Low Doses of Ouabain Protect from Serum Deprivation–Triggered Apoptosis and Stimulate Kidney Cell Proliferation *via* Activation of NF-κB

Juan Li, Sergey Zelenin, Anita Aperia, and Oleg Aizman

Karolinska Institutet, Department of Woman and Child Health, Astrid Lindgren Children's Hospital, Stockholm, Sweden

It now generally is agreed that Na,K-ATPase, in addition to its role in the maintenance of Na^+ and K^+ gradients across the cell membrane, plays a role in communicating information from the extracellular environment to intracellular signaling pathways. It was reported recently that interaction between ouabain-bound Na,K-ATPase and the 1,4,5-trisphosphate receptor (IP_3R) triggers slow calcium oscillations and activation of NF-κB. Here it is demonstrated that this signaling pathway can serve to prevent cell death and promote cell growth. Rat renal proximal tubular cells in primary culture first were grown in the presence of 10% serum and then exposed to 0.2% serum for 24 h to induce apoptosis. Serum starvation increased the apoptotic index from 1.21 ± 0.26 to 14.01 ± 1.17%. Ouabain in concentrations that did not inhibit Na,K-ATPase activity (1 to 10 nM) completely abolished the apoptotic effect of serum starvation. Ouabain protection from apoptosis was not observed when release of calcium from intracellular stores *via* the IP_3R was prevented. It was shown that the NH_2 terminal tail of the Na,K-ATPase α subunit plays a key role in ouabain-triggered calcium oscillations. It was found that ouabain did not protect from apoptosis in serum-deprived cells that expressed a mutant Na,K-ATPase α subunit with deletion of the NH_2 terminal tail. Ouabain exposure (10 nM for 24 h) significantly increased translocation of NF-κB from cytoplasm to nucleus. Helenalin, an inhibitor of NF-κB, abolished the antiapoptotic effect of ouabain. Ouabain (0.1 to 10 nM) also was found to stimulate proliferation and increase the viability of kidney cells. These effects were abolished when release of calcium *via* the IP_3R was prevented.

J Am Soc Nephrol 17: 1848–1857, 2006. doi: 10.1681/ASN.2005080894

Na,K-ATPase is a ubiquitous integral membrane protein that maintains the Na^+ and K^+ gradients across the plasma membrane of animal cells (1). Recent studies suggest that Na,K-ATPase, in addition to being the major determinant of intracellular ion composition, may act as a signal transducer (2–5).

Ouabain, a steroid derivative, is a specific ligand of Na,K-ATPase that dose-dependently inhibits the activity of Na,K-ATPase (6). Noninhibitory doses of ouabain activate the signaling function of Na,K-ATPase. The signaling cascade that is triggered by Na,K-ATPase is complex, and several different pathways have been implicated (7–9). We recently reported that ouabain can trigger an interaction between Na,K-ATPase and the inositol 1,4,5-trisphosphate (IP_3) receptor, which results in low-frequency Ca^{2+} oscillations and activation of the transcription factor NF-κB. This phenomenon was observed in rat renal proximal tubular cells in primary culture and in a kidney cell line (10).

A number of recent studies have demonstrated that ouabain in noninhibitory doses can promote cell proliferation (11–13). Because NF-κB has an antiapoptotic effect (14), we speculated that ouabain also might act to protect from apoptosis. Here we demonstrate that noninhibitory doses of ouabain can protect rat renal proximal tubular cells from serum deprivation–triggered apoptosis, albeit not from pharmacologically staurosporine–triggered apoptosis. We show that protection from serum-triggered apoptosis depends on NF-κB activation. Normal kidney development is critically dependent on a well-controlled balance between cell proliferation and apoptosis (15). Accumulating evidence suggests that ouabain is a mammalian hormone, produced in the adrenal glands and the hypothalamus (16,17). Therefore, ouabain might play an important role as a modulator of kidney growth and development.

Materials and Methods

Cells

Rat proximal tubule (RPT) cells were prepared from kidneys of 20-d-old male Sprague-Dawley rats as described previously (18). The kidneys were removed and placed in cold PBS. The cortical layers were dissected and placed in a PBS solution that contained collagenase (7 mg in 20 ml of PBS) for 15 min at 37°C and triturated gently using fire-polished Pasteur pipette. The reaction was stopped by washing the cells twice in PBS that contained 1% trypsin inhibitor. After washing, the equal volumes of cell suspension were plated on 60-mm Petri dishes that contained glass coverslips. Cells were cultured in supplemented DMEM (20 mM HEPES, 24 mM $NaHCO_3$, 10 μg/ml penicillin, 10

Received August 31, 2005. Accepted April 10, 2006.

Published online ahead of print. Publication date available at www.jasn.org.

Address correspondence to: Dr. Oleg Aizman, Karolinska Institutet, Astrid Lindgren Children's Hospital, Q2:09, SE-171 76 Stockholm, Sweden. Phone: +46-08-51777375; Fax: +46-08-51777328; E-mail: oleg.aizman@kbh.ki.se.

ISSN: 1046-6673/1707-1848

μg/ml streptomycin, and 10% FBS) on glass coverslips for 24 h in 5% CO_2 at 37°C. In the serum deprivation protocols, cells were incubated with 0.2% FBS for 24 h before the experiment. In some protocols, a kidney cell line (COS-7), derived from fetal monkey kidney, was used.

Expression Plasmids and Cell Transfection

A full-length rat Na,K-ATPase α1-subunit (accession no. NP_036636.1) was constructed and cloned into pEGFP-C2 vector (α1-GFP). This construct was modified to obtain a mutant cDNA that encodes rat Na,K-ATPase α1-subunit with truncation of 38 NH_2-terminal residues (α1M38-GFP). These constructs were sequenced to confirm that GFP was added in-frame at the 5′ end of the Na,K-ATPase α1-subunit. The region of truncation of the NH_2-terminus was decided on the basis of the structure/functional analysis reported elsewhere (19). RPT cells that were grown on 12-mm glass coverslips were transiently transfected on culture day 2 with either α1-GFP or α1M38-GFP plasmids that contained 0.2 μg/well DNA construct using Effectene (Qiagen, Hilden, Germany) according to the manufacturer's specifications. Cells were examined with a Leica TCP SP inverted confocal microscope 24 h after transfection. GFP fluorescence was excited at 488 nm and detected with a 510- to 530-nm band pass filter. After immunostaining with GFP antibody, approximately 50% of cells were found to be GFP positive.

Measurements of Cell Proliferation

RPT cells were cultured on 60-mm culture dishes for 24 h. On culture day 2, when cells were approximately 50% confluent, the culture medium was changed to DMEM that contained 5% FBS. [^{3}H]thymidine 1 μCi/well was added, and cells were incubated for an additional 24 h in DMEM 5% FBS in the absence or presence of various ouabain concentrations, washed twice with PBS, and then lysed in 1 M NaOH. The cell lysate was used to measure radioactivity by scintillation counting (LKB; Wallac, Turku, Finland). Protein content was determined using a kit from Bio-Rad Laboratories (Hercules, CA) following the manufacturer's instructions. All experiments were performed in triplicate.

COS-7 cell proliferation was determined as described above with some modifications. COS-7 cells (10,000 cells/well) were cultured in 96-well flat-bottom tissue culture plates in DMEM supplemented with 10% FBS for 24 h. The medium was changed to DMEM with 1% FBS for an additional 24 h with or without indicated ouabain concentrations. Cells were incubated with 2.5 μCi/well [^{3}H]thymidine during the last 5 h of culture. Cells were harvested by using automatic harvester, and [^{3}H]thymidine incorporation was measured using liquid scintillation counting.

