)₂cAMP was set as 100%, and the amount of each compound (OLF, Dh-OLF, and progesterone) secreted after 24 h in (Bu)₂cAMP-supplemented medium was set relative to 100%. For all experiments above, at the end of the incubation, medium was collected and stored frozen at −80 °C before analysis. Approximately 1 × 10⁶ cells/flask were harvested by scraping with a rubber policeman and centrifuged; the cell pellet was washed twice with PBS and placed in distilled, deionized H2O (ddH2O) for sonication. After sonication three times (30 s each time), disrupted cells were centrifuged, and the released contents in ddH2O were collected and frozen at −80 °C for further use. The collected cell medium and cell contents in water were further purified by solid-phase extraction and HPLC with two different chromatographic gradients to isolate and purify the endogenous compounds as described below.

Isolation and purification of OLF and Dh-OLF. We isolated and purified OLF and Dh-OLF from human adrenocortical cells as described previously (4). We diluted the cell extract (or cell contents in the case of cell pellet analysis) with two volumes of ddH2O, precipitated the denatured proteins with 10 g/L 5-sulfosalicylic acid (90 °C for 10 min), and immediately added 1 mol/L Tris-Cl (pH 8.0) to a final concentration of 10 mmol/L to neutralize the pH. We then centrifuged the extract and decanted and filtered the supernatant. Initial purification was performed with reversed-phase C₁₈ Sep-Pak extraction cartridges. We then eluted the compounds of interest with two volumes of 200 mL/L CH₃CN in ddH₂O. The CH₃CN was evaporated, and the samples were reconstituted in ddH₂O and filtered.

Chromatographic separation of OLF and Dh-OLF involved two HPLC chromatographic steps. HPLC was performed with a C₁₈ reversed-phase µBondapak column (3.9 × 300 mm; 10-µm particle size) connected to a Waters 600E system controller and a Waters 996 photodiode array detector. HPLC fractions were collected with a Waters Fraction Collector (Millipore Corp.) and evaporated with a RC 10.22 Centrifugal Vacuum Concentrator connected to a RCT 60 Refrigerated Trap (both from Jouan). The first HPLC step involved a linear gradient of 20–80% CH₃CN in water over 30 min, and eighty 0.5-mL (1 min) fractions were collected, dried, and reconstituted in ddH₂O. The fractions eluting at 4–6 min into the linear gradient were measured by ouabain and dihydroouabain EIA before any further analysis. These fractions from the linear gradient were then rechromatographed using an isocratic mobile phase of 100 mL/L CH₃CN in ddH₂O for 40 min to separate OLF (29 min) and Dh-OLF (27.5 min) from each other, as described previously (4). All fractions of interest were resuspended in plasma and then analyzed by the appropriate EIA.

Na⁺,K⁺-ATPase catalytic activity inhibition assay. This assay was used to measure the effect of OLF and Dh-OLF on phosphate release in the hydrolysis of ATP, based on the method of Chan and Swaminathan (33) with minor modifications (25), as we detailed previously, to increase the sensitivity of the assay for samples of small volume. Briefly, to 20 µL of sample/calibrator (containing the desired concentration of calibrator), equal volumes of porcine cerebrocortical Na⁺,K⁺-ATPase solution (1 U/L) and ATP (10 mmol/L) were added and incubated at 37 °C for 10 and 15 min, respectively. Sample, calibrator, and ATP solutions were all resuspended in Tris-Cl buffer (pH 7.8). We then added molybdate solution (150 µL), and color development was allowed to proceed for a maximum of 30 min before measurement at 340 nm. The color intensity is proportional to ATP breakdown and, therefore, Na⁺,K⁺-ATPase activity. All samples were corrected for background, averaged, and normalized to ouabain-sensitive Na⁺,K⁺-ATPase activity (100% inhibition at 1 mmol/L ouabain). The percentage of inhibition of Na⁺,K⁺-ATPase activity represents the proportion of ouabain-sensitive ATPase activity that is inhibitable by either OLF or Dh-OLF.

