Endogenous cardiac steroids (ECS) are putative ligands of the inhibitory binding site of the membrane sodium pump (Na\(^+\),K\(^+\)-ATPase). There is growing evidence that cardiotonic steroids may promote the growth of cardiac and vascular myocytes, including evidence indicating growth stimulation at concentrations in the same range as circulating ECS concentrations. We investigated four parameters to determine whether ouabain, a proposed ECS, promotes growth of immortalized rat proximal tubule epithelial cells: cell count by hemocytometer; metabolic activity as reflected in the mitochondrial conversion of the tetrazolium salt, 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, to its formazan product (MA); DNA synthesis reflected as histone phosphorylation state detected using anti-phosphohistone 3 antibody (HP). Maximum stimulatory responses were observed at 1 nM ouabain (MA, 20.3% increase, \(p < 0.01\); DNA, 28.4% increase, \(p < 0.001\); HP, maximum response at 0.5 h, 50% increase, \(p < 0.001\)). We observed that growth stimulation was associated with stimulation of ERK1/2 phosphorylation (ERK-P), and both growth and ERK-P could be blocked by the MEK inhibitor (U0126, 100 nM). Western blot analysis revealed that the only \(\alpha\) isoform of Na\(^+\),K\(^+\)-ATPase that could be detected in these cultures was the ouabain-resistant \(\alpha1\) isoform. Measurement of ouabain inhibition of ion transport in these cultures using \(^{86}\)Rb\(^+\) uptake revealed the predominance of the expected ouabain-resistant isoform (IC\(_{50} = 24 \mu M\)) and an additional minor (15%) ouabain-sensitive inhibition with IC\(_{50} = 30 \mu M\). Similar bi-modal transport inhibition curves were obtained in freshly dissected rat proximal tubules. These results indicate that renal epithelial cells may be a sensitive target of the ERK1/2-activating and growth-promoting effects of ouabain even in the presence of ouabain-resistant Na\(^+\),K\(^+\)-ATPase.

There is evidence that cardiotonic steroids (CS)\(^\dagger\) are a normal product of the mammalian adrenal cortex. At present, two CS, ouabain and marinobufagenin, appear to best fulfill criteria for endogenous mammalian adrenocortical cardiotonic steroids (ECS) (1–5). Plant-derived CS-glycosides are widely used therapeutically to increase myocardial performance by inhibition of active transmembrane cation transport that results from binding to the membrane-inserted sodium, potassium-ATPase (Na\(^+\),K\(^+\)-ATPase) (4, 6–8). Recently, evidence has been generated indicating that, in addition to an ion transport inhibitory role, CS also activate a signaling function that influences growth and proliferation of cardiac and vascular myocytes (9, 10). Although the growth-promoting effect of CS appears to depend on initiation of signaling complexes that result from binding of CS to Na\(^+\),K\(^+\)-ATPase, stimulation of growth has been shown to be independent of inhibition of cation transport (11, 12).

These observations raise the possibility that the usual physiological function of ECS lies not only in an ion transport inhibitory role but in influencing growth and proliferation of target cells. The kidney has long been considered a likely target of ECS (13, 14). However, the effect of CS on renal epithelial cell growth and proliferation has not been examined. Modulation of growth and hypertrophy is an important element of renal development and the renal response to altered environment in both young and adult animals (15, 16). Parts of the kidney are also prone to injury from metabolic or osmotic stresses (17, 18), and compensatory growth of adjacent uninjured tissue may be a normal element in adaptation to such renal injury. Thus, the existence of signaling mechanisms to drive renal growth globally or in response to local injury places importance on a full understanding of the regulation of renal growth and hypertrophy. Endogenous cardiotonic steroids may contribute to this adaptation.