WST-1 assay (Chemicon International, Temecula, CA) measures the increase in metabolic activity and is an index of expansion in the number of viable cells. Cells (10,000 cells/well) were cultured in a 96-well plate (100 μl/well culture medium) for 24 h and exposed for 24 h to different ouabain concentrations or to medium alone (control). WST-1 reagent (20 μl) was added directly to culture wells and incubated for 1 h. The absorbance was measured at 450 nm with a 96-well plate reader. The optical density values were normalized to baseline values and presented as percentage of control.

Trypan blue dye exclusion test was used to evaluate cell viability. After treatment with indicated ouabain concentrations for 24 h, cells were harvested and the number of dead cells was determined by microscopic examination.

Detection and Quantification of Apoptotic Cells

For determination of apoptotic index, we used ApopTag Red In Situ Apoptosis Detection kit (Chemicon International). Cells were plated on 24-well plates that contained 12-mm glass coverslips. For achieving approximately the same number of cells, equal volumes of cell suspension were added to each well. On culture day 2, when cells had achieved approximately 50% confluence, the medium was changed and cells were cultured with 10 or 0.2% FBS for 24 h and treated with various ouabain concentrations. The terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling assay was conducted according to the manufacturer's instructions. Nuclei were counterstained with 4′,6-diamidino-2-phenylindole (DAPI; 1.5 μg/ml) added to PBS at last wash. Cells were mounted in Immu-Mount (Thermo Shandon, Midland, Canada). Only cells in primary culture were used for apoptosis studies, because it was found in pilot studies that serum deprivation for 48 h had only a marginal effect on apoptosis in cell lines.

Cells were observed with Leica TCS SP inverted confocal scanning laser microscope using ×40/1.4 N.A. oil-immersion objective. ApopTag Red fluorescence was excited at 543 nm and detected with a 560- to 620-nm band pass filter. Images were recorded using Leica software. DAPI-stained cells were viewed with an ultraviolet light source. Cells were considered apoptotic when they exhibited DAPI and ApopTag Red staining and characteristic apoptotic morphology (cell shrinkage, pyknotic nuclei, and apoptotic bodies).

Apoptotic index (AI; the number of apoptotic cells/total number of cells counted × 100%) was determined by counting the number of ApopTag-positive cells over the total number of cells, determined with DAPI staining. In each preparation, eight to 10 randomly selected areas were examined, and in each area, between 100 and 200 DAPI-stained cells were counted.

NF-κB Activity

NF-κB translocation to nucleus was used as an index of NF-κB activation and studied with immunocytochemistry and subcellular fractionation followed by Western blot. Immunocytochemistry was performed as described previously (10). NF-κB was probed with rabbit anti-human polyclonal NF-κB p65 antibody (1:200; (Santa Cruz Biotechnology, Santa Cruz, CA); secondary antibody was anti-rabbit Alexa 546 (1:3000). Green fluorescence protein was probed with mouse polyclonal anti-GFP antibody (1:200; Institute for Biodiagnostics, River Falls, WI) and goat anti-mouse Alexa 546 (1:6000; Molecular Probes, Eugene, OR) as a secondary antibody. The immunolabeled cells were observed with a Leica TCS SP inverted confocal scanning laser microscope using ×40/1.4 N.A. objective. Preparations in which the primary antibody was omitted from the staining protocol were used as negative controls. NF-κB translocation to nucleus was calculated semiquantitatively as the ratio between the mean fluorescence signal intensity in a given area in the nucleus and cytosol (10). In each preparation, six to seven randomly selected images that contained approximately 100 cells were observed in a confocal microscope, and all cells in these images were analyzed. Calculations were performed using software from Scion Image (Scion Corp., Frederick, MD) by a person who was blind to the protocol.

Western blot was performed as described previously (10). NF-κB was probed with rabbit anti-human polyclonal NF-κB p65 (1:2000; Santa Cruz Biotechnology) in nuclear extracts. IκB was probed with a rabbit anti-human antibody (1:2000; Santa Cruz Biotechnology) in cytosolic extracts.

Subcellular Fractionation

RPT cells were washed with ice-cold PBS, then 200 μl of Buffer A (10 mM HEPES [pH 7.9], 10 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, and a cocktail of protease inhibitors and 2% NP 40) was added. The mixture was vortexed and incubated on ice for 10 min and centrifuged at 500 ×

g for 5 min; the supernatant was cytosolic protein. The pellet was resuspended in 65 μl of buffer B (50 mM HEPES, 10% glycerol, 300 mM NaCl, 50 mM KCl, and a cocktail of protease inhibitors). The mixture was vortexed and incubated on ice for 30 min and centrifuged at 13,000 × *g* for 10 min; the supernatant was nuclear protein. The protein content was measured using BioRad protein assay reagent. When the extracts were not used immediately, they were stored at −80°C.

Rubidium Uptake

The dose-dependent effect of ouabain on Na,K-ATPase activity was determined by measuring ouabain-sensitive $^{86}Rb^+$ uptake as described previously (20). Na,K-ATPase–dependent $^{86}Rb^+$ uptake was calculated as the difference between $^{86}Rb^+$ uptake in the absence of ouabain (total uptake) and in the presence of 2 mM ouabain (ouabain-insensitive uptake).

Chemicals

All chemicals were obtained from Sigma (St. Louis, MO) unless otherwise stated. Chemicals were used in the following concentrations in all experiments: Staurosporine 0.5 μM, ouabain 0.1 nM to 100 μM, cyclopiazonic acid 0.5 μM, 2-aminoethoxydiphenyl borate (2-APB) 5 μM, helenalin 1 to 2 μM (Biomol International, Exeter, UK).

Statistical Analyses

Statistical analysis was performed with STATISTICA 6.0 software (Statsoft, Tulsa, OK). In some groups, *t* test was considered appropriate, but in the majority of cases, one-way ANOVA followed by the *post hoc* test was used to test the significance of differences. The values are expressed as means ± SEM. $P < 0.05$ was considered statistically significant.

Results

Effect of Low Ouabain Concentrations on Na,K-ATPase Activity

We first made a dose-response study of the effect of ouabain on Na,K-ATPase activity in RPT cells to ensure that noninhibitory and nontoxic doses of ouabain would be used in later experiments. Rat renal Na,K-ATPase has a low ouabain sensitivity, and millimolar concentrations of ouabain are required for full inhibition of the enzyme. Cellular uptake of $^{86}Rb^+$ was used as an index of Na,K-ATPase activity. RPT cells were pretreated with indicated ouabain concentrations for 30 min. We found no detectable changes in $^{86}Rb^+$ uptake between control cells and cells that were pretreated with 1 nM to 1 μM of ouabain (Figure 1C, insert). Higher concentrations of ouabain dose-dependently decreased $^{86}Rb^+$ uptake (Figure 1C).

Noninhibitory Doses of Ouabain Protects from Serum Deprivation–Triggered but not from Staurosporine-Triggered Apoptosis

The effect of ouabain on apoptosis first was studied in RPT cells that were grown in medium supplemented with 10 or 0.2% FBS, as described in Materials and Methods. Cells that were grown in 10% FBS had a low incidence of apoptosis (AI 1.21 ± 0.26%). Serum deprivation (0.2% FBS for 24 h) caused a dramatic increase in the number of apoptotic cells (AI 14.01 ± 1.17%). Representative confocal images of apoptotic cells that were identified with terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling staining are shown in Figure 1. Ouabain in nanomolar concentrations completely abolishes the apoptotic effect of serum deprivation (AI in the presence of 1 nM ouabain 3.75 ± 0.36%; AI in the presence of 10 nM ouabain 1.57 ± 0.27%; Figure 1, A and B). This effect was not unique in RPT cells, because it also was observed in primary culture of rat astrocytes (data not shown). To investigate whether the protective effect of ouabain was specific for serum deprivation–triggered apoptosis, we incubated RPT cells with 0.5 μM staurosporine, a broad-spectrum inhibitor of protein kinases and potent apoptosis inducer, for 24 h in the presence or absence of ouabain. Staurosporine caused a significant increase in the number of apoptotic cells (AI 17.53 ± 2.77%). Ouabain had no protective effect on staurosporine-triggered apoptosis (AI in the presence of 1 nM ouabain 24.30 ± 5.20%; AI in the presence of 10 nM ouabain 21.50 ± 4.50%; Figure 1, A and B).