Results

Development of Antisera and Immunoassays

We used commercially purchased ouabain and dihydroouabain without further purification for the production of antibodies in rabbits. The resulting titers of purified antisera were 1 × 10⁶ for ouabain antisera and 8 × 10⁵ for dihydroouabain antisera. Titers are expressed as the reciprocals of the serum dilutions that produced an absorbance at 492 nm (Δ₄₉₂ nm) of 0.2 in an ELISA with BSA-conjugated antigen on the solid phase. Except for the chemical saturation of the lactone ring, ouabain and dihydroouabain are structurally identical. To distinguish between these two compounds and to render
them immunogenic at or near the lactone ring position, each glycoside was conjugated to KLH and BSA through the rhamnose sugar moiety. The rhamnose ring was opened, and the two aldehyde moieties created were coupled to NH₂ groups on KLH and BSA (Fig. 1). We tested these antisera for specificity toward structurally related compounds. As shown in Table 1, antisera raised against dihydroouabain demonstrated cross-reactivities of only 1.4% with ouabain and <0.05% with its aglycone, ouabagenin. Furthermore, the antisera did not detect (<0.01%) digoxin or its aglycone, digoxigenin, and only slightly detected dihydriodigoxin (<0.05% cross-reactivity). The antisera raised to ouabain demonstrated cross-reactivities of 18% with dihydroouabain and 10% with ouabagenin. Thus, the antiserum developed to measure Dh-OLF seem more specific than the one developed to measure OLF for structural differences in the lactone ring. Shown in Fig. 2 are typical calibration curves for OLF (Fig. 2A) and Dh-OLF (Fig. 2B) for assays using the antiserum indicated and the plant-derived ouabain or dihydroouabain, respectively, as the calibrators. The within-run imprecision (CV) for the OLF immunoassay was 7.5%, 6.4%, and 7.1% for ouabain concentrations of 0.34, 1.02, and 3.40 nmol/L, respectively. For the Dh-OLF assay, the within-run imprecision (CV) was 3.8%, 12%, and 6.5% at comparable concentrations and for six determinations each. The lower limit of detection, defined as 2 SD above the mean of the zero calibrator, was 72 pmol/L for the OLF and 60 pmol/L for the Dh-OLF assay.

**SECRETION OF Dh-OLF AND OLF FROM HUMAN ADRENAL CELL CULTURES**

Human adrenal cells (H295R-1) grown in serum-free medium for 24 h produced 0.18 ± 0.03 pmol of ouabain-equivalent OLF and 0.39 ± 0.04 pmol of dihydroouabain-equivalent Dh-OLF per 10⁶ cells (n = 3 separate experiments; Table 2). We used previously described purification techniques (4) to isolate and purify OLF and Dh-OLF simultaneously from the medium of human adrenal cell cultures. In the isocratic HPLC system described above, ouabain coeluted with OLF (29) and Dh-OLF coeluted with one of the newly discovered isomers of plant-derived dihydroouabain (dho-B; Fig. 3) (25). Under these conditions (100 mL/L CH₃CN), OLF eluted at 29 min, whereas Dh-OLF eluted at 27.5 min, and each was detected by use of their respective EIAs. Consistent with our previous findings for human plasma and bovine adrenal cortex (4), we did not detect any immunoreactivity with the dihydroouabain EIA at the elution position (23.5 min; Fig. 4A) corresponding to the other recently discovered isomer of the plant-derived dihydroouabain (dho-A; Fig. 3) (25).

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**Table 1. Cross-reactivities of ouabain and dihydroouabain antibodies.***

<table>
<thead>
<tr>
<th>Substance</th>
<th>Cross-reactivity, %</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Anti-ouabain</td>
</tr>
<tr>
<td>Dihydroouabain</td>
<td>18</td>
</tr>
<tr>
<td>Ouabain</td>
<td>100</td>
</tr>
<tr>
<td>Ouabagenin</td>
<td>10</td>
</tr>
<tr>
<td>Digoxin</td>
<td>0.66</td>
</tr>
<tr>
<td>Dihydriodigoxin</td>
<td>6.70</td>
</tr>
<tr>
<td>Digoxigenin</td>
<td>3.30</td>
</tr>
<tr>
<td>Cholesterol</td>
<td>0.03</td>
</tr>
<tr>
<td>Bufalin</td>
<td>10</td>
</tr>
</tbody>
</table>

*Cross-reactivity is expressed as the molar concentration of the calibrator (ouabain or dihydroouabain) at 50% c/c₀ over the molar concentration of the compound at 50% c/c₀ multiplied by 100%. Ouabain cross-reactivity was measured by ouabain EIA, and dihydroouabain cross-reactivity was measured by dihydroouabain EIA. Cross-reactivity for the following compounds was <0.05% to ouabain and dihydroouabain: pregnenolone, corticosterone, progesterone, deoxycorticosterone, aldosterone, cortisol, hydroxyprogesterone, uracil.
Inhibition of Na\(^+\),K\(^+\)-ATPase by Dh-OLF and OLF

