Effects of Angiotensin II on Sodium Potassium Pumps, Endogenous Ouabain, and Aldosterone in Bovine Zona Glomerulosa Cells

Jui R. Shah, James Laredo, Bruce P. Hamilton, John M. Hamlyn

Abstract—Angiotensin (Ang) II stimulates secretions of aldosterone and an endogenous ouabain-like steroid (EO) from bovine adrenal zona glomerulosa (BAG) cells. The BAG cell sodium pump, a possible target of EO, affects aldosterone secretion although little is known about this pump. Here, we describe the effects of Ang II on the characteristics of this transporter and steroid secretions. Under serum-free conditions, 3H-ouabain bound to a single class of sites on BAG cells. Binding of label was time and concentration dependent, was sensitive to extracellular potassium ions, and was displaced by ouabain and digoxin with EC50 of ≈218 and ≈232 nmol/L, respectively. Sodium pump–mediated 86Rb uptake was inhibited by ouabain (EC50 ≈301 nmol/L). Ang II dose dependently augmented secretions of EO and aldosterone, increased ouabain-sensitive 86Rb uptake and 3H-ouabain binding, and increased the affinity for 3H-ouabain binding (Kd, from 205 to 80 nmol/L) with no change in the maximal number of sodium pumps (5.45 ± 10^6) per cell. Losartan blocked all effects of Ang II except EO secretion, which was inhibited by PD123319. We conclude that BAG cells express sodium pumps in high density and bind ouabain to a single class of low-affinity sites. The characteristics of the sodium pumps protect BAG cells from EO autotoxicity but may exclude them from mediating feedback inhibition of EO secretion. The effects of Ang II on sodium pump activity, ouabain binding affinity, and aldosterone secretion are mediated via Ang II type 1 receptors, whereas Ang II type 2 receptors augment EO secretion. The role of the Ang II–mediated increase in the ouabain sensitivity of BAG cell sodium pumps in the secretions of aldosterone and EO remains to be elucidated. (Hypertension. 1999;33[part II]:373-377.)

Key Words: cardiac glycosides • adrenal • Na,K-ATPase • secretion • cell culture

Steroid hormones of adrenocortical origin are important regulators of cardiovascular, renal, and neural function. In addition to the classic steroid hormones, recent evidence has suggested that the adrenal cortex may secrete ≥1 counterparts to the cardenolide ouabain.1–5 Endogenous “ouabain” (EO) is a specific, high-affinity, reversible inhibitor of sodium pumps.2,3,5 Moreover, the classic secretagogues, angiotensin (Ang) II and adrenocorticotropic hormone, raise EO secretion from primary adrenocortical cultures.6 Previously, we showed that Ang II stimulates aldosterone and EO secretions from bovine adrenal zona glomerulosa (BAG) cells via distinct receptor subtypes.7 Moreover, inhibition of sodium pumps in adrenal cells may augment aldosterone secretion.8 The apparent links between Ang II, EO, sodium pumps, and aldosterone secretion led us to characterize the sodium pump of BAG cells and investigate the effects of Ang II on the pump in concert with measurements of steroid secretion.

Methods

Adrenocortical Cell Culture
Bovine adrenal glands were obtained from a local slaughterhouse and transported to the laboratory on ice in phosphate-buffered solution. Adrenocortical cells were dispersed and cultured according to established methods. Full details are given elsewhere.7,9,10 Cells were plated at ≈4 million (for secretion experiments) or 1 million (for binding studies) per well. All experiments were performed with at least triplicate cell cultures.

Steroid Secretion Experiments and Radioimmunoassays
Details are given elsewhere.10 After incubation, the secretion media was collected and assayed for aldosterone and EO. They were rinsed with buffer and dissolved in 1% sodium dodecyl sulfate (SDS) for assay of total cell protein (BCA) and estimation of cell-associated radioactivity when appropriate. Aldosterone was measured with a commercially available radioimmunoassay (RIA) kit (Diagnostic Products Corp). The RIA has no cross-reactivity to ouabain or EO. Intra-assay and interassay coefficients of variation were 6.8% and 8.2%, respectively. EO was measured by RIA by use of extracted...
samples and a polyclonal rabbit antiouabain serum (R8) described elsewhere.\textsuperscript{11,12} The assay is highly cross-reactive with EO\textsuperscript{1} but exhibits no significant cross-reactivity (<0.01\%) with cortisol, aldosterone, adrenocorticotropic hormone, and Ang II. Typical intra-assay and interassay coefficients of variation were 6.9\% and 13.6\%, respectively.