Ouabain-Mediated Protection from Serum Deprivation–Triggered Apoptosis Is Ca^{2+} Dependent

We have shown that depletion of intracellular Ca^{2+} stores in the endoplasmic reticulum (ER) abolishes ouabain-induced Ca^{2+} signaling (10). In the studies shown in Figure 2, RPT cells were pretreated for 24 h with a sarco-ER Ca^{2+}-ATPase inhibitor, cyclopiazonic acid (CPA; 0.5 μM), to deplete the intracellular stores of calcium. In cells that were pretreated for 24 h with CPA, the Ca^{2+} concentration in ER indeed was reduced (supplementary data). This treatment completely abolished the antiapoptotic effect of ouabain (AI in the presence of 1 nM ouabain 17.42 ± 1.03%; AI in the presence of 10 nM ouabain 18.91 ± 3.07%; Figure 2A). CPA alone, in the concentration of 0.5 μM, did not have any effect on AI in serum-deprived RPT cells (Figure 2A). Regulated Ca^{2+} release from intracellular ER Ca^{2+} stores occurs *via* IP_3 receptors (IP_3R) or *via* ryanodine receptors. IP_3R are expressed abundantly in RPT cells, whereas ryanodine receptors do not seem to be of any functional importance in these cells (10). The membrane-permeable substance 2-APB is an inhibitor of IP_3R-evoked Ca^{2+} release as well as a blocker of store-operated calcium-channels. IP_3R has been reported to be blocked completely by 1 to 20 μM 2-APB, whereas store-operated calcium-channel is inhibited by 50 to 100 μM 2-APB (21,22). Exposure of RPT cells to 5 μM 2-APB completely prevented the antiapoptotic effect of ouabain in serum-deprived cells (AI in the presence of 1 nM ouabain 13.89 ± 3.67%; AI in the presence of 10 nM ouabain 13.60 ± 5.17%; Figure 2B). 2-APB alone did not have any significant effect on serum deprivation–triggered apoptosis in RPT cells (Figure 2B). Taken together, these results strongly indicate that ouabain protection from serum deprivation–triggered apoptosis depends on Ca^{2+} release from the intracellular stores.

Partial Truncation of Na,K-ATPase α1 N-terminus Abolishes Ouabain Protection from Serum Deprivation–Triggered Apoptosis

In cells that express a truncated form of Na,K-ATPase α1, where the first 32 amino acid residues from the N-terminus of the catalytic α1 subunit of sodium pump (Na,K-ATPase α1 M32) were deleted, ouabain no longer will trigger Ca^{2+} oscillations and activation of NF-κB (23). Because this finding sug-

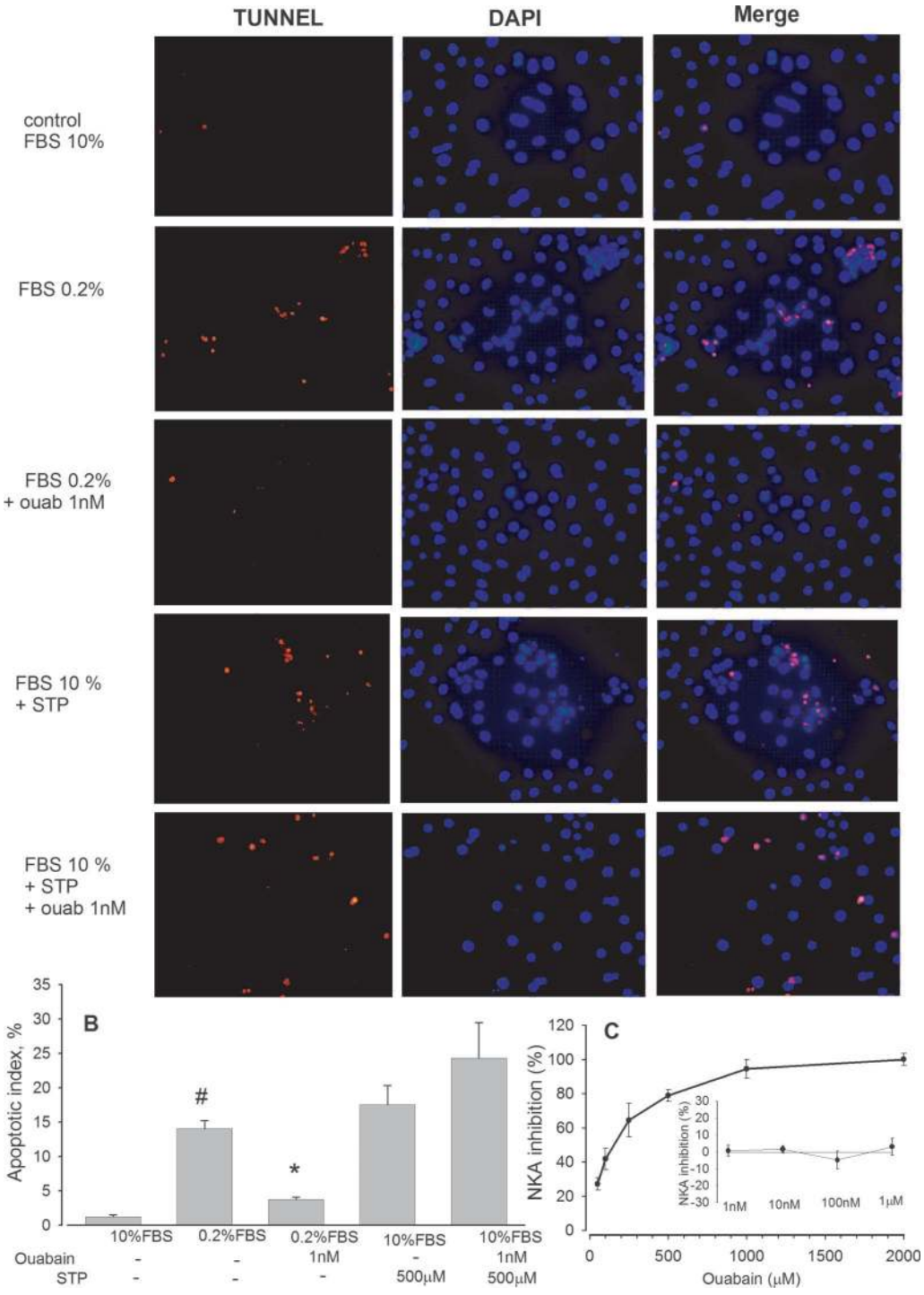

Figure 1. Ouabain protects from serum deprivation–triggered apoptosis. (A) Representative fluorescence images of rat proximal tubule (RPT) cells that were stained by terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay to detect apoptotic cells and by 4′,6-diamidino-2-phenylindole stained to detect all cells and merge images. Cells were considered apoptotic when they exhibited typical ApopTag Red staining and characteristic apoptotic morphology (supplementary data). Apoptotic index (AI), the number of apoptotic cells/total number of cells counted × 100%, was determined by analyzing five to seven randomly selected areas with 100 to 200 cells in each area from six independent experiments. (B) Ouabain in nanomolar concentrations completely reversed serum deprivation–triggered apoptosis. Serum deprivation increases the AI from 1.21 ± 0.26 to 14.01 ± 1.17%. Values are mean ± SEM; $n = 6$. $^{\#}P < 0.001$, cells that were treated with 10 *versus* 0.2% FBS; $^{*}P < 0.001$ cells that were treated with 0.2 *versus* 0.2% FBS + ouabain. Ouabain had no effect on staurosporine-triggered apoptosis. AI mean ± SEM, $n = 3$. $P > 0.05$. One-way ANOVA followed by the *post hoc* test were used. (C) Dose-dependent inhibition of Na,K-ATPase activity by ouabain measured as ouabain-sensitive $^{86}Rb^{+}$ uptake in RPT cells (mean ± SEM; $n = 5$). Ouabain in concentrations of 1 nM to 1 μM did not inhibit ouabain-sensitive $^{86}Rb^{+}$ uptake in RPT cells (insert). Values are mean ± SEM; $n = 4$.