In the present study, we have investigated the ability of ouabain to influence growth of cultured renal epithelial cells that express only an \(\alpha1\) isoform of Na\(^+\),K\(^+\)-ATPase that is highly resistant to inhibition by ouabain and other CS (K\(_i\) = 1.4 \times 10\(^{-4}\) M) (19–21). The doses of ouabain we have investigated for their growth-promoting effects correspond to normal plasma ouabain levels (22, 23). Here we report that nanomolar concentrations of ouabain are able to stimulate mitosis and proliferative cell growth of rat renal epithelial by an ERK kinase-dependent signaling pathway. This work suggests that endogenous CS may have an important biological function even in rodent species previously considered resistant to these steroids.

**Experimental Procedures**

**Cell Culture**

Rat renal proximal convoluted tubule epithelial cells (PCT) were originally obtained from Dr. Ulrich Hopfer, Case Western Reserve Medical School, and we have maintained them for the past 5 years. The epithelial phenotype of the cell line is well documented, and the cells demonstrate numerous morphological and functional characteristics of differentiated PCT cells (24, 25). Cells were cultured in Dulbecco’s modified Eagle’s medium/P12 (1:1) supplemented with 15 mM HEPES,
then exposed for 8 or 24 h to 1 nM ouabain or to medium alone in control renal epithelial cells. Cells were cultured in 96-well plates for 24 h and luted and counted by hemocytometer. (Bars represent mild trypsinization and gentle shaking. Cell suspensions were dispersed and then exposed for 8 h to different concentrations of ouabain or to medium alone in control cultures. Cell number was determined using the CellTiter 96® AQueous One Solution reagent and then exposed for 8 h to different concentrations of ouabain or to medium alone in control cultures. At the same time, BrdUrd was added to each well. The quantitation of BrdUrd incorporation into newly synthesized DNA of proliferating cells was estimated immunologically using specific monoclonal anti-BrdUrd and horseradish peroxidase-conjugated secondary antibodies.

**Preparation of Rat Renal Proximal Tubules**

Proximal tubule (PT) fragments were isolated from 8-week-old male rats (WKY strain bred in our colony) using the method described by Seri et al. (26). After induction of isoflurane anesthesia, the kidneys were removed and the cortex dissected and minced on ice to a paste-like consistency. This minced cortex was incubated with Hanks’ balanced salt solution supplemented with 0.075 g/100 ml Type I collagenase, 500 μM pyruvic acid, 1 mmol/liter lactic acid, and 1 mmol/liter sodium butyrate, pH 7.4. After of 45 min of incubation at 37 °C with continuous bubbling with 95% O2, 5% CO2, tissue fragments were placed on ice and then poured sequentially through sieves of 100-, 70-, and 40-μm pore size to obtain a PT-enriched suspension. The PTs were washed three times by centrifugation at 600 rpm at 4 °C in a Beckman CS-6R centrifuge. After the final centrifugation, PTs were resuspended in culture medium and placed into an incubator for adaptation prior to ouabain-sensitive ion transport activity assay.

**Measurement of PCT Proliferation and Growth**

Cell Proliferation—To determine the effects of ouabain on cell number, plates simultaneously seeded with cells were distributed into groups from which cells were counted by hemocytometer either before or after 8 or 24 h of ouabain treatment. Untreated cultures (medium without ouabain) were used as controls. Cells were detached from culture plate wells with mild trypsinization and gently shaken to disperse them. They were then diluted in culture medium for counting by hemocytometer using an inverted phase contrast microscope. To provide a measurement of cell growth reflecting the increasing metabolic activity occurring as cells grow we used the CellTiter 96® AQsolutions Solution cell proliferation assay (catalog number G3580), as recommended by the manufacturer (Promega). Briefly, cells were cultured in a 96-well plate (100 μl of culture medium/well) for 24 h and then exposed for 8 h to different concentrations of ouabain or to medium alone in control cultures. The growth of cells as reflected by metabolism of the tetrazolium salt, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, was determined using the CellTiter 96® AQsolutions One Solution reagent. Titer reagent (20 μl) was added directly to culture wells and incubated for 3 h, and then absorbance at 490 nm was measured with a 96-well plate reader to quantify the amount of formazan product, which reflects the number of cells in culture (27). Data were normalized to a signal obtained for control, untreated cultures.

