Novel Receptors for Ouabain: Studies in Adrenocortical Cells and Membranes

Stephen C. Ward, Bruce P. Hamilton, John M. Hamlyn

Abstract—Sodium-potassium pumps (Na pumps) are the only known plasma membrane receptors for cardiac glycosides. However, adrenocortical cells secrete an endogenous ouabain via an unknown mechanism that is subject to feedback inhibition via the cell surface. In addition, recent studies suggest that the induction of sustained hypertension by ouabain analogs in rats may be independent of Na pump inhibition. Accordingly, we used bovine adrenocortical cells and membranes to search for novel binding sites for ouabain. In high extracellular potassium solutions, the binding of ouabain to the Na pumps of cultured cells was suppressed, yet residual specific binding of $^3$H-ouabain was observed. In high extracellular potassium, Scatchard analyses revealed a novel class of ouabain binding sites with high affinity ($K_d=50$ nmol/L, $B_{\text{max}}=2.5 \times 10^5$ sites/cell) that was distinct from the low-affinity Na pump sites ($K_d=1$ mmol/L, $B_{\text{max}}=4.5 \times 10^6$ sites/cell). Analysis of the kinetics for the dissociation of $^3$H-ouabain from intact cells revealed components whose $t_{0.5}$ values were 6.5 minutes, 3.3 hours, and 33 hours and associated with novel sites, Na pumps, and lysosomal recycling, respectively. Studies with isolated membranes under ligand conditions where the participation of Na pumps was minimized revealed specific ouabain binding to novel sites that was saturable, time-dependent, of high affinity ($K_d=15$ nmol/L), and of low density (apparent $B_{\text{max}}=0.23$ pmol/mg, c.f., Na pumps = $10.2$ pmol/mg). Ouabain binding to the novel sites was stimulated by high concentrations of KCl but was not affected by aldosterone or cortisol up to 30 nmol/L. Novel sites were not detected in skeletal muscle or liver membranes. Photoaffinity studies followed by SDS-PAGE showed ouabain-protectable labeling of membrane polypeptides with apparent molecular weights of 143, 113, and 65 kDa. We conclude that adrenocortical cells express ouabain receptors that are distinct from Na pumps. These novel receptors may be involved in the regulation and/or secretion of endogenous ouabain. (Hypertension. 2002; 39[part 2]:536-542.)

Key Words: digitalis ■ ouabain ■ adrenal gland ■ sodium pump ■ glycosides

The therapeutic properties of the cardiac glycosides (CG) have long been recognized. More recently, a mammalian analog of ouabain, endogenous ouabain (EO), has been described and shown to circulate in elevated amounts in many patients with hypertension. Plasma membrane sodium-potassium pumps (Na pumps) are the only recognized receptor for the CGs. Moreover, the acute cardiotoxic and vasotonic effects of the CGs and the raised arteriolar tone in hypertension have been attributed to diminished Na pump activity with secondary elevation of intracellular calcium.

However, 3 recent lines of evidence have suggested the existence of other functional CG receptors. For example, whereas the prolonged infusion of ouabain induced hypertension in the rat, comparable infusions of digoxin did not, even though both CGs are near equipotent inhibitors of rat Na pumps. Second, and most paradoxical, the relationship between the hypertensinogenic potency of a series of ouabain analogs and their efficacy as Na pump inhibitors was found to be an inverse one. Third, EO secretion from cultured adrenocortical cells has been suggested to be regulated by a form of feedback inhibition mediated by the accumulation of subnanomolar concentrations of EO in the conditioned culture medium. However, plasma membrane Na pumps seem unlikely to be the primary feedback mediators because of their relatively low affinity for ouabain (≈300 nmol/L) under physiological conditions. These considerations prompted us to look for novel classes of binding sites for ouabain (NOR) in cells and membranes derived from the cortex of bovine adrenal glands.