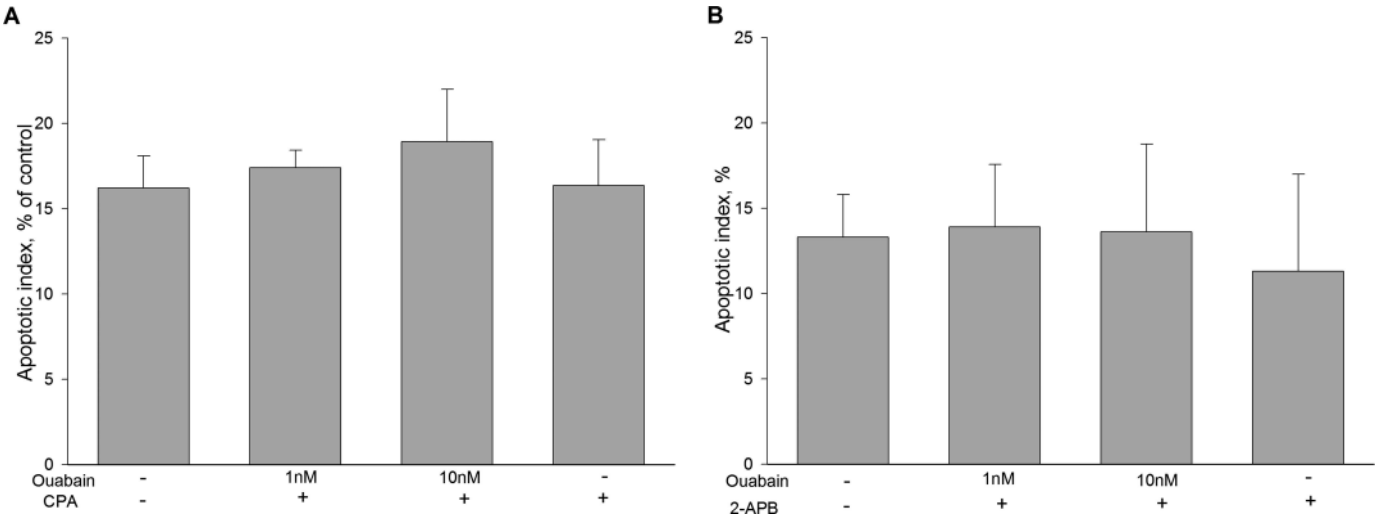

Figure 2. Ouabain protection from serum deprivation–triggered apoptosis is Ca^{2+} dependent. (A) Inhibition of the sarco–endoplasmic reticulum Ca^{2+}-ATPase (SERCA) pump by cyclopiazonic acid (CPA; 0.5 μM) abolished the antiapoptotic effect of ouabain. AI (mean ± SEM) was calculated from five to seven randomly selected areas with 100 to 200 cells in each area from three independent experiments. One-way ANOVA followed by the *post hoc* test were used. $P > 0.05$, no significance. (B) Inhibition of 1,4,5-trisphosphate receptor (IP_3R) by 2-aminoethoxydiphenyl borate (2-APB; 5 μM) prevented the antiapoptotic effect of ouabain on serum deprivation–triggered apoptosis. AI mean ± SEM; $n = 7$. One-way ANOVA followed by the *post hoc* test were used. $P > 0.05$, no significance.

gested that first 32 amino acids of the NH_2 terminus tail of the Na,K-ATPase catalytic α1-subunit play a key role in Na,K-ATPase–IP_3R interaction, we next studied the effect of ouabain on serum-deprived RPT cells that were transfected with Na,K-ATPase α1M32 or with wild-type rat α1 Na,K-ATPase. Both α1M32 and wild-type α1 had been fused to GFP to allow identification of transfected cells. A number of studies have shown that removal of the N-terminus tail of the α1 subunit does not alter overall Na,K-ATPase activity (19,24–26). Cells that expressed wild-type or truncated α1 Na,K-ATPase had similarly low AI in 10% of FBS ($AI_{GFP\text{-}NKA\alpha1WT}$ 1.56 ± 0.64%; $AI_{GFP\text{-}NKA\alpha1M32}$ 2.84 ± 0.79%; Figure 3C). After 24 h of serum deprivation, AI was increased to the same extent in cells that expressed GFP-Na,K-ATPaseα1WT and GFP-Na,K-ATPaseα1M32 ($AI_{GFP\text{-}NKA\alpha1WT}$ 17.54 ± 2.23%; $AI_{GFP\text{-}NKA\alpha1M32}$ 18.32 ± 1.51%; Figure 3C). The antiapoptotic effect of ouabain was preserved in cells that expressed wild-type GFP-Na,K-ATPaseα1WT (AI in the presence of 1 nM ouabain 2.14 ± 0.83%; AI in the presence of 10 nM ouabain 1.41 ± 0.69%; Figure 3C). In contrast, in cells that expressed truncated GFP-Na,K-ATPaseα1M32, ouabain had no significant protective effect on serum deprivation–triggered apoptosis (AI in the presence of 1 nM ouabain 16.56 ± 2.43%; AI in the presence of 10 nM ouabain 16.15 ± 3.39%; Figure 3, B and C).

Ouabain Activates NF-κB

We showed previously that 250 μM ouabain triggers a translocation of NF-κB to the nucleus within 30 min and that this effect depends on the interaction between Na,K-ATPase and IP_3R (10,23). Under nonstimulated conditions, NF-κB is located predominantly in the cytoplasm in association with the inhibitory protein IκB. Upon activation, IκB dissociates from this complex and NF-κB is translocated to the nucleus (27). In control cells, the NF-κB immunosignal was preferentially detected in cytoplasm (Figure 4, A and B). After 24 h of exposure to 1 or 10 nM ouabain, the nuclear/cytosolic ratio of the immunosignal was significantly increased (Figure 4, A and B).

In another protocol, subcellular fractionation was performed on cells that were exposed to 0.2% FBS alone or to 0.2% FBS and 10 nM of ouabain. Immunoblotting of nuclear and cytosolic fractions showed a significant increase in NF-κB and a decrease in cytosolic IκB (Figure 4, C and D). Short-term exposure to a low ouabain concentration (10 nM) failed to activate NF-κB (Figure 4E). The effect was detected initially after 2 h and, maximum was achieved after 12 h.

Inhibition of NF-κB Prevents Antiapoptotic Effect of Ouabain

To examine the role of NF-κB activation for ouabain protection from serum deprivation–triggered apoptosis, we preincubated RPT cells with helenalin, an NF-κB inhibitor that exert its effect by inhibiting the DNA-binding activity of NF-κB (28). Helenalin (1 μM) abolished the antiapoptotic effect of ouabain (AI in the presence of 1 nM ouabain 20.20 ± 1.26%; AI in the presence of 10 nM ouabain 15.83 ± 1.03%; Figure 4, F and G). Helenalin alone, in this low concentration, did not have any significant effect on serum deprivation– triggered apoptosis (Figure 4, F and G).