Both OLF and Dh-OLF isolated from H295R-1 cells inhibited the catalytic activity of porcine cerebrocortical Na\(^+\), K\(^+\)-ATPase in vitro. Conditioned medium from five culture flasks, each containing \(10^6\) cells, was pooled and processed as described to extract OLF and Dh-OLF. Purified OLF (fraction eluting at 29 min) and Dh-OLF (fraction eluting at 27.5 min) were each resuspended in ddH\(_2\)O to measure ouabain-sensitive sodium pump inhibition or in plasma to measure immunoreactivity. Neighboring fractions (10–40 min) were treated similarly, and both immunoreactivity and sodium pump inhibition data demonstrated that only the fractions eluting at 29 and 27.5 min had measurable immunoreactivities to ouabain or dihydroouabain antisera (Fig. 4A) in addition to inhibiting sodium pump catalytic activity (Fig. 4B). Specifically, 29% of the ouabain-sensitive catalytic activity of the sodium pump was inhibited at 0.03 \(\mu\)mol/L OLF and 17% was inhibited at 0.07 \(\mu\)mol/L Dh-OLF (Fig. 4B).

Stimulation of OLF and Dh-OLF secretion by (Bu)\(_2\)cAMP

Cells were grown for a total of 48 h, of which growth in serum-free medium supplemented with (Bu)\(_2\)cAMP was for 24 h. At the end of the incubation, we collected the conditioned medium and extracted the compounds of interest. Stimulation of steroidogenesis by (Bu)\(_2\)cAMP, as expected, was demonstrated by an increase in progesterone secretion into the medium (Fig. 5A). Progesterone, as well as other common steroids, is readily separable from the OLF or Dh-OLF in our HPLC isolation procedures (unpublished data). We observed that 24 h after (Bu)\(_2\)cAMP treatment, the secretion of Dh-OLF into the medium was increased by 50% compared with unstimulated cells (Fig. 5B). Although there seemed to be a slight increase in OLF secretion in (Bu)\(_2\)cAMP-stimulated cells, this increase was not statistically significant (Fig. 5B). We also observed that progesterone, OLF, and Dh-OLF (Table 2) were found mainly in the conditioned medium, i.e., when collected and processed, the cells did not contain measurable amounts of these compounds. Additionally, in a parallel experiment we collected and processed the conditioned medium and cell pellet after 12 h without stimulation (cells of both experiments originated from the same passage) and were unable to measure detectable quantities of OLF and Dh-OLF (Table 2). This may be attributable to limitations in the sensitivities of the assays and, therefore, does not exclude possible secretion of OLF and Dh-OLF after 12 h.

### Table 2. Production of OLF and Dh-OLF by H295R-1 cells.

<table>
<thead>
<tr>
<th>Mammalian factor</th>
<th>Concentration in medium, pmol/10(^6) cells</th>
<th>Concentration in cell pellet, pmol/10(^6) cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12 h</td>
<td>24 h</td>
</tr>
<tr>
<td>OLF</td>
<td>&lt;0.04(^{\text{a,c}})</td>
<td>0.18 ± 0.03(^{\text{d}})</td>
</tr>
<tr>
<td>Dh-OLF</td>
<td>&lt;0.03(^{\text{a,c}})</td>
<td>0.39 ± 0.04(^{\text{d}})</td>
</tr>
</tbody>
</table>

\(^{\text{a}}\) OLF and Dh-OLF amounts were measured by the OLF and Dh-OLF EIAs, respectively. Unity molar immunoreactivity of OLF and Dh-OLF with ouabain and dihydroouabain antibodies, respectively, was assumed as described in our previous work (4) to calculate moles of hormone-like factor secreted into the medium and retained in the cell pellet. For each compound, the amount measured was after 24 h of cell growth in serum-free medium and in the absence of (Bu)\(_2\)cAMP.

\(^{\text{b}}\) Each measurement was performed in duplicate, and the average of two experiments was calculated.

\(^{\text{c}}\) The detection limits per 10\(^6\) cells were calculated from the detection limits of the respective OLF and Dh-OLF EIAs.