Methods

Adrenocortical Cell Preparation and Culture

The isolation and culture protocol followed that used previously by us. In brief, the adrenal medulla was surgically removed from fresh bovine adrenal glands, and the cortex was sliced using a Stadie-Riggs microtome and dispersed using collagenase. Percoll-treated cells were cultured and used within 3 to 4 days.
Preparation of Bovine Adrenocortical Membranes
Fresh adrenal glands were obtained from a slaughterhouse. All procedures were performed at 0 to 4°C. After removal of fat, the medulla was dissected, and the cortical tissue was scraped from the capsule with a scalpel blade. The cortical tissue was weighed (~8 g wet weight per adrenal cortex), suspended in 10 vol/g tissue homogenization buffer (HB; containing (in mmol/L) 250 sucrose, 2.5 EDTA, 2.5 EGTA, 1 ATP-Na, 25 imidazole-Tris [pH 7.6]), and homogenized for 30 seconds with a polytron. After centrifugation of the homogenate (20 minutes, 1500g), the supernatant was collected and centrifuged (15 minutes, 7500g). The pellet containing nuclei and most mitochondria was discarded, and the supernatant was centrifuged (60 minutes, 44 000 g). The resultant clear supernatant was discarded. The membrane pellets were suspended in 10 vol of HB, recentrifuged as before, and reconstituted with 1 vol of HB.

Preparation of Membranes From Other Bovine Tissues
Membranes from bovine liver (caudate lobe) and skeletal muscle (cheek) were prepared from 23 g each using the same homogenization protocol as described for adrenal glands.

³H-Ouabain Binding to Bovine Adrenocortical Cells
Bovine adrenocortical (BAC) cells were incubated as indicated with various concentrations of [³H]-ouabain (20 to 30 Ci/mmole; Amersham Pharmacia Biotech) in standard electrolyte solution (SES) containing (in mmol/L) 145 NaCl, 5 KCl, 1 CaCl₂, 5.5 MgCl₂, 5.6 glucose, and 1.8 HEPES-NaOH [pH 7.4] in an incubator with 95% air/5% CO₂. Where indicated in some experiments, equimolar amounts of NaCl were replaced with KCl. Specific binding was determined with parallel incubations containing excess unlabeled ouabain. Bound ligand was separated by washing the cells 3 times with ice-cold SES. The cells were solubilized with 1% SDS. Portions were used for ³H counting and protein determination (BCA method).

³H-Ouabain Binding to Membranes
Studies were carried out in a total volume of 300 µL as follows: 50 µL membranes in HB, 100 µL [³H]-ouabain in EDTA/EGTA buffer (in mmol/L, 1 EDTA, 1 EGTA, 10 TES-tris [pH 7.4]), and 150 µL of either Mg²⁺/Pi buffer (phosphorylating conditions) containing (in mmol/L) 5 Tris-phosphate, 5 MgCl₂, and 50 TES-imidazole [pH 7.4] or EDTA/EGTA buffer (nonphosphorylating conditions). The reactions were started by incubation at 37°C, terminated by transfer to ice for 15 minutes, and subjected to rapid filtration through Whatman GF/B glass filter paper using a cell harvester. After washes, the filter paper was soaked in scintillation fluid overnight and the associated label was counted by scintillation spectrometry. Membrane protein was assayed as described for cells. In membrane experiments (and, where indicated, certain experiments with BAC cells), the data for the time course and substrate dependence were fit iteratively (least squares) to an equation of the following form:

\[ B = \frac{B_{\text{max}}(X)}{K + X} \]  

For the time course, X=incubation time, K=t½ or half time of binding. For substrate dependence, X=concentration of ³H-ouabain, and K represents the Kᵦ (apparent dissociation constant). In both cases, B=³H-ouabain specifically bound, and Bₘₐₓ=number of receptors available. Computer iteration was used to determine the K and Bₘₐₓ.

Dissociation of ³H-Ouabain From BAC Cells
Primary BAC cell cultures were preincubated with ³H-ouabain (100 nmol/L) in DMEM for 3 to 5 hours at 37°C. The cells were washed and trypsinized in SES at room temperature. Unadhered cells were centrifuged for 10 minutes at 200g (4°C), and the cell pellet was resuspended in Bio-Gel P-2 prehydrated with DMEM. The mixture was loaded into a column (1 cm diameter, 1.3 cm long, 1 mL volume) and brought to 37°C. The column was perfused with DMEM (37°C) at the indicated flow rates, and the effluent was collected using a fraction collector. In some experiments, trypsinization preceded the incubation with ³H-ouabain. The data were fit iteratively to Equation 2 using least squares.