Ouabain Stimulates Cell Proliferation

To study the rate of DNA synthesis, we determined the rate of [^{3}H]thymidine incorporation. Ouabain 0.1 to 10 nM significantly stimulated RPT cell proliferation (125.5 ± 5.5% for 0.1 nM, 135.5 ± 9.6% for 1 nM, and 119.9 ± 9.1% for 10 nM ouabain; Figure 5A). Higher doses of ouabain (100 nM to 250 μM) had either no effect or an inhibitory effect (data not shown). Ouabain-mediated stimulation of cell proliferation was

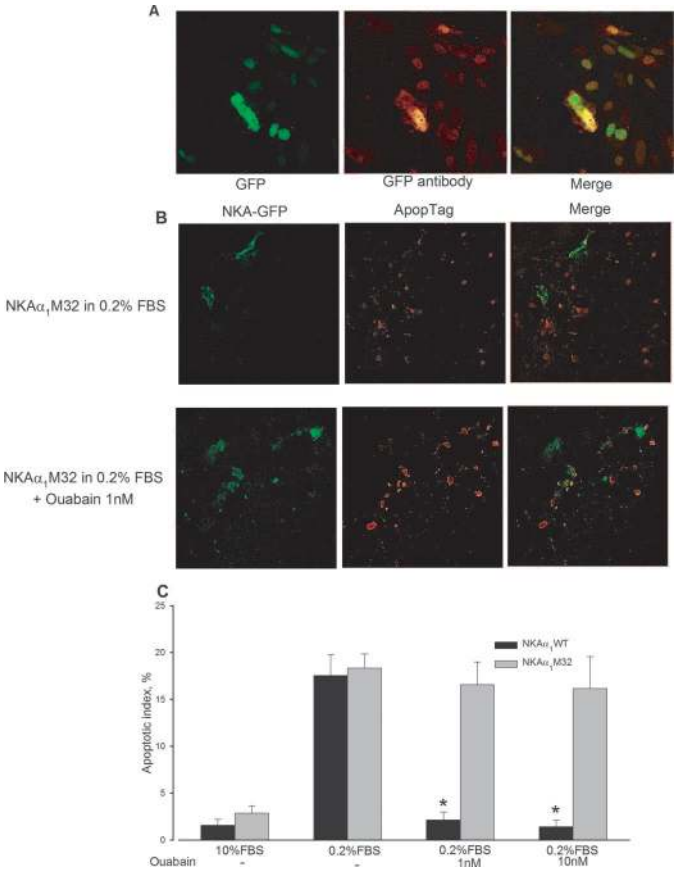

Figure 3. Partial truncation of Na,K-ATPase α1 N-terminus abolishes ouabain protection from serum deprivation–triggered apoptosis. (A) Representative confocal image of RPT cells that were transfected with GFP-tagged Na,K-ATPase α1 (green, left) and stained with anti-GFP antibody (middle, red). Merged image is on the right. (B) Representative confocal images of RPT cells that were transfected with GFP-tagged Na,K-ATPase, where the N-terminus tail was partially truncated ($NKA_{\alpha 1}M32$). Cells were treated as indicated. RPT cells that expressed GFP-tagged $NKA_{\alpha 1}M32$ were identified by GFP fluorescence (green, left) and stained using TUNEL assay for apoptosis (middle, red). Merged image is on the right. A membrane signal is difficult to distinguish because the images were recorded with an open pinhole to optimize detection of apoptotic cells. (C) Ouabain failed to protect from serum deprivation–triggered apoptosis in RPT cells that expressed GFP-tagged Na,K-ATPase, where the N-terminus tail was partially truncated ($NKA_{\alpha 1}M32$), but not in cells that expressed wild-type GFP-tagged Na,K-ATPase ($NKA_{\alpha 1}WT$). ▨, cells that expressed $NKA_{\alpha 1}WT$; ■, cells that expressed $NKA_{\alpha 1}M32$. AI mean ± SEM; $n = 4$. *$P < 0.001$, one-way ANOVA followed by the *post hoc* test.

Ca^{2+} dependent. Pretreatment of RPT cells with 5 μM 2-APB or 0.5 μM CPA for 24 h completely abolished the stimulatory effect of ouabain on cell proliferation (Figure 5, C and D). Using Trypan blue exclusion assay, we found that 24 h of exposure to ouabain (0.1 nM to 250 μM) did not have any toxic effect on cell viability (data not shown). We also determined the effect of ouabain on the rate of metabolic activity measured as an increase in the overall activity of the mitochondrial dehydrogenases. Ouabain 0.1 to 10 nM significantly stimulated RPT cell metabolic activity (143.4 ± 22.5% for 0.1 nM, 157.1 ± 9.0% for 1 nM, and 117.9 ± 9.0% for 10 nM ouabain; Figure 5B). These effects of low-dose ouabain were not unique for rat kidney cells because it was also observed in COS-7 monkey kidney cells (121.4 ± 6.1% for 0.1 nM, 118.7 ± 5.5% for 1 nM, and 128.7 ± 8.6% for 10 nM ouabain; Figure 5E).

Discussion

We recently reported that ouabain can act as an inducer of regular, low-frequency intracellular calcium oscillations that elicit activation of the transcription factor NF-κB (10). These studies were performed on rat RPT cells in primary culture and on COS cells, a cell line derived from fetal monkeys. Here we demonstrate that protection from apoptosis and enhanced cell proliferation are downstream effects of this signaling pathway. Normal kidney development is critically dependent on a well-controlled balance between cell proliferation and apoptosis. Aberrations in these processes lead to a variety of kidney malformations (15). Therefore, our findings that ouabain in nanomolar concentrations regulates kidney cell proliferation and apoptosis have implications for the understanding of normal kidney development. Because ouabain is a potent inhibitor of Na,K-ATPase activity, it can be argued that effects of ouabain are mediated by inhibition of Na,K-ATPase activity. The concentrations of ouabain that in this study induced the antiapoptotic effect and stimulated cell proliferation, however, did not cause any measurable inhibition of Na,K-ATPase activity.

Our results indicate that the antiapoptotic effect of ouabain depends on IP_3R-mediated Ca^{2+} release from intracellular stores and subsequently activation of NF-κB. Depletion of intracellular ER Ca^{2+} stores and inhibition of the IP_3R abolished the ouabain-induced slow Ca^{2+} oscillations and prevented the antiapoptotic effect of ouabain. Calcium oscillations with a periodicity in the range of 1 min to several minutes will activate NF-κB (29), a pleiotropic regulator of many genes that are involved in regulation of cell growth, differentiation, and apoptosis. NF-κB is known to inhibit apoptosis through induction of antiapoptotic proteins and/or suppression of proapoptotic genes (14). Constitutive NF-κB activation, observed in many malignant tumors, protects the cells from apoptotic stimuli (30,31). Our results indicate that NF-κB activation is required for protection against serum deprivation–triggered apoptosis. By using two different methodologic approaches, we demonstrate that nanomolar concentrations of ouabain activate NF-κB and that this activation is essential for the antiapoptotic effect of ouabain. The selective NF-κB inhibitor helenalin prevented ouabain-mediated NF-κB activation and completely abolished the antiapoptotic effect of ouabain.