\(^{\text{d}}\) Each measurement was performed in duplicate, and the average of three experiments was calculated.

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Fig. 3. HPLC elution profile of cardiac glycosides.

HPLC elution profile (100 mL/L CH\(_3\)CN; isocratic mode) of micromolar concentrations of the plant-derived cardenolides dihydroouabain [2.5 \(\mu\)mol/L dho-A and 3.0 \(\mu\)mol/L dho-B, estimated by molar absorptivity at 196 nm, as detailed previously (20)] and ouabain (5.0 \(\mu\)mol/L, estimated by weight). Absorbance was monitored at 196 nm, which is the absorbance maximum of dihydroouabain (dho-A and dho-B).

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Fig. 3. HPLC elution profile of cardiac glycosides.
The objective of this study was to investigate the production of OLF and Dh-OLF by the human adrenal cell line H295R-1 and to develop immunoassays sufficiently specific to distinguish between OLF and Dh-OLF. We succeeded in developing two competitive EIAs and used them in combination with chromatographic separation and inhibition of Na\(^+\),K\(^+\)-ATPase to demonstrate the ability of a human adrenocortical cell line to produce both of these compounds de novo. These cells secrete substantially more Dh-OLF than OLF, with the former being cAMP-dependent.

Using plant-derived ouabain and dihydroouabain as immunogens, we developed antisera to detect OLF and Dh-OLF and confirmed the presence of OLF and Dh-OLF by use of EIAs in combination with several chromatographic separation steps. The antisera were sensitive to the only structural difference between the two compounds, i.e., an oxidized or reduced lactone ring. The selectivity was accomplished using the approach originally reported by Butler et al. (28) to couple cardenolide compounds to KLH/BSA through their rhamnose sugar moieties (Fig. 1). This technique maximally exposes the lactone ring portion of the cardenolides, thus giving the structural specificity needed to distinguish between the oxidized (OLF) and reduced (Dh-OLF) lactone rings (Fig. 6). The antisera directed against the saturated lactone ring (dihydroouabain) seems more specific to the structural difference on the lactone ring than antisera raised against the oxidized ring (ouabain), e.g., the cross-reactivity of antibodies to dihydroouabain was 2% with ouabain, whereas the cross-reactivity of antibodies to ouabain was 18% with dihydroouabain (Table 1). Although the anti-

![Fig. 4. Separation of OLF and Dh-OLF by HPLC measured by EIA and corresponding biological activity.](image)

(A), HPLC elution profile of mammalian cardenolides and detection by EIA. OLF (eluting at 29 min) and Dh-OLF (eluting at 27.5 min) were purified by use of isocratic 100 mL/L CH\(_3\)CN in water. OLF (486 pg of ouabain equivalents) and Dh-OLF (981 pg of dihydroouabain equivalents) were detected by the ouabain and dihydroouabain EIAs, respectively. (B), inhibition of the sodium pump catalytic activity by OLF and Dh-OLF. OLF (0.03 \(\mu\)mol/L) and Dh-OLF (0.07 \(\mu\)mol/L), which eluted at 29 and 27.5 min, respectively, in the above gradient and were identified by immunoreactivity, inhibited 29% and 17% of ouabain-sensitive sodium pump catalytic activity, respectively. Neighboring fractions (10–40 min) were processed similarly and assayed for immunoreactivity with the two antibodies and for inhibition of the catalytic activity of the sodium pump. No other immunoreactive fractions were detected, and no fractions inhibited the sodium pump catalytic activity.

**Discussion**

The objective of this study was to investigate the production of OLF and Dh-OLF by the human adrenal cell line H295R-1 and to develop immunoassays sufficiently specific to distinguish between OLF and Dh-OLF. We succeeded in developing two competitive EIAs and used them in combination with chromatographic separation and inhibition of Na\(^+\),K\(^+\)-ATPase to demonstrate the ability of a human adrenocortical cell line to produce both of these compounds de novo. These cells secrete substantially more Dh-OLF than OLF, with the former being cAMP-dependent.