\[ Y = Ae^{-BS} + Ce^{-DX} \]

A and C are the Y intercepts for the first and second component (fmol ³H-ouabain/unit time noted). B and D are the dissociation rate constants (k⁻¹) for the first and second components, respectively. The half-time of dissociation was determined by Equation 3.

\[ t_{1/2} = \ln(2)/k^{-1} \]

The amount associated with each component at the start of the washout (t₀) was determined by integrating Equation 3 from 0 to ∞, to yield the expression:

\[ \text{Amount at } t_0 = Y \text{ intercept } / k^{-1} \]

Photoaffinity Labeling of Membrane Proteins
The basic protocol followed that described by Rogers and Lazdunski. Bovine adrenocortical (BAC) membranes were incubated (final volume 1 mL) in EDTA/EGTA buffer containing ³H-ouabain (200 nmol/L final concentration) with or without excess unlabeled ouabain (60 µmol/L final concentration) to determine nonspecific and total binding, respectively. The reaction was performed for 1 hour at 37°C to achieve equilibrium binding to NOR, transferred to an ultraviolet quartz cuvette and irradiated with an Oriell 150 W xenon arc lamp for 30 minutes. After irradiation, 8 vol of HB was added. The samples were washed 3 times by centrifugation (15 minutes, 14 000 g, 4°C), and the membrane pellets were resuspended in 100 µL sample buffer containing SDS and loaded onto precast 8 to 16% polyacrylamide gels with molecular weight markers (6.5 to 200 kDa). After SDS-PAGE (35 mA, 4 hours), half of the gel was cut and stained with Coomassie Brilliant Blue. The remaining gel was cut vertically to separate sample lanes, sliced into 2-mm horizontal pieces, and soaked in 0.5 mL of 5% periodic acid for 36 hours. Scintillation fluid (3 mL) was added, and each slice was soaked overnight and counted by scintillation spectrometry. The apparent molecular weights of labeled polypeptides were determined by computer interpolation using the migration of molecular weight standards run simultaneously in the same gels.

Materials
Ouabain octahydrate was from Sigma Chemical Company. Other reagents were of analytical grade or better according to American Chemical Society criteria.

Results

Time Course of ³H-Ouabain Binding to BAC Cells
The term “cell associated” ³H-ouabain is used where appropriate because the exact location of tracer (surface bound versus intracellular) is not distinguished by our experimental approach. Initial experiments showed that the time course for the specific association of ³H-ouabain with cells in normal SES was pseudo-linear up to 8 hours (not shown) as described in other recent studies. The data were fit by Equation 1 and yielded a t½ of 73.5 hours and a Bₘₐₓ equivalent to 4.2×10⁶ sites/cell. In experiments in which the concentration of ³H-ouabain was varied, specific binding was well fit by a single component hyperbolic saturation curve (apparent Kᵦ=356±87 nmol/L, Bₘₐₓ=18.2±3.5 pmol/mg or ~2.7±0.5×10⁶ sites/cell).

Effects of Extracellular Potassium on ³H-Ouabain Binding to BAC Cells
Increasing [K⁺], dramatically inhibits the binding of ³H-ouabain to cellular Na pumps. Under these conditions, the
interaction of \(^{3}\text{H}\)-ouabain with novel classes of binding sites should be more visible to ligand binding methods. As shown in Figure 1, the association of \(^{3}\text{H}\)-ouabain with BAC cells decreased dramatically as [K\(^{+}\)]\(_{o}\) was raised to 20 mmol/L, the amount of \(^{3}\text{H}\)-ouabain associated with the cells began to increase. Raising [K\(^{+}\)]\(_{o}\) further led to a decline in ouabain binding so that amounts bound at 10 and 40 mmol/L were similar. The inset shows the \(^{3}\text{H}\)-ouabain associated with the cells in excess of that mediated by Na pumps. The excess component of ouabain binding was maximal at [K\(^{+}\)]\(_{o}\) = 20 to 25 mmol/L. The unexpected appearance of ouabain binding stimulated by K\(^{+}\) suggested the involvement of a novel class of ouabain binding sites whose characteristics differed from known Na pumps.