\textbf{\textsuperscript{3}H-Ouabain Binding Studies}
After removal of the culture medium, the cells were washed twice with buffer (containing in mmol/L NaCl 145, KCl 5, CaCl\textsubscript{2} 2, MgCl\textsubscript{2} 1, glucose 10, HEPES 10-NaOH, pH 7.4). Buffer containing \textsuperscript{3}H-ouabain (10 to 300 nmol/L) either alone or with pharmacological reagents was added to the cells and incubated at 37°C in 5\% CO\textsubscript{2} for 4 or 8 hours. For nonspecific binding, 30 \mu mol/L cold ouabain was present in the buffer, and specific binding was taken as the difference between total and nonspecific binding. Competition curves for \textsuperscript{3}H-ouabain binding were constructed by use of 10 nmol/L \textsuperscript{3}H-ouabain in the presence of increasing amounts of cold ouabain. At the end of the experiment, cells were rinsed with buffer and dissolved in 1\% SDS. Total cell protein (BCA) and cell-associated radioactivity were determined.

\textbf{\textsuperscript{86}Rb Uptake Studies}
The culture medium was removed, and the cells were washed twice with a secretion buffer containing 2 mmol/L RbCl. Secretion buffer fortified with 10 mmol/L \textsuperscript{86}RbCl and various pharmacological agents was incubated with cells at 37\% in 5\% CO\textsubscript{2} for 4 hours. The medium was aspirated, and cells were rinsed 3 times in 2 \mu L ice-cold buffer with 2 mmol/L RbCl and mixed with 1 mL of 1\% SDS for assay of total cell protein and cell-associated \textsuperscript{86}Rb (gamma counter). Pump activity was the difference between uptake in the absence and presence of 0.1 nmol/L ouabain. Pump fluxes are expressed as nanomoles of Rb plus potassium per milligram per 4 hours.

\textbf{Materials}
PD123319-ditrifluoroacetate (DTFA) was a gift of Dr David Taylor (Warner-Lambert, Ann Arbor, Mich). DTFA was removed by high-performance liquid chromatography before use. Losartan (DuP 753) was a gift of Dr Ronald Smith (Du Pont Merck). Collagenase type I and DNase type I were from Worthington Biochemicals. Percoll was from Pharmacia. Fetal bovine serum and Dulbecco’s modified Eagle’s medium were from GIBCO-BRL. \textsuperscript{3}H-ouabain was from New England Nuclear. Other chemicals were from Sigma Chemical Co.

\textbf{Statistical Analyses}
Evaluations of significance were performed with ANOVA and Fisher’s specific test. Results were expressed as mean±SEM, and $P<0.05$ was used to indicate significance unless otherwise noted.

\textbf{Results}
\textbf{Characterization of BAG Cell Sodium Pumps}
Figure 1 shows the time course, concentration dependence, and potassium sensitivity of \textsuperscript{3}H-ouabain binding to BAG cells. In the time course and concentration experiments, specific binding was >90\% of the total. Specific binding increased fairly linearly with time. The concentration dependence of specific binding was unexpectedly linear, showing no clear evidence of saturation even at 300 nmol/L. These results suggested that the sodium pumps in these cells had fairly low affinity for ouabain. In addition, specific ouabain binding was reduced dose dependently by extracellular potassium over the range of 0.01 to 10 mmol/L. The dose-response curve for potassium ions was shifted $\approx 7.5$-fold rightward when binding was performed in 100 instead of 10 nmol/L \textsuperscript{3}H-ouabain.

To explore the binding affinity of the sodium pumps for cardiac glycosides, the cells were coincubated with increasing doses of cold cardiac glycosides in the presence of tracer amounts (10 nmol/L) of \textsuperscript{3}H-ouabain. As shown in Figure 2, binding of \textsuperscript{3}H-ouabain was displaced in a concentration-dependent manner by both ouabain and digoxin with $IC_{50}$ of $\approx 218$ and $\approx 232$ nmol/L, respectively. In each case, the Hill coefficients were nearly unity, indicating the interaction of ouabain and digoxin with a single class of \textsuperscript{3}H-ouabain binding sites.

Rb$^+$ ions substitute for K$^+$ in stimulating ATP hydrolysis and sodium pump–mediated transport into cells. Preliminary experiments showed that BAG cells accumulated \textsuperscript{86}Rb and that the ouabain-sensitive component comprised >80\% of the uptake over 4 hours (data not shown).
Effects of Ang II Receptors on Ouabain Binding and Sodium Pump Activity

The effect of Ang II on the binding of $^3$H-ouabain to BAG cells is shown in Figure 2. Ang II dose dependently increased the specific binding of $^3$H-ouabain to BAG cells from a basal of 6.14 fmol/mg at 100 nmol/L Ang II. The EC$_{50}$ for Ang II stimulation of $^3$H-ouabain binding was 1 nmol/L. This value is similar to the EC$_{50}$ for the stimulation of corticosteroid secretions, suggesting that the same receptor may be involved in both effects.