BrdUrd Uptake—The Oncogene Research Products BrdUrd proliferation assay was used according to the accompanying manufacturer’s directions. Briefly, cells were cultured in a 96-well plate for 24 h and then exposed for 8 h to different concentrations of ouabain or to medium alone in control cultures. At the same time, BrdUrd was added to each well. The quantitation of BrdUrd incorporation into newly synthesized DNA of proliferating cells was estimated immunologically using specific monoclonal anti-BrdUrd and horseradish peroxidase-conjugated secondary antibodies.

**Analysis of Mitosis by Immunostaining with Antiphospho-Histone H3 Antibody**—Cells were cultured in 8-well Lab-Tech chamber slides (Nalge Nunc International) for 24 h and then incubated with vehicle or

**Fig. 1.** A, ouabain stimulates increased cell number in cultured rat renal epithelial cells. Cells were cultured in 96-well plates for 24 h and then exposed for 8 or 24 h to 1 nM ouabain or to medium alone in control cultures. Cell number was determined by dispersal of the attached cells by mild trypsinization and gentle shaking. Cell suspensions were diluted and counted by hemocytometer. (Bars indicate ±S.E.; *, p < 0.05, n = 4–5 per group.) B, ouabain (OU) stimulates PCT cells growth in a dose-dependent manner. Cells were cultured in 96-well plate for 24 h and then exposed for 8 h to different concentrations of ouabain or to medium alone in control cultures. The number of living cells was determined using the CellTiter 96® AQsolutions One Solution reagent (Promega). Reagent was added directly to culture wells and incubated for 3 h, and then absorbance at 490 nm was measured with a 96-well plate reader to quantify the amount of formazan product, which is proportional to the number of cells in culture (27, 36). Data are normalized to a signal obtained for control cultures. (Bars indicate ±S.E.; *, p < 0.05, **, p < 0.01; n = 6.) C, ouabain stimulates DNA synthesis in PCT cells. Cells were cultured in 96-well plate for 24 h and then exposed for 8 h to different concentrations of ouabain or to medium alone in control cultures. At the same time, BrdUrd was added to each well. The quantitation of BrdUrd incorporation into newly synthesized DNA of proliferating cells was estimated immunologically using specific monoclonal anti-BrdUrd and horseradish peroxidase-conjugated secondary antibodies.
with 1 nM ouabain for 15 min, 30 min, 1 h, 2 h, and 5 h. After fixation in 100% methanol at −20 °C for 45 min, the cells were washed three times for 5 min each with 0.1% Triton X-100 in phosphate-buffered saline followed by 1 h incubation with blocking buffer (3% bovine serum albumin/0.1% Triton-X100). Then they were incubated for 1 h with antibody to Ser-10-phospho-histone H3 (28), a marker of mitosis (Upstate Biotechnology, Lake Placid, NY), washed with 0.1% Triton-X100 in phosphate-buffered saline, incubated for 1 h with secondary antibody (Alexa 594 goat anti-rabbit IgG, Molecular Probes A-11037), stained with DAPI, and mounted with 150 μl of Antifade™ (Molecular Probes, S-7461). The slides were analyzed and imaged under fluorescence microscopy. Data are represented as the percentage of total cells per unit area positive for red staining. Each treatment was done in four chambers, and a minimum of 500 cells/chamber was counted.

Western Blots

PCTs were rinsed with phosphate-buffered saline and then lysed with 100 μl of lysis buffer (8 M urea, 40 mM Tris-base, 4% CHAPS). Cell lysates were scraped and then homogenized on ice for 1 min with a motorized microcentrifuge tube pestle. After centrifugation for 10 min at 6,000 × g and 4 °C, the supernatant was aliquoted and stored at −20 °C. Protein content was measured using the BCA protein assay (Pierce). The proteins (10 μg/well) were separated by SDS-PAGE electrophoresis on 8% gels. Proteins were blotted onto nitrocellulose membranes (Osmonics Inc.) followed by immunodetection with specific antibodies against phospho-ERK1/2 (New England Biolabs), ERK1/2 (Santa Cruz Biotechnology), or against the α1, α2, and α3 isoforms of Na,K-ATPase (provided by Dr. Thomas Pressley, Texas Tech University HSC) (29). Western blots were detected with the ECL-Plus detection reagent kit (Amersham Biosciences). In the pERK1/2 and total ERK1/2 experiments, autoradiograms were imaged by a flat-bed scanner, and band intensity was converted into densitometry units using NIH Image 1.68 software.