In subsequent experiments, cells were incubated in SES containing [K\(^{+}\)]\(_{o}\) = 0 or 15 mmol/L, and the concentration dependence of \(^{3}\text{H}\)-ouabain binding was examined under the 2 conditions. In the absence of K\(^{+}\)\(_{o}\), (Figure 2A), the binding of ouabain was concentration dependent and saturable. Scatchard analyses (Figure 2A, inset) revealed that ouabain bound to a single class of high-affinity sites (apparent K\(_{d}\) = 8.4 \pm 1.5 nmol/L, B\(_{\text{max}}\) = 21.3 \pm 2.0 pmol/mg) corresponding to 3.2 \pm 0.3 \times 10^6 sites/cell. In SES with high [K\(^{+}\)]\(_{o}\) (Figure 2B), the amount of ouabain specifically bound was dramatically lower (Figure 2A) and the concentration dependence of the binding was pseudolinear. Scatchard analysis (Figure 2B, inset) now revealed 2 classes of binding sites (apparent K\(_{d}\) = 66 and 1240 nmol/L, B\(_{\text{max}}\) = 1.5 and 23.7 pmol/mg) with 2.3 \times 10^6 and 3.57 \times 10^6 sites/cell, respectively. The low-affinity sites in high [K\(^{+}\)]\(_{o}\) were ascribed to cell surface Na pumps because their maximal density was comparably high to that in Figure 2A. The second class of binding sites that were characterized by their high affinity and low density under the high K\(^{+}\)\(_{o}\) conditions has not been previously described.

**Figure 1.** Effect of [K\(^{+}\)]\(_{o}\) on the association of \(^{3}\text{H}\)-ouabain with BAC cells. Cells were incubated for 4 hours at 37°C with \(^{3}\text{H}\)-ouabain (50 nmol/L) in SES containing the indicated concentrations of potassium (KCl replacing NaCl). Data are mean \pm SEM (n = 3). SEM values are \(<\) symbol diameter. The solid line is the result of iterative fit using the first 3 (0 to 10 mmol/L K\(^{+}\)) and last 4 points (80 to 100 mmol/L K\(^{+}\)) to an equation of the form Y = Y\(_{\text{max}}\) / (1 + ([K\(^{+}\)] / K\(_{50}\))^n), where n is the Hill coefficient and KCI and EC\(_{50}\) have their usual meanings. The derived EC\(_{50}\) for K\(^{+}\) was 1.58 \pm 0.10 mmol/L. Insets, Apparent stimulatory effect of K\(^{+}\) on the association of \(^{3}\text{H}\)-ouabain with NOR. The data are the difference between the measured data and the equation-derived values.

**Figure 2.** Effect of extracellular potassium on the concentration dependence of \(^{3}\text{H}\)-ouabain binding to BAC cells. A, \(^{3}\text{H}\)-ouabain binding in potassium-free SES. B, \(^{3}\text{H}\)-ouabain binding in SES with 15 mmol/L potassium. Shown are means \pm SEM (n = 3). Where not shown, SEM values are \(<\) symbol diameter. Insets, respective Scatchard plots.

**Dissociation of \(^{3}\text{H}\)-Ouabain From BAC Cells**

To provide additional evidence for a second class of ouabain binding sites in cells, we followed the dissociation kinetics of prebound \(^{3}\text{H}\)-ouabain. Two sets of perfusion conditions were used. Where fast washout kinetics were desired, the flow rate was 1.5 mL/min, the fraction time was 2 minutes, and the cells were not prewashed after incubation with \(^{3}\text{H}\)-ouabain. For slow washout kinetics, the flow rate was 0.13 mL/min, the fraction time was 30 minutes, and the cells were prewashed. In each case, the column volume was 1 mL. Figure 3 shows the washout kinetics of \(^{3}\text{H}\)-ouabain using fast and slow perfusion conditions. Both data sets were well fit by Equation 2, and the Table shows the derived values for the variables.

Under rapid perfusion conditions, 2 values for t\(_{1/2}\) were found. The very fast component (t\(_{1/2}\) = 0.5 minutes) represents the physical washout kinetics of the column under the...
Membranes were incubated with 3H-ouabain (200 nmol/L) in –branes. A, Na pump mediated binding. B, Binding to NOR.