Na,K-ATPase and the IP_3R form a cell-signaling microdomain that, in the presence of ouabain, generates Ca^{2+} oscillations (23). Results from this study indicate that the interaction between ouabain-bound Na,K-ATPase and IP_3R is essential for the antiapoptotic effect of ouabain. The NH_2 terminus tail of the Na,K-ATPase catalytic α1-subunit plays a key role in this interaction (23). In cells that expressed Na,K-ATPase α1-subunit

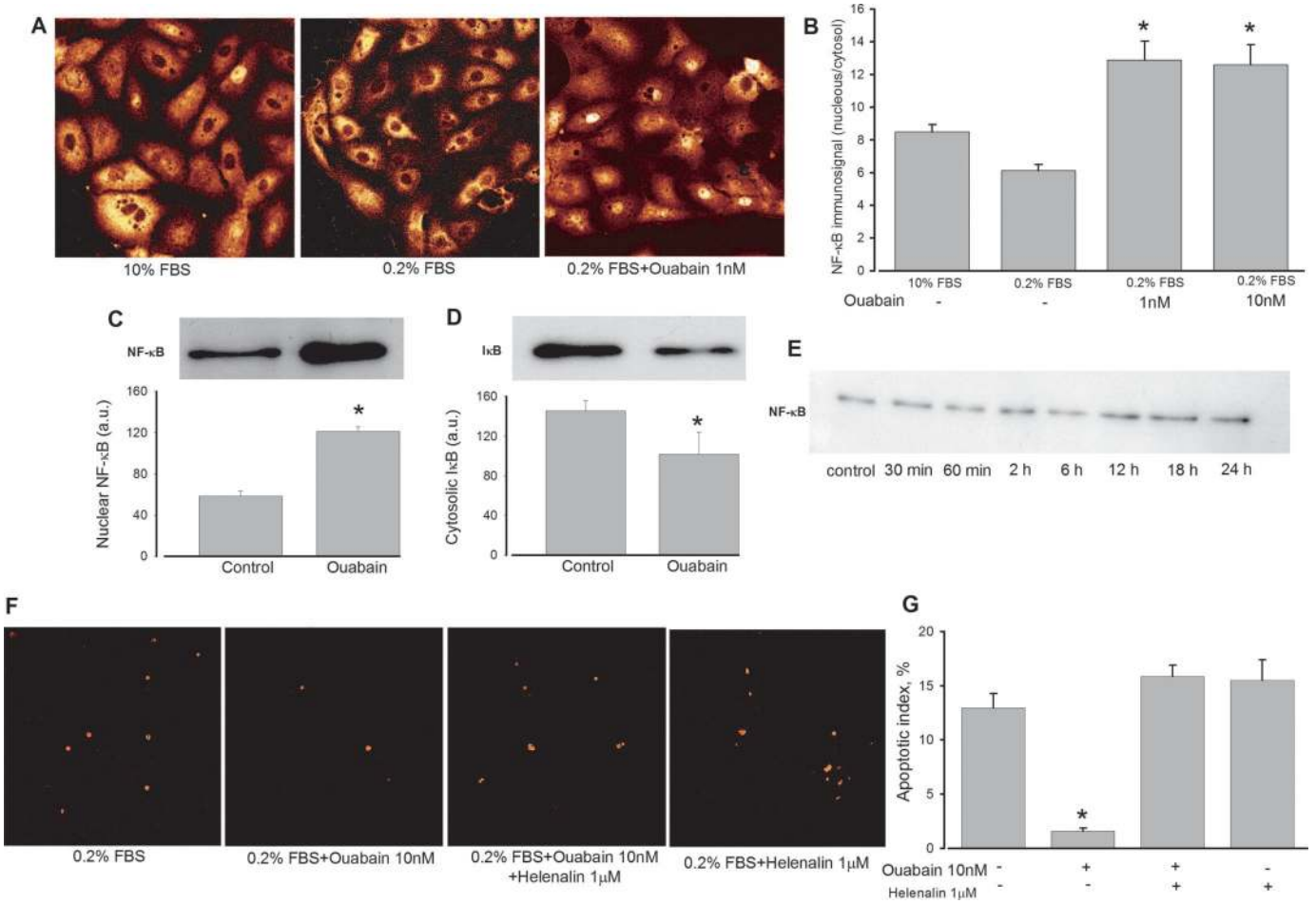

Figure 4. Inhibition of NF-κB prevents antiapoptotic effect of ouabain. (A) Representative confocal images of NF-κB immunofluorescence signal in RPT cells. Nuclear staining was increased in cells that were treated with 10 nM ouabain. (B) The ratio of nucleus to cytosol NF-κB immunofluorescence signal in RPT cells that were exposed to 1 and 10 nM ouabain. In each experiment, six to seven randomly selected areas with 100 to 200 cells in each were analyzed. Values are mean ± SEM; $n = 3$; one-way ANOVA followed by the *post hoc* test, $^*P < 0.05$. (C and D) Representative Western blot and densitometric analysis of three independent experiments showing changes in nuclear NF-κB (C) and cytosolic IκBα protein (D) in cells exposed to 10 nM ouabain. $^*P < 0.05$, *t* test. (E) Representative Western blot showing time-course changes in nuclear NF-κB in cells that were exposed to 10 nM ouabain. (F) Representative confocal images of RPT cells that were stained by TUNEL assay to detect apoptotic cells. RPT cells were treated with 10 nM ouabain in the absence or presence of NF-κB inhibitor helenalin (1 μM) or just with helenalin. (G) An inhibitor of NF-κB, helenalin (1 μM), abolished the antiapoptotic effect of ouabain on serum deprivation–triggered apoptosis. Helenalin alone did not have any significant effect on serum deprivation–triggered apoptosis. Values are mean ± SEM; $n = 3$. $^*P > 0.05$, one-way ANOVA followed by the *post hoc* test.

with a truncated NH_2 terminus tail, ouabain failed to protect from serum deprivation–triggered apoptosis.

The calcium-dependent antiapoptotic effect of ouabain may seem paradoxic in view of the fact that a number of recent studies have demonstrated that calcium release from the ER *via* the IP_3R results in a mitochondrial overload of calcium and cell death (32–35). This proapoptotic effect is triggered by the binding of cytochrome C to IP_3R, which leads to an uncontrolled release of calcium (36,37). In contrast, ouabain-triggered calcium release occurs as highly constant repetitive calcium transients with a periodicity in the minute range. Therefore, the pre- or proapoptotic effect of IP_3R activation depends on the pattern of calcium release. The question of whether ouabain may exert a feedback control on the proapoptotic effects of cytochrome C remains to be determined. It should be noted, though, that ouabain failed to protect apoptosis that was induced by 0.5 μM staurosporine for 24 h. This dose of staurosporine is associated with a massive release of cytochrome C.

Low concentrations of ouabain also were found to stimulate the proliferation of kidney cells from rat and monkey. Our observation that ouabain stimulates cell growth is in line with findings from several other laboratories that showed that ouabain stimulates growth of smooth muscle cells and renal epithelial cells from both rodent and human species (11–13,38,39). These effects have been attributed to the activation of several intracellular signaling pathways, among them activation of Ras/MAPK-ERK kinase/mitogen-activated protein kinase cascade (3,4). Here we provide evidence that the proliferation effect of ouabain also can be transduced *via* a signaling pathway that involves calcium release from intracel-