Ouabain-sensitive Ion Transport Activity Assay

Uptake of 86Rb+ was used as an indicator of Na+,K+-ATPase ion transport activity. Cells were cultured to confluence and trypsinized. These cells were grown to higher density than in the growth stimulation experiments to have a more abundant supply of cells, and therefore a greater transport signal, for the 86Rb+ uptake assay. Trypsinization was followed by centrifugation and rinsing with cell culture medium. The cells were then resuspended in cell culture medium and placed into an incubator for adaptation. The cell number per ml was calculated using a hemocytometer. After 30 min of adaptation, cells were aliquoted to 106 cells/sample tube and preincubated for 30 min with different concentrations of ouabain. Then, 86Rb+ uptake was initiated by the addition of 1 mCi of 86Rb+ as the chloride salt (Amersham Biosciences) to each sample, and the reaction was stopped after 30 min by placing tubes on ice with addition of 1 ml of ice-cold phosphate-buffered saline followed by rapid centrifugation at 4 °C and 2500 rpm. Supernatant was aspirated, and the washing procedure was repeated. Cell pellets were counted in γ counter (Cobra Quantum, Packard). Ouabain-insensitive 86Rb+ uptake was determined in the presence of 1 mM ouabain.

Statistical Analysis

The results are expressed as mean ± S.E. One-way analysis of variance with Dunnett’s test or Student-Newman-Keuls test was performed using GraphPad Prism version 3 for Windows (GraphPad Software). Values were considered to be statistically different when p < 0.05. Nonlinear regression analysis was performed to analyze the dose-response curves of inhibition of 86Rb+ uptake by ouabain in PCT and rat kidney.

RESULTS

Ouabain and Cell Proliferation in Cultured Rat Proximal Tubule Cells—To study the effect of ouabain on PCT proliferation, we assessed four parameters associated with cell proliferation: cell count, metabolic activity, DNA synthesis, and histone phosphorylation.

Cell Count—To determine the effects of 1 nM ouabain on cell number, cells were counted by hemocytometer before and after ouabain treatment. The results of this experiment are shown in Fig. 1A and indicate increased cell numbers at both 8 and 24 h in the treated cultures.

Metabolic Activity of Cells—We assessed metabolic activity of cells using an assay based on the reduction of the tetrasolium salt 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide by cellular metabolism. Bioreduction of tetrasolium salts probably reflects the integrated pyridine nucleotide dependent redox state of the cell (27). Therefore, the amount of formazan product is proportional to the metabolic activity of cells in culture and provides a widely used, although indirect, measurement of cell growth (30). The results of this experiment are shown in Fig. 1B. The number of cells, as reflected by increased formazan generation, markedly increased in cultures treated with ouabain at dose ranges 0.1–10 nM.

DNA Synthesis—During S phase, dividing cells undergo DNA synthesis and genomic replication. If the DNA precursor BrdUrd is added to the cell culture, cells undergoing division incorporate BrdUrd into their DNA. The incorporated BrdUrd can then be detected by a quantitative cellular enzyme immunoassay using monoclonal antibodies against BrdUrd (31). Again, ouabain at dose ranges 0.1–10 nM stimulated DNA synthesis in PCT (Fig. 1C).