Figure 4. Time dependence of 3H-ouabain binding to membranes. A, Na pump-mediated binding. B, Binding to NOR. Membranes were incubated with 3H-ouabain (200 nmol/L) in Mg2+/Pi (A) or EDTA/EGTA (B) buffer for the indicated time at 37°C. Data are means±SEM. SEM values not shown are < the symbol diameter.

Table 1. Component Analysis for the Dissociation of 3H-Ouabain From BAC Cells

<table>
<thead>
<tr>
<th>Perfusion Rate</th>
<th>t1/2</th>
<th>Y Intercept</th>
<th>k⁻¹</th>
<th>Total at Time 0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fast</td>
<td>0.5 min</td>
<td>1360 ± 180 fmol/2 min</td>
<td>1.41 ± 0.07 min⁻¹</td>
<td>480 fmol</td>
</tr>
<tr>
<td></td>
<td>6.5 min</td>
<td>8.9 ± 1.4 fmol/2 min</td>
<td>0.11 ± 0.02 min⁻¹</td>
<td>40 fmol</td>
</tr>
<tr>
<td>Slow</td>
<td>3.3 h</td>
<td>9.1 ± 2.3 fmol/2 min</td>
<td>0.21 ± 0.11 min⁻¹</td>
<td>300 fmol</td>
</tr>
<tr>
<td></td>
<td>32.8 h</td>
<td>116 ± 186 fmol/30 min</td>
<td>0.021 ± 0.008 min⁻¹</td>
<td>11 000 fmol</td>
</tr>
</tbody>
</table>

Values for k⁻¹ were derived by iterative fitting of the data to Equation 2. Equations 3 and 4 were used to compute the other values. Values shown are means±SEM where indicated.

In contrast to the behavior of adrenocortical membranes, the ouabain binding to NOR in liver membranes (Figure 5B) was barely detectable (ie, 0.006 pmol/mg).

In adrenocortical membranes, Scatchard analysis showed that a single class of Na pump sites mediated ouabain binding with an apparent Kd and Bmax of 5.2 ± 1.0 nmol/L and 18.1 ± 0.8 pmol/mg membrane protein, respectively. In adrenocortical membranes, NOR-mediated ouabain binding was also well explained by a single class of sites (Figure 5B), with Kd and Bmax equal to 16.1 ± 2.4 nmol/L and 0.28 ± 0.01 pmol/mg protein, respectively. It may be noted that the linear Scatchard plot for NOR gives no indication of the presence of the 64-fold greater number of Na pump sites in the binding reaction relative to NOR. The inability of the Scatchard analysis to reveal binding information for the Na pump under these conditions is a reflection of the dramatic decline in the binding affinity of Na pumps for ouabain (ie, from ~5 nmol/L to >50 μmol/L) under the conditions in which NOR was measured.

Figure 5 shows the effect of KCl on ouabain binding by the Na pump and NOR in adrenocortical membranes. KCl inhibited ouabain binding to the Na pump (IC50 = 0.7 ± 0.02 mmol/L). In contrast, the effect of KCl on NOR was complex because low [KCl] inhibited ouabain binding (IC50 = 0.5 ± 0.02 mmol/L).

Figure 6 shows the effect of KCl on ouabain binding by the Na pump and NOR in adrenocortical membranes. KCl inhibited ouabain binding to the Na pump (IC50 = 0.7 ± 0.02 mmol/L). In contrast, the effect of KCl on NOR was complex because low [KCl] inhibited ouabain binding (IC50 = 0.5 ± 0.02 mmol/L).
whereas higher concentrations were stimulatory (EC₅₀ = 8.7±5.9 mmol/L).

**Photoaffinity Labeling Studies**

Three major polypeptides (143, 113, and 65 kDa) became covalently labeled by ³H-ouabain under reaction conditions that strongly favored high-affinity binding to NOR (Figure 7). The labeling of the 3 polypeptides was highly reproducible, specific, and dependent on both photolysis time and the concentration of ³H-ouabain. There was minimal or no labeling of the 95-kDa Na pump α-subunit as expected. At present, the identity of the 143-kDa polypeptide is unclear. It is possible that it represents the covalent attachment of ouabain to cross-linked αβ subunits. No labeling was detected in the Na pump β-subunit (∼45 kDa), and, in the absence of photolysis, no labeling was found in any polypeptide. Calculations suggested that the efficiency of photolytic labeling was 1 to 2% for NOR. The labeling was reduced dramatically by silver staining, hence separate gel lanes were used for counting. The silver sensitivity suggests that ³H-ouabain may become covalently attached to 1 or more amino acids via a thioester linkage.