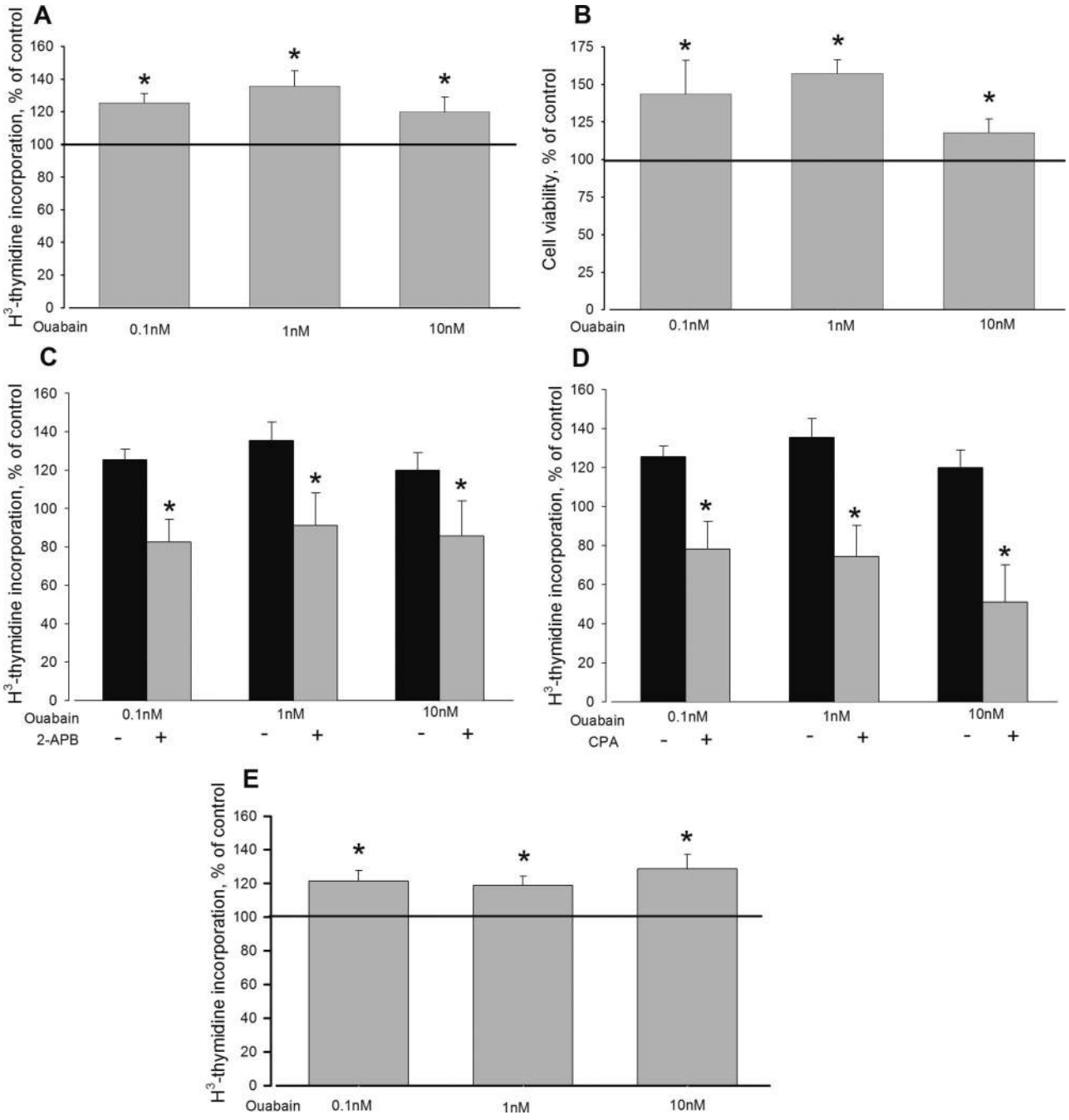

Figure 5. Ouabain-mediated stimulation of cell proliferation is Ca^{2+} dependent. (A) Ouabain in concentrations of 0.1 to 10 nM significantly stimulated RPT cell proliferation as measured by [^{3}H]thymidine incorporation. Incorporation of radiolabeled [^{3}H]thymidine into DNA is plotted as percentage of incorporation relative to ouabain-untreated cells. Values are mean ± SEM; $n = 5$. *$P < 0.01$. (B) Ouabain in concentrations of 0.1 to 10 nM stimulated RPT cell viability as determined by WST-1. The results were plotted as percentage of changes compared with ouabain-untreated cells. Values are mean ± SEM; $n = 4$. *$P < 0.01$. (C) Inhibition of IP_3R by 2-APB (5 μM) abolished the effect of ouabain on cell proliferation. Values are mean ± SEM; $n = 4$. $P > 0.05$. (D) Inhibition of SERCA pump by CPA (0.5 μM) abolished the effect of ouabain on cell proliferation. Values are mean ± SEM; $n = 4$. $P > 0.05$. (E) Ouabain in concentrations of 0.1 to 10 nM significantly stimulated COS-7 cell proliferation as measured by [^{3}H]thymidine incorporation. Incorporation of radiolabeled [^{3}H]thymidine into DNA is plotted as percentage of incorporation relative to ouabain-untreated cells. Values are mean ± SEM; $n = 5$. *$P < 0.01$. One-way ANOVA followed by the *post hoc* test were used.

lular stores *via* the IP_3R. Notably, a recent study from Xie's group demonstrated a cross-talk between the Ras/MAPK-ERK kinase/mitogen-activated protein kinase and an IP_3R–calcium signaling pathway in ouabain-stimulated cells (40).

Ouabain protection from apoptosis may be an endogenous defense mechanism. There now is strong evidence indicating that ouabain is a mammalian hormone that, like other steroid hormones, is synthesized and released from the adrenal gland

and the hypothalamus (16,17,41). Circulating levels of ouabain in mammals is estimated to be in the picomolar range and has been reported to be significantly increased in conditions in which extensive cell growth and differentiation are required, including pregnancy (42), early postnatal life (43), and after unilateral nephrectomy (44), a condition that is associated with compensatory growth of the remaining kidney.

Acknowledgments

This work was supported by grants from the Swedish Research Council, Karolinska Institutet research foundation, and Sällskapet Barnavård.

We thank Eivor Zettergren, Louise Gustafsson, and Andrei Chagin for experimental assistance.