Using plant-derived ouabain and dihydroouabain as immunogens, we developed antisera to detect OLF and Dh-OLF and confirmed the presence of OLF and Dh-OLF by use of EIAs in combination with several chromatographic separation steps. The antisera were sensitive to the only structural difference between the two compounds, i.e., an oxidized or reduced lactone ring. The selectivity was accomplished using the approach originally reported by Butler et al. (28) to couple cardenolide compounds to KLH/BSA through their rhamnose sugar moieties (Fig. 1). This technique maximally exposes the lactone ring portion of the cardenolides, thus giving the structural specificity needed to distinguish between the oxidized (OLF) and reduced (Dh-OLF) lactone rings (Fig. 6). The antisera directed against the saturated lactone ring (dihydroouabain) seems more specific to the structural difference on the lactone ring than antisera raised against the oxidized ring (ouabain), e.g., the cross-reactivity of antibodies to dihydroouabain was <2% with ouabain, whereas the cross-reactivity of antibodies to ouabain was 18% with dihydroouabain (Table 1). Although the anti-

![Fig. 5. Secretion of progesterone, OLF, and Dh-OLF by human adrenocortical cells (H295R-1) after stimulation by (Bu)\(_2\)cAMP.](image)

(A), (Bu)\(_2\)cAMP-stimulated progesterone secretion. Progesterone secretion into the medium was measured after 24 h of growth in serum-free medium with (stimulated) and without (unstimulated) added (Bu)\(_2\)cAMP. Two samples of medium were withdrawn (50 and 25 \(\mu\)L) and analyzed by RIA. Duplicate measurements were taken, and the mean was calculated from both samples. (B), (Bu)\(_2\)cAMP-stimulated Dh-OLF secretion into the medium. OLF and Dh-OLF secreted into the medium were measured by the ouabain and dihydroouabain EIAs, respectively, after cell growth in serum-free medium with and without the addition of (Bu)\(_2\)cAMP. Duplicate measurements were taken, and the mean of three experiments (n = 3) was calculated. The SE is represented by error bars.
genic response was designed to maximize specificity to the lactone rings as epitopes, some selectivity for the sugar portion was evident, as reported by others (34) for antibodies developed against ouabain and digoxin.

Because we separated the oxidized and reduced species chromatographically before analysis by immunoassay or by inhibition of Na\(^{+},K^{+}\)-ATPase, the issue of antibody cross-reactivity becomes less critical in our study. However, this is an important consideration in development of assays that would measure these compounds directly in a nonpurified medium such as serum or other biological fluids. The concentration working ranges and analytical sensitivities of both EIAs (Fig. 2) were adequate to measure the amounts of OLF and Dh-OLF secreted by the cell cultures after processing and purification as in our experiments. However, for applications involving direct measurements of these compounds from serum or other biological fluids, further refinement of the two assays will be necessary to obtain clinically relevant reproducibility and specificity. In addition, the lower limits of detection for the two EIAs (72 pmol/L for OLF and 60 pmol/L for Dh-OLF) seem adequate for direct measurements of these compounds in serum based on the wide OLF concentration range (10–800 pmol/L) described by Vakkuri et al. (34) and on the OLF (380 ± 42 pmol/L) and Dh-OLF (5000 ± 460 pmol/L) concentrations in human serum we reported in the blood of mammals (4). It is important to note that in this study we calibrated the two EIAs using the plant-derived compounds and then used immunoequivalence to assess the concentrations of both OLF and Dh-OLF. Previous work, based on independent estimates of molar absorbity by spectrophotometry, suggests there is a one-to-one correspondence in the reactivity of antibodies raised to ouabain or dihydroouabain toward OLF or Dh-OLF, respectively (4). We therefore believe that measurements in this present study reasonably approximate the amounts of these compounds secreted by the cell cultures.

In addition to the selectivity of the antibodies developed, we were able to authenticate the identity of OLF and Dh-OLF from H295R-1 cells by showing that after multiple separation steps, both endogenous compounds have HPLC elution profiles (Fig. 4A) identical to those of ouabain and dihydroouabain, respectively (Fig. 3). We raised the dihydroouabain antiserum against the mixture of both dihydroouabain components, dho-A and dho-B (see Fig. 1). It is interesting to note that in secretions from our human cell cultures, the dihydroouabain antiserum was able to detect only a Dh-OLF component corresponding to the plant-derived dho-B, and not dho-A (Fig. 4A). This is consistent with our previous findings; we were unable to detect any immunoreactivity in the position at which dho-A secreted by bovine adrenal cells elutes in a mobile phase of 100 mL/L CH\(_3\)CN in water (23.5 min; see Fig. 3) (4). Possible explanations include that the detection limit of the assay was too high to detect a Dh-OLF compound at the dho-A elution position or that humans simply do not secrete the dho-A form of the Dh-OLF compound.