**Specificity and Distribution of NOR**

Neither aldosterone nor cortisol (30 μmol/L) affected ouabain binding by the Na pump or NOR. Membranes from bovine skeletal muscle and liver (Figure 5) showed specific, saturable, and high-affinity ³H-ouabain binding to Na pumps under phosphorylating conditions. However, in the absence of phosphorylating conditions, no NOR-mediated binding could be reliably detected in membrane preparations from either tissue.

**Discussion**

The major results of the present study include the following observations. First, a novel class of binding sites for ouabain has been detected on the surface of BAC cells. These sites are saturable, specific for ouabain, and of high affinity. The sites are expressed at low density and have dissociation kinetics distinct from the Na pumps in the BAC cells. Second, studies in isolated membranes confirmed the characteristics of these novel binding sites and facilitated further characterization. Third, the novel binding sites are tissue specific and were not found in membranes from liver or skeletal muscle. The absence of NOR from the latter tissues indicates that NOR does not reflect the binding of ouabain with membrane lipids. Thus, the characteristics of these novel binding sites in the adrenal cortex suggest that they are mediated by a single class of polypeptide receptors for ouabain.

The rationale for the present studies originated from several observations. First, the prolonged administration of ouabain and digoxin produced opposite effects on long-term blood pressure in normal rats. Second, the potency of ouabain analogs as Na,K-ATPase inhibitors was correlated inversely with their effects on long-term blood pressure. Third, studies with BAC cells showed that EO secretion slowed unexpectedly with extended incubation times and that this could be ameliorated by increasing the volume of the external medium. Hence, EO secretion was suggested to be regulated by negative feedback, possibly triggered by the accumulation of EO or a cosecreted product in the extracellular fluid. Moreover, as BAC cell Na pumps exhibit low affinity for ouabain under physiological conditions, a direct role in EO feedback control was unlikely. Instead, the aforementioned observations, when taken together, pointed to the existence of 1 or more novel classes of functional ouabain binding sites.

Previous studies in BAC cells showed a single class of high-density ³H-ouabain binding sites (∼4.5×10⁶ sites/cell) that were linked functionally with Na pumps. However, we had noted that a second class of ouabain binding sites with a density <5% of the Na pump number would have escaped detection by routine binding methods. Accordingly, the discovery of novel ouabain binding sites in this work that are present at ∼1% of the density of Na pumps is attributable to the combination of 2 experimental conditions. The first used conditions that dramatically lowered the ouabain binding...
affinity of Na pump (>50 μmol/L), whereas the second used low subsaturating concentrations of 3H-ouabain (<200 nM) that further minimized any residual tendency for ouabain to bind to Na pumps. Under these conditions, ouabain binding to the Na pump was effectively silenced and a second set of ouabain binding sites then became readily detectable. Surprising, the new ouabain binding sites were found to have a high affinity for ouabain and, as expected, were present at a low density when compared with the number of Na pumps. Consistent with the existence of the new binding sites, the washout kinetics of 3H-ouabain from the cells revealed a novel fast component of dissociation that has not been previously described and whose kinetics were distinct from those ascribed to Na pumps and the lysosomal recycling of ouabain.

Several lines of evidence presented here distinguish the novel ouabain binding sites from classical Na pumps. First, it is well accepted that Na pumps bind ouabain with the highest affinity when phosphorylated at the active site. Ordinarily, the hydrolysis of ATP by the Na pump in the presence of sodium and magnesium ions leads to the formation of a phosphorylated intermediate. After discharge of sodium ions to the extracellular phase, the phosphorylated Na pump undergoes a conformational change to a state (E2P) that favors the binding of ouabain with the highest affinity. The E2P state can also be readily generated in the presence of magnesium and inorganic phosphate. Thus, by manipulation of the ligand conditions that either promote (Na$^+$+Mg$^{2+}$+ATP or Mg$^{2+}$+inorganic phosphate) or preclude (divalent cation chelators and the absence of the aforementioned ligands) phosphorylation, it is possible to shift stably the binding affinity for ouabain over many orders of magnitude (eg, see Hamlyn et al). Hence, it is possible to render Na pumps highly visible or invisible to 3H-ouabain. Traditionally, most of the experiments that have looked at the membrane binding of 3H-ouabain have used some form of phosphorylating conditions to maximize the detection and quantification of Na pumps. Therefore, it is ironic that the opposite strategy, ie, the use of conditions that minimize the binding of ouabain by Na pumps, is the underpinning of the present result and that NOR continued to exhibit high-affinity ouabain binding under these conditions.