Further experiments in which the concentration of $^3$H-ouabain was systematically varied showed that the effect of Ang II increased the binding of label at all concentrations tested (Figure 3). Moreover, Scatchard analysis (Figure 3, inset) showed that the binding of $^3$H-ouabain to BAG cells under basal conditions was well fit to a single class of binding sites with an apparent $K_d$ of 205 nmol/L, and a $B_{max}$ of 35.6 fmol/µg protein. In the presence of 10 nmol/L Ang II, a single class of binding sites was again detected whose dissociation constant and maximal binding were 79 nmol/L and 35.9 fmol/µg, respectively. Thus, Ang II increased the sensitivity of the sodium pumps to ouabain 3-fold without changing their number.

Figure 4 shows the effects of specific blockade of Ang II type 1 and 2 receptors on $^3$H-ouabain binding and sodium pump activity (as estimated by ouabain-sensitive $^{86}$Rb uptake). Ang II augmented $^3$H-ouabain binding and increased sodium pump activity. In parallel incubations, Ang II stimulated ($P<0.05$) the secretions of aldosterone (basal, 0.82±0.08 pmol·mg$^{-1}$·2 h$^{-1}$; Ang II, 5.68±0.57 pmol·mg$^{-1}$·2 h$^{-1}$) and EO (basal, 0.64±0.04 pmol·mg$^{-1}$·2 h$^{-1}$; Ang II, 1.5±0.09 pmol·mg$^{-1}$·2 h$^{-1}$), except for EO secretion, all effects of Ang II were blocked by losartan. In contrast, PD123319 specifically inhibited Ang II–stimulated EO secretion. Neither losartan nor PD123319 affected the measured parameters in the absence of Ang II (not shown).
**Discussion**

The present investigation was prompted by several observations. First, BAG cells were shown to secrete aldosterone and EO. Second, EO was known to be a high-affinity and specific inhibitor of the sodium potassium pump. Third, many reports indicated that ouabain and other sodium pump inhibitors may affect aldosterone secretion. Fourth, part of the effect of Ang II on aldosterone secretion was suggested to involve inhibition of adrenocortical sodium pumps. Fifth, the kinetics of EO secretion implied autoinhibition, perhaps mediated by sodium pumps. Sixth, the characteristics of the sodium pumps in primary cell cultures of BAG cells used by our laboratory and others were not known. Collectively, these observations suggested that the secretion and subsequent binding of EO to adrenocortical sodium pumps might have multiple consequences. Accordingly, we investigated the sodium pump of the BAG to evaluate its properties and possible roles in the Ang II–stimulated secretions of EO and aldosterone. Measurements of aldosterone secretion were used for correlative and comparative purposes and to demonstrate the functional integrity of this secretory system.

Under basal conditions, both the time and concentration dependence of 3H-ouabain binding were pseudolinear (Figure 1). This is consistent with the use of ligand concentrations that are significantly less than the receptor affinity. More than 90% of the total binding was specific. The concentration dependence of specific binding showed no evidence of saturation even at 300 nmol/L. Changes in potassium ion concentration within the physiological range reduce the dependence of specific binding showed no evidence of saturation even at 300 nmol/L. Changes in potassium ion concentration within the physiological range reduce the affinity for cardiac glycoside binding to the sodium pump. The marked potassium ion sensitivity of the interaction of 3H-ouabain with BAG cells and the weakened effect of potassium at the highest concentration of 3H-ouabain used show that specific binding is mediated exclusively by sodium pumps. Further analysis of the relationship showed that the competition between labeled and cold ouabain occurred at a single class of binding sites whose apparent affinity for ouabain and digoxin was surprisingly low (Figure 2). These results, consistent with the temporal and concentration data in Figure 1, have other implications. We suggested previously that feedback inhibition of EO secretion may be mediated by autoreceptors. The present results suggest that these autoreceptors are not sodium pumps. For example, the rate of EO secretion falls to 10% of the initial rate as the concentration of secreted EO rises into the range of 0.2 to 0.5 nmol/L. On the basis of a binding affinity for ouabain of 200 nmol/L and with equivalent potency assumed, an EO concentration of 0.5 nmol/L would bind and inhibit 0.25% of the bulk sodium pumps. Another possibility is that a small number of specialized pumps are involved. In some cell types, small numbers of sodium pumps with high ouabain affinity are clustered in functional microdomains over portions of the sarcoplasmic reticulum. In vascular smooth muscle cells, nanomolar concentrations of ouabain interact specifically with these pumps to affect stored calcium, while leaving other sodium pumps involved in the control of bulk cytosolic sodium unaffected. If such specialized sodium pumps or other types of ouabain receptors were present in BAG cells, they would comprise <5% of the specific binding sites measured and would escape detection by our methods.