Histone H3 Phosphorylation—Since a culture increases in

Fig. 2. Percent of mitotic cells increases in ouabain-treated cultures. A, images of cells co-stained for mitosis marker, phospho-histone-H3 (bottom panel, red), and DAPI (upper panel, blue). Arrows show cells in which the red mitotic marker (phosphohistone-H3) co-localizes with mitotic chromosome condensation (DAPI). As shown in B, PCT cells were incubated with vehicle or with 1 nM of ouabain as indicated. At various intervals after addition of ouabain, cells were fixed and stained with DAPI for nuclei and with phosphohistone-H3 antibody for identification of mitotic cells. The percent of mitotic cells was counted as described under “Experimental Procedures.” (Bars indicate ±S.E.; *, p < 0.01. **, p < 0.001.)
Ouabain Promotes Rat Renal Epithelial Cell Growth

PCT raises the question of which Na⁺,K⁺-ATPase isoforms are expressed in rat PCT cells. In other studies, we have shown that, in vitro, the rat proximal tubule expresses exclusively the α1 isoform of Na⁺,K⁺-ATPase that is highly resistant to inhibition by ouabain (37). Detection of the expression of the α2 or α3 isoforms in PCT might provide an explanation for the remarkable dose sensitivity of the observed growth response to ouabain. Here we confirm by immunoblotting analysis that the ouabain-resistant α1 isoform is detectable in these cultures as well as in blots made by loading an equal amount of brain protein lysate. As expected, Western blots from brain lysates also produced appropriately sized immunoreactive bands, indicating the presence of α2 and α3 isoforms. However, no α2 and α3 isoforms could be detected in the PCT cell line (Fig. 4A).

Dependence of Growth Stimulation on Inhibition of Na⁺,K⁺-ATPase Ion Transport—Although early findings suggested a role for Na⁺,K⁺-ATPase in the regulation of cell growth and expression of various genes, only in recent years has evidence accumulated that accompanies the onset of mitosis, and therefore, represents a convenient marker for dividing cells (32–34). We have shown here (Fig. 2), that the percent of mitotic cells determined by the abundance of cells staining positive for phosphorylated histone H3 markedly increased in 30 min after treatment with ouabain and declined thereafter. Taken together, these data provide four independent measures, indicating that ouabain stimulates cell proliferation in rat PCT at low doses corresponding to circulating concentrations of endogenous CS.

Intracellular Signaling Involved in Regulation of Ouabain-induced Proliferation—Regulatory mechanisms controlling proliferation include intracellular protein kinases that can transduce signals detected on the cell surface into changes in gene expression. Most prominent among the known signal transduction pathways that control these events are the mitogen-activated protein kinase (MAPK) cascades whose components are evolutionarily conserved in structure and organization (35). It was shown in cardiac myocytes and vascular smooth muscle cells that regulation of cell growth by ouabain includes signaling through the Raf/MEK/MAPK cascade that includes activation of p42/44 MAPK (ERK1/2) (9, 10). Ouabain-induced DNA synthesis and ERK1/2 phosphorylation were both inhibited by the MEK inhibitor (10). Here we show that ouabain stimulation of cell growth in PCT occurs concurrently with rapid activation of ERK1/2. The time course of ERK1/2 activation by 1 nM of ouabain is shown in Fig. 3A. Ouabain induced marked activation of ERK1/2 that reached a peak at 30 min and declined thereafter. Blockade of this pathway with 0.1 μM of the MEK inhibitor U0126 (36) prevents both ERK1/2 activation and the growth response to ouabain (Fig. 3, A and C). Total ERK1/2 levels were unaffected by ouabain and U0126 (Fig. 3B).

Sodium Pump Isoforms Expressed by Immortalized Rat Proximal Tubule Epithelial Cells—Our evidence that low nanomolar quantities of ouabain produce growth and cell division in rat PCT raises the question of Na⁺,K⁺-ATPase isoforms are expressed in rat PCT cells. In other studies, we have shown that, in vitro, the rat proximal tubule expresses exclusively the α1 isoform of Na⁺,K⁺-ATPase that is highly resistant to inhibition by ouabain (37). Detection of the expression of the α2 or α3 isoforms in PCT might provide an explanation for the remarkable dose sensitivity of the observed growth response to ouabain. Here we confirm by immunoblotting analysis that the ouabain-resistant α1 isoform is detectable in these cultures as well as in blots made by loading an equal amount of brain protein lysate. As expected, Western blots from brain lysates also produced appropriately sized immunoreactive bands, indicating the presence of α2 and α3 isoforms. However, no α2 and α3 isoforms could be detected in the PCT cell line (Fig. 4A).