Second, whereas high concentrations of potassium invariably suppress ouabain binding to Na pumps, they enhanced ouabain binding to NOR in both intact cells and isolated membranes. Third, a small portion of the total bound ouabain that dissociated from the cells was kinetically distinct from the unbinding of label from Na pumps. Fourth, Scatchard analyses showed that the maximum number of cell Na pumps was similar under zero and high K$^+$ conditions even though NOR was visible only under the latter conditions. Fifth, during photolysis, the 113- and 65-kDa polypeptides were labeled reproducibly by 3H-ouabain. There was little evidence for specific labeling of the Na pump α-subunit as expected because the binding reaction that preceded the photolysis step was conducted under conditions that favored the high-affinity interaction of 3H-ouabain with NOR. Moreover, each of the 3 polypeptides mentioned above became specifically labeled. The 113- and 65-kDa polypeptides differ significantly in size from the α (95 kDa) and β (45 kDa) subunits of the Na pump and the αβ combination (140 kDa). However, additional work with specific photoaffinity ouabain analogs and possibly other methods will be required to confirm or refute the suggestion that the 113- and/or 65-kDa polypeptides contribute to NOR. Nevertheless, when taken together, the available results indicate that NOR and Na pumps represent distinct classes of binding sites for ouabain and that these exist simultaneously in BAC cells and membranes. Moreover, NOR and Na pumps do not seem to be interconvertible entities.

The rationale for NOR discovery in the adrenal cortex included possible roles in transmembrane transport of EO, feedback inhibition of EO secretion and/or its biosynthesis, or some combination thereof. As NOR seems to be well explained by a single discrete binding affinity (Figure 5), it is plausible that NOR might mediate both EO transport and feedback inhibition of its secretion. With respect to EO biosynthesis, >95% of the 3H remained firmly associated with ouabain in the cell dissociation experiments in agreement with other work. The minimal back metabolism of ouabain suggests, albeit indirectly, that NOR is not involved in EO biosynthesis. Additional work will be required to evaluate the possibilities definitively.

Membrane proteins (oATPs) that transport CGs and bile salts have been described. In addition, members of the multidrug resistance (MDR) family export cytotoxic drugs, including digoxin, from cancer cells and may participate in corticosteroid secretion. However, NOR-mediated ouabain binding was unaffected by supraphysiological concentrations of cortisol and aldosterone, indicating that NOR does not recognize the classical mammalian planar steroids in which the AB, BC, and CD steroid rings are all trans fused. Typically, NOR was not detectable in liver or skeletal muscle under assay conditions where it was reproducibly quantifiable in adrenocortical membranes. In the present experiments, NOR-mediated ouabain binding in liver membranes was ~50-fold lower than in membranes from the adrenal cortex. The low level of NOR expression in liver, coupled with the observation that the MDR family of transporters generally interact with ouabain with millimolar affinity, suggests that NOR and MDR are not directly related. Novel receptors with somewhat higher affinity for CGs were described in membranes from dog heart but now seem to reflect either the copresence of other unrecognized Na pump isoforms and/or a 31.5-kDa sarcoplasmic reticulum protein that affects the opening of ryanodine-sensitive calcium channels. The intracellular location and small size of the sarcoplasmic reticulum protein may distinguish it from NOR.

In summary, studies with adrenocortical cells and membranes demonstrate a receptor for ouabain that is distinct from classical Na pumps. The localization of this receptor in the adrenal cortex and its specificity for ouabain suggest that it may have a role in the transport and/or regulation of polar CGs. Moreover, the existence of novel binding sites for ouabain should fuel further interest in the association of EO with hypertension and congestive heart failure and their therapy.
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