Another factor of relevance to BAG cell function is the high density (5.45×10⁶) of sodium pumps per cell estimated from Scatchard analysis (Figure 3). This is reminiscent of tissues such as nerve, heart, and kidney in which the high copy number typically is associated with large inward sodium fluxes and high rates of aerobic metabolism. Moreover, it is consistent with other reports that have noted high Na,K-ATPase activity in glomerularosa tissue.

What is the possible significance of high pump turnover, low affinity, and high pump densities? In other systems in which a ligand may be present continuously, rapid receptor turnover can serve as a means to alter signaling. The concentration of EO leaving the dog adrenal is 0.75 nmol/L, roughly 4- to 5-fold higher than that entering the gland. Moreover, the local intra-adrenal concentrations of EO, in the vicinity of the secretory cells especially during Ang II stimulation, may be 10-fold higher than those measured in the mixed venous effluent. Even under these conditions, our calculations suggest that 12% to 15% of the available pumps could be occupied by EO. This level of pump blockade would be expected to cause physiologically relevant increases in cell sodium and stored calcium that would augment signaling by Ang II and other stimulants without toxicity. The affinity of the adrenocortical sodium pump for cardiac glycosides in cows, coupled with a high pump density, is poised for physiological regulation by EO while forestalling the potential for autotoxity in the adrenocortical environment.

Previous reports of the effects of Ang II on sodium pump activity in adrenocortical cells are varied. In rat glomerulosa cells and bovine adrenal cells, Ang II was thought to inhibit sodium pumps. Exposure of cells to Ang II does not appear to affect the Na,K-ATPase activity in isolated membranes. Consistent with this impression, our results showed that Ang II, via Ang II type 1 receptors, increased both the affinity of BAG sodium pumps for ouabain and their transport activity (Figure 4) without upregulation or down-regulation of surface membrane pump number (Figure 3). The increase in pump activity in response to Ang II probably originates from an increase in cell sodium, which is known to enhance the formation of a phosphorylated intermediate of the pump whose affinity for ouabain is heightened simultaneously.

The increased EO secretion occurring at a time when the ouabain affinity of the BAG cell sodium pump is increased, along with the demand for heightened active transport, seems paradoxical (Figures 3 and 4). One possibility is that intracellular EO might augment Ang II–stimulated aldosterone secretion. In rat adrenal cells, aldosterone secretion was increased during inhibition of sodium pumps by both Ang II and ouabain. In other species, some studies reported inhibition, stimulation, or transient stimulation followed by inhibition of aldosterone secretion in response to ouabain. Studies that observed inhibition of aldosterone secretion have in general used pharmacological concentrations of ouabain that would inhibit >50% of the available sodium pumps. These conditions are often lethal because the activity of the
residual pumps cannot increase sufficiently to normalize outward sodium flux and cell potassium.

Two recent sets of observations suggest that aldosterone secretion can be augmented by concentrations of ouabain that could inhibit no more than a small portion of the bulk sodium pumps. In the rat, continuous infusions of ouabain for 5 weeks resulted in circulating plasma levels ranging from ~1 to 5 nmol/L. Under these conditions, plasma aldosterone was dose dependently elevated, whereas plasma renin activity was unchanged. Thus, low concentrations of ouabain approaching the physiological range of EO provide a strong in vivo stimulus to aldosterone secretion in the rat. A similar result, albeit with pharmacological concentrations of ouabain, has been observed with rat BAG cells in vitro. In addition, Tamura et al. have shown that ouabain at 10 nmol/L cally confirmed Conn’s syndrome. These observations, been described in 'aldosterone secretion significantly.

Elevated circulating levels of EO and aldosterone have been described in ~50% of hypertensive patients with surgically confirmed Conn’s syndrome. These observations, along with those mentioned above and the accumulating evidence that the secretions of EO and aldosterone are regulated differently, raise the possibility that the hyperaldosteronism in some patients with Conn’s syndrome may be secondary to a primary overproduction of EO. New classes of agents that block ouabain and EO binding may distinguish among these patients.

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References
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