Both OLF and Dh-OLF isolated from H295R-1 cells inhibited the catalytic activity of porcine cerebrocortical Na\(^{+},K^{+}\)-ATPase in vitro (Fig. 4B). In a previous study using OLF and Dh-OLF extracted from bovine adrenal cortex and Na\(^{+},K^{+}\)-ATPase preparations from porcine cerebral cortex, we used inhibition curves over a wide range of concentrations to show that OLF is more potent than Dh-OLF in inhibiting the catalytic activity of the sodium pump (25). These relative potencies for inhibition of Na\(^{+},K^{+}\)-ATPase reflect the relative inhibitory potencies of the plant-derived counterparts; ouabain (0.66 ± 0.095 μmol/L) has a lower IC\(_{50}\) than dihydroouabain-B (1.63 ± 0.12 μmol/L) (25). Although we did not isolate enough material in the present study to obtain complete inhibition curves, our data do show a similar pattern of inhibitory potency between OLF and Dh-OLF. For example, chromatographic isolates of OLF and Dh-OLF inhibited the catalytic activity of the sodium pump (29% inhibition by 0.03 μmol/L ouabain immunoequivalents for OLF and 17% inhibition by 0.07 μmol/L dihydroouabain immunoequivalents for Dh-OLF; Fig. 4B). These results are within 10% of the potencies we reported previously for the

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Fig. 6. Lactone ring-dependent specificity of antibodies to ouabain and dihydroouabain.

Ouabain-specific antibodies had 100% cross-reactivity with ouabain, but only 18% with the structurally almost identical dihydroouabain (left). Dihydroouabain-specific antibodies reacted 100% to dihydroouabain but reacted with only 1% of the ouabain exposed to the dihydroouabain antiserum. At the top are the structures of the oxidized (left) and reduced (right) lactone rings of ouabain and dihydroouabain, respectively.
inhibitory activities of OLF and Dh-OLF isolated from bovine adrenal glands (4).

Human adrenal H295R-1 cells grown in serum-free medium produce both OLF and Dh-OLF (Table 2). Compared with the culture incubation medium, the cell pellets had undetectable amounts of either OLF or Dh-OLF. In addition, 100 mL of unconditioned NuSerum medium processed as described earlier contained no detectable OLF and Dh-OLF immunoreactivity in our EIAs for OLF and Dh-OLF, respectively. In a previous study by Doris et al. (10), the authors showed that ouabain can accumulate in adrenocortical cells and then be released into the cell culture medium. To ensure that we are measuring OLF and Dh-OLF produced by the human adrenal cells and not “recycled” mammalian cardenolides absorbed during incubation, we demonstrated that OLF and Dh-OLF were not detectable in the cell pellets before their secretion into the medium. We are therefore confident that our measured OLF and Dh-OLF are indeed produced de novo. In a recent study we demonstrated the isolation and purification of a dihydrodiginin-like compound (Dh-OLF) from bovine adrenal tissues and human serum (4). Subsequently, we found that Y-1 murine adrenal cells in culture produced 43 pg (0.074 pmol) of OLF and 300 pg (0.5 pmol) of Dh-OLF/10^6 cells (24). This present study now indicates that human adrenal cells produce comparable amounts of these compounds de novo: 103 pg (0.18 ± 0.03 pmol; n = 3) of OLF and 230 pg (0.39 ± 0.04 pmol; n = 3) of Dh-OLF/10^6 cells. Although more detailed studies are needed to define the relative amounts of the OLFs secreted between species, it is notable that Dh-OLF seems to be consistently present at higher amounts than OLF as is dihydrodiginin-like immunoreactive factor compared with digoxin-like immunoreactive factor (4, 24). It is interesting to note that a recent study from our laboratory demonstrated the incorporation of radiolabeled carbon into the structure of OLF and Dh-OLF by adrenal cells in culture (35). This supports the concept of de novo synthesis of OLF and Dh-OLF by adrenal cells.