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emerged that this plasma membrane enzyme can communicate with the nucleus through second messenger signaling pathways. Work suggesting signal transduction occurring by an action of ouabain of a subset of sodium pumps to bind and respond to low concentrations of ouabain. Much higher concentrations of ouabain were required to effectively inhibit the majority of $^{86}\text{Rb}^+$ uptake, as expected in rat renal epithelial cells (19–21). Further analysis of inhibition of $^{86}\text{Rb}^+$ uptake by ouabain in PCT was performed using nonlinear regression analysis of the inhibition curves obtained across a wide range of ouabain concentrations. The data was fitted to two models with one and two classes of binding sites, respectively. We have shown that the two-site model fits our data better than the one-site model (p < 0.0001). Results of this analysis are presented in Tables I and II. To confirm this unexpected observation, ouabain-induced inhibition of $^{86}\text{Rb}^+$ in freshly harvested rat PT fragments was examined. The dose-response curve obtained for PT is similar to that for PCT cultures and is shown in Fig. 4B. Nonlinear regression analysis again indicated that a two-site model fits the ion transport inhibition data better than a single-site model (Tables I and II).

**DISCUSSION**

The major finding of this study is that the cardiotonic steroid-glycoside, ouabain, which has been proposed to be an endogenous mammalian steroid product of the adrenal cortex (40–42), induces renal epithelial cell growth and proliferation via the mitogen-activated protein kinase cascade (ERK1/2) at concentrations corresponding to the reported circulating level of ouabain (22, 23). These effects occur in cells that express exclusively the rat Na$^+$/K$^+$/ATPase $\alpha 1$ isoform that is highly resistant to inhibition by ouabain. These findings provide new evidence that Na$^+$/K$^+$/ATPase may have a signal transduction function that may be additional to and possibly separate from its ion transport function and that may not require the erosion of transmembrane ion gradients sustained by Na$^+$/K$^+$/ATPase for its effect (11, 12, 38).

The highly conserved CS binding site is present on all $\alpha$ subunit catalytic isoforms of the ubiquitous membrane sodium pump, Na$^+$/K$^+$/ATPase. Isoforms of Na$^+$/K$^+$/ATPase are the only established targets for digitalis CS-glycosides, although all sodium pumps are not inhibited equally. Potency is both species- and isoform-dependent (19–21,43). In addition to CS inhibition of coupled sodium and potassium transport, there is considerable recent evidence suggesting that the sodium pump may act as a cell signaling receptor activated by CS binding and responding by coordination of intracellular signaling pathways. This has led to an emerging concept that the sodium pump has dual functions: an ion transport function and a signal transduction function stimulating cell growth and hypertrophy in which cardiotoxic steroids function as activating ligands at lower concentrations than required to inhibit ion transport (4, 11, 12). Indeed, our observation that the only catalytic $\alpha$ isoform of Na$^+$/K$^+$/ATPase expressed in cultured rat PCT is the highly ouabain-resistant $\alpha 1$ isoform raises the question of whether the growth responses observed can reasonably be expected to result from ouabain binding to this isoform. The existing rat renal Na$^+$/K$^+$/ATPase literature does not provide an entirely clear background to assess this question. In part, this is because a diverse range of assays and starting materials have been used that include ouabain inhibition of the ATP hydrolytic activity of partially purified enzyme (44–46) and ion transport measurements using radioactive cations to assess the ouabain sensitivity of freshly dissected renal tissue (47). These methods are not directly comparable with the experiments we have performed and often did not investigate effects at low ouabain concentrations. Our observations (Fig. 4 B and Table I) suggest a minor population of Na$^+$/K$^+$/ATPase units in rat proximal tubule cells that retain high sensitivity to

![Image](61x362 to 303x737)
Ouabain inhibition so that 10–15% of the maximum ion transport is inhibited by concentrations of ouabain ranging from 10^{-10} to 10^{-6} M. Statistical analysis of our extensively replicated experiments provide support for the existence of two classes of ouabain-inhibitable cation transport activity. However, the experimental methodology used to measure ion transport lacks precision and requires use of a single pool of cells to perform simultaneous examination across replicates covering a large range of ouabain concentrations. Additional experimental methods will be required to provide irrevocable evidence of two classes of ouabain inhibition of ion transport suggested by curve fitting analysis of our data.