References

1. Skou JC, Esmann M: The Na,K-ATPase. *J Bioenerg Biomembr* 24: 249–261, 1992
2. Xie Z: Ouabain interaction with cardiac Na/K-ATPase reveals that the enzyme can act as a pump and as a signal transducer. *Cell Mol Biol (Noisy-le-grand)* 47: 383–390, 2001
3. Xie Z, Askari A: Na(+)/K(+)-ATPase as a signal transducer. *Eur J Biochem* 269: 2434–2439, 2002
4. Xie Z, Cai T: Na+-K+-ATPase-mediated signal transduction: From protein interaction to cellular function. *Mol Interv* 3: 157–168, 2003
5. Yu SP: Na(+), K(+)-ATPase: The new face of an old player in pathogenesis and apoptotic/hybrid cell death. *Biochem Pharmacol* 66: 1601–1609, 2003
6. Allen DG, Eisner DA, Wray SC: Birthday present for digitalis. *Nature* 316: 674–675, 1985
7. Kometiani P, Liu L, Askari A: Digitalis-induced signaling by Na+/K+-ATPase in human breast cancer cells. *Mol Pharmacol* 67: 929–936, 2005
8. Liu J, Periyasamy SM, Gunning W, Fedorova OV, Bagrov AY, Malhotra D, Xie Z, Shapiro JI: Effects of cardiac glycosides on sodium pump expression and function in LLC-PK1 and MDCK cells. *Kidney Int* 62: 2118–2125, 2002
9. Sophocleous A, Elmatzoglou I, Souvatzoglou A: Circulating endogenous digitalis-like factor(s) (EDLF) in man is derived from the adrenals and its secretion is ACTH-dependent. *J Endocrinol Invest* 26: 668–674, 2003
10. Aizman O, Uhlen P, Lal M, Brismar H, Aperia A: Ouabain, a steroid hormone that signals with slow calcium oscillations. *Proc Natl Acad Sci U S A* 98: 13420–13424, 2001
11. Abramowitz J, Dai C, Hirschi KK, Dmitrieva RI, Doris PA, Liu L, Allen JC: Ouabain- and marinobufagenin-induced proliferation of human umbilical vein smooth muscle cells and a rat vascular smooth muscle cell line, A7r5. *Circulation* 108: 3048–3053, 2003
12. Aydemir-Koksoy A, Abramowitz J, Allen JC: Ouabain-induced signaling and vascular smooth muscle cell proliferation. *J Biol Chem* 276: 46605–46611, 2001
13. Dmitrieva RI, Doris PA: Ouabain is a potent promoter of growth and activator of ERK1/2 in ouabain-resistant rat renal epithelial cells. *J Biol Chem* 278: 28160–28166, 2003
14. Nakanishi C, Toi M: Nuclear factor-kappaB inhibitors as sensitizers to anticancer drugs. *Nat Rev Cancer* 5: 297–309, 2005
15. Sariola H, Philipson L: Bridge over troubled waters. *Nat Med* 5: 22–23, 1999
16. Kawamura A, Guo J, Itagaki Y, Bell C, Wang Y, Haupert GT Jr, Magil S, Gallagher RT, Berova N, Nakanishi K: On the structure of endogenous ouabain. *Proc Natl Acad Sci U S A* 96: 6654–6659, 1999
17. Schoner W: Endogenous cardiac glycosides, a new class of steroid hormones. *Eur J Biochem* 269: 2440–2448, 2002
18. Larsson SH: Short-term primary cultures in studies of growth regulation in rat proximal tubule cells. *Am J Kidney Dis* 17: 631–633, 1991
19. Daly SE, Lane LK, Blostein R: Structure/function analysis of the amino-terminal region of the 1 and 2 subunits of Na,K-ATPase. *J Biol Chem* 271: 23683–23689, 1996
20. Cheng XJ, Fisone G, Aizman O, Aizman R, Levenson R, Greengard P, Aperia A: PKA-mediated phosphorylation and inhibition of Na(+)-K(+)-ATPase in response to beta-adrenergic hormone. *Am J Physiol* 273: C893–C901, 1997
21. Bootman MD, Collins TJ, Mackenzie L, Roderick HL, Berridge MJ, Peppiatt CM: 2-Aminoethoxydiphenyl borate (2-APB) is a reliable blocker of store-operated Ca2+ entry but an inconsistent inhibitor of InsP3-induced Ca2+ release. *FASEB J* 16: 1145–1150, 2002
22. Peppiatt CM, Collins TJ, Mackenzie L, Conway SJ, Holmes AB, Bootman MD, Berridge MJ, Seo JT, Roderick HL: 2-Aminoethoxydiphenyl borate (2-APB) antagonises inositol 1,4,5-trisphosphate-induced calcium release, inhibits calcium pumps and has a use-dependent and slowly reversible action on store-operated calcium entry channels. *Cell Calcium* 34: 97–108, 2003
23. Miyakawa-Naito A, Uhlen P, Lal M, Aizman O, Mikoshiba K, Brismar H, Zelenin S, Aperia A: Cell signaling microdomain with Na,K-ATPase and inositol 1,4,5-trisphosphate receptor generates calcium oscillations. *J Biol Chem* 278: 50355–50361, 2003
24. Burgener-Kairuz P, Horisberger JD, Geering K, Rossier BC: Functional expression of N-terminal truncated alpha-subunits of Na,K-ATPase in *Xenopus laevis* oocytes. *FEBS Lett* 290: 83–86, 1991
25. Ohta T, Noguchi S, Nakanishi M, Mutoh Y, Hirata H, Kagawa Y, Kawamura M: The 'lysine cluster' in the N-terminal region of Na+/K(+)-ATPase alpha-subunit is not involved in ATPase activity. *Biochim Biophys Acta* 1059: 157–164, 1991
26. Shanbaky NM, Pressley TA: Mammalian alpha 1-subunit of Na(+)-K(+)-ATPase does not need its amino terminus to maintain cell viability. *Am J Physiol* 267: C590–C597, 1994
27. Senftleben U, Cao Y, Xiao G, Greten FR, Krahn G, Bonizzi G, Chen Y, Hu Y, Fong A, Sun SC, Karin M: Activation by IKKalpha of a second, evolutionary conserved, NF-kappaB signaling pathway. *Science* 293: 1495–1499, 2001
28. Lyss G, Knorre A, Schmidt TJ, Pahl HL, Merfort I: The anti-inflammatory sesquiterpene lactone helenalin inhibits the transcription factor NF-kappaB by directly targeting p65. *J Biol Chem* 273: 33508–33516, 1998
29. Hu Q, Deshpande S, Irani K, Ziegelstein RC: [Ca(2+)](i) oscillation frequency regulates agonist-stimulated NF-kappaB transcriptional activity. *J Biol Chem* 274: 33995–33998, 1999
30. Cacicedo JM, Yagihashi N, Keaney JF Jr, Ruderman NB, Ido Y: AMPK inhibits fatty acid-induced increases in NF-

kappaB transactivation in cultured human umbilical vein endothelial cells. *Biochem Biophys Res Commun* 324: 1204–1209, 2004
31. Rossler OG, Bauer I, Chung HY, Thiel G: Glutamate-induced cell death of immortalized murine hippocampal neurons: Neuroprotective activity of heme oxygenase-1, heat shock protein 70, and sodium selenite. *Neurosci Lett* 362: 253–257, 2004
32. Blackshaw S, Sawa A, Sharp AH, Ross CA, Snyder SH, Khan AA: Type 3 inositol 1,4,5-trisphosphate receptor modulates cell death. *FASEB J* 14: 1375–1379, 2000
33. Khan AA, Soloski MJ, Sharp AH, Schilling G, Sabatini DM, Li SH, Ross CA, Snyder SH: Lymphocyte apoptosis: Mediation by increased type 3 inositol 1,4,5-trisphosphate receptor. *Science* 273: 503–507, 1996
34. Patterson RL, Boehning D, Snyder SH: Inositol 1,4,5-trisphosphate receptors as signal integrators. *Annu Rev Biochem* 73: 437–465, 2004
35. Hung CC, Ichimura T, Stevens JL, Bonventre JV: Protection of renal epithelial cells against oxidative injury by endoplasmic reticulum stress preconditioning is mediated by ERK1/2 activation. *J Biol Chem* 278: 29317–29326, 2003
36. Boehning D, Patterson RL, Sedaghat L, Glebova NO, Kurosaki T, Snyder SH: Cytochrome c binds to inositol (1,4,5) trisphosphate receptors, amplifying calcium-dependent apoptosis. *Nat Cell Biol* 5: 1051–1061, 2003
37. Hirota J, Furuichi T, Mikoshiba K: Inositol 1,4,5-trisphosphate receptor type 1 is a substrate for caspase-3 and is cleaved during apoptosis in a caspase-3-dependent manner. *J Biol Chem* 274: 34433–34437, 1999
38. Akimova OA, Bagrov AY, Lopina OD, Kamernitsky AV, Tremblay J, Hamet P, Orlov SN: Cardiotonic steroids differentially affect intracellular Na+ and [Na+]i/[K+]i-independent signaling in C7-MDCK cells. *J Biol Chem* 280: 832–839, 2005
39. Kometiani P, Li J, Gnudi L, Kahn BB, Askari A, Xie Z: Multiple signal transduction pathways link Na+/K+-ATPase to growth-related genes in cardiac myocytes. The roles of Ras and mitogen-activated protein kinases. *J Biol Chem* 273: 15249–15256, 1998
40. Yuan Z, Cai T, Tian J, Ivanov AV, Giovannucci DR, Xie Z: Na/K-ATPase tethers phospholipase C and IP3 receptor into a calcium-regulatory complex. *Mol Biol Cell* 16: 4034–4045, 2005
41. Scheiner-Bobis G, Schoner W: A fresh facet for ouabain action. *Nat Med* 7: 1288–1289, 2001
42. Vakkuri O, Arnason SS, Pouta A, Vuolteenaho O, Leppaluoto J: Radioimmunoassay of plasma ouabain in healthy and pregnant individuals. *J Endocrinol* 165: 669–677, 2000
43. Calvino MA, Pena C, Rodriguez de Lores Arnaiz G: Endogenous modulators of brain Na+,K(+)-ATPase at early postnatal stages of rat development. *Int J Dev Neurosci* 16: 97–101, 1998
44. Yamada K, Goto A, Hui C, Yagi N, Nagoshi H, Sasabe M, Sugimoto T: Role of ouabainlike compound in rats with reduced renal mass-saline hypertension. *Am J Physiol* 266: H1357–H1362, 1994

Supplemental information for this article is available online at http://www.jasn.org/.