The discovery of Dh-OLF is recent (24), and little information is available on its biosynthesis. The present study indicates that cAMP stimulates the secretion of Dh-OLF but not OLF in human adrenal cells. Cells grown for 24 h in serum-free medium supplemented with (Bu)_2cAMP secreted proportionately more Dh-OLF than OLF. Cells in which steroidogenesis was stimulated by (Bu)_2cAMP secreted more progesterone into the medium, as expected (Fig. 5A). Additionally, to demonstrate the viability of the adrenocortical cells, we measured steroid production, i.e., progesterone (Fig. 5A) and aldosterone [data not shown and Refs. (31, 36)]. To date, those working to characterize the secretion of the mammalian cardenolides by cell cultures have used the oxidized compounds, e.g., OLF or the digitalis-like factors, as the secretory products. If the structural difference between OLF and Dh-OLF is limited to the chemical state of the lactone ring, as is currently suspected, both compounds are likely to have similar biosynthetic origins for the better part of their structures. In most studies, the biosynthesis of these kinds of endogenous factors has been investigated by stimulating steroid synthesis in cultured adrenal cells and observing changes in the secretion of the endogenous glycosides/cardenolides. Several stimuli that increase steroidogenesis have been shown to stimulate the secretion of OLF in mammalian adrenocortical tissues or cultures (9, 37). In 1998, Hinson et al. (3) showed increased secretion of a ouabain-like compound (OLC) by perfused rat adrenal glands after stimulation of steroidogenesis by adrenocorticotropic hormone (ACTH). In another study, stimulation of OLC synthesis occurred 10 min after administration of ACTH and indicated that OLC is acutely regulated in rat adrenals (6). Previous work has shown that ACTH and angiotensin II stimulate secretion of OLF as well as aldosterone and cortisol from bovine adrenals (38, 39). Similarly, other work has shown that the use of both progesterone and pregnenolone as precursors in the biosynthetic pathway noticeably increased the secretion of OLF in adrenal cells, but that cortisol as a precursor only slightly increased OLF secretion into the medium (40). Additional evidence on the origin and biosynthesis of endogenous ouabain in adrenals has been provided by studies of Hamlyn et al. (29) that show secretion of endogenous ouabain from adrenal glands after the medium was supplemented with pregnenolone. Their results also suggested that although pregnenolone seems to be a substrate for both endogenous ouabain and aldosterone biosynthesis, a divergence in the biosynthetic pathway of these steroids seems to occur before progesterone synthesis. However, Perrin et al. (40) have reported that progesterone also increased the biosynthesis of OLC and that pregnenolone, via progesterone, may represent a precursor in the biosynthesis of OLC in adrenal glands. Their argument was supported by results showing that both pregnenolone and progesterone increase OLC secretion in primary cultures of bovine adrenal cells.

The possibility of a second-messenger mechanism being involved in regulating secretion of these compounds has also been considered. A study by Shah et al. (9) seemed to indicate that neither cAMP or protein kinase C is involved in the signaling pathway of endogenous ouabain synthesis. Therefore, although it has been shown that ACTH stimulates endogenous ouabain secretion, preliminary evidence suggests that cAMP does not seem to serve as the cellular mediator. This has led to the conclusion that the mechanisms regulating secretion of aldosterone and endogenous ouabain are distinct. Hamlyn et al. (29) showed that (Bu)_2cAMP increased the production of aldosterone in adrenal cells while not significantly increasing secretion and production of endogenous ouabain. In the present study, we investigated the role of the second messenger cAMP in stimulating Dh-OLF synthesis in human adrenal cells by supplement-
The concentration of OLF was unaltered, consistent with the increase in progesterone secretion. We also showed that the penetrable form of cAMP. We were able to stimulate cell culture medium with (Bu)_2cAMP, a membrane-
of OLF may be controlled by cAMP-mediated stimulation. This evidence also points to a possible product–precursor relationship between OLF and Dh-OLF in human cells.

In conclusion, this report suggests that OLF and Dh-OLF are synthesized de novo by a human adrenal cell culture, that immunoassays sufficiently specific to distinguish between OLF and Dh-OLF can be developed, that mammalian cells produce only one isomer of Dh-OLF in lieu of the two plant-derived isomers of dihydrououabain, and that the secretion of Dh-OLF is more sensitive to cAMP stimulation than is OLF. Our findings suggest the possibility of a product–precursor relationship between these two compounds, and this warrants additional investigation. The availability of a human cell line that secretes both OLF and Dh-OLF now provides a working model for characterizing the biosynthesis and secretory regulation of these two compounds in humans.

We are deeply grateful to Rebecca Combs for invaluable technical assistance. This work was supported in part by NIH Grants HL R01-36172 and HL R01-59404 (to R.V.) and National Science Foundation Grant OSR-9452895 (to R.V.).

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