One possible source of heterogeneity among rat renal epithelial Na^+-K^+-ATPase subunits that all contain the ouabain-resistant α1 isoform may derive from at least two members of the FXYD family of proteins that have recently been identified as being expressed in renal epithelial cells. These proteins multimerize into Na^+-K^+-ATPase and modulate aspects of the function of the ion transporter (48). Since one of these, the γ subunit of Na^+-K^+-ATPase, was originally identified as forming part of the ouabain binding site (49), it is possible that differential participation of one or more of the FXYD proteins or their variant isoforms contributes to the creation of a subset of Na^+-K^+-ATPase with increased ouabain sensitivity. Others have reported the presence of two isoforms of Na^+-K^+-ATPase in the rat nephron that differ in sensitivity of their ATP hydrolytic activity to inhibition by ouabain, although the molecular basis of this sensitivity has not been uncovered (50). Further experiments will be required to investigate this speculation.

The effective stimulation of growth by ouabain in cultured, ouabain-sensitive canine vascular myocytes was observed at concentrations from 10^{-8} to 10^{-6} M that are expected to cause little or no change in intracellular ion concentrations (10). In contrast, effects of ouabain on growth and proliferation in cultured rat cardiac myocytes occur at higher doses (10^{-4} to 10^{-5} M), which may reflect an action on ouabain-resistant isoforms of Na^+-K^+-ATPase that are expressed in rat myocytes (9, 38, 51, 52). In the present work, we have shown that nanomolar concentrations of ouabain stimulate the growth of rat renal proximal tubule-derived epithelial cells. Therefore, responses in rat kidney occur at concentrations that are 4–5 orders of magnitude below the range stimulating growth in rat cardiac myocytes but are similar to those stimulating growth of canine vascular myocytes.

The present data provide new evidence of important biological effects of CS in rat kidney tissue at physiological concentrations. If these responses are biologically relevant, their relevance may arise through growth-promoting effects induced by low levels of circulating ECS that may play an important role in signaling mechanisms driving renal growth globally or may enhance growth responses to local injury. Dramatic compensatory renal growth occurs in the remaining kidney of uninephrectomized subjects. Such dramatic nephron loss and the acutely reduced renal filtration associated with it may lead to short term alterations, such as volume expansion, that stimulate production of ECS. In addition to the dramatic growth responses evinced by remaining renal tissue after uninephrectomy, parts of the kidney may be subject to less extensive injury from metabolic (53) or osmotic stresses (54). A balance between apoptosis and compensatory growth of surviving adjacent tissue are normal elements in adaptation to such renal injury, and ECS may modulate the growth response occurring in response to localized renal injury.

The issue of how ouabain produces growth and cell division in a rat cell culture preparation containing only the highly ouabain-resistant rat α1 isoform of Na^+-K^+-ATPase remains to be fully resolved. Our findings indicate that nanomolar concentrations of ouabain are capable of stimulating strong and rapid activation of MAP kinase signaling pathways accompanied by mitotic cell growth. Similar observations have been made in rat and canine myocytes, and there is evidence that ouabain binding to Na^+-K^+-ATPase may confer on the enzyme a signal transduction function (9–12, 38, 51, 52). The involvement of Na^+-K^+-ATPase as a signal transducer of the ouabain-induced growth response in rat renal epithelial cells or of an alternative signaling mechanism, perhaps with parallels to the aldosterone mediated activation of epidermal growth factor receptor (55), remain to be more fully investigated.

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