Original Paper

Ouabain Increases Gap Junctional Communication in Epithelial Cells

Arturo Ponce Isabel Larre Aida Castillo Refugio García-Villegas
Adrián Romero Catalina Flores-Maldonado Jacqueline Martinez-Rendón
Rubén Gerardo Contreras Marcelino Cerejido

Department of Physiology, Biophysics and Neurosciences, Center For Research and Advanced Studies (Cinvestav), México D. F., México

Key Words

Sodium pump • Gap junctions • Connexin • Neurobiotin • MDCK

Abstract

Background/Aims: The finding that endogenous ouabain acts as a hormone prompted efforts to elucidate its physiological function. In previous studies, we have shown that 10 nM ouabain (i.e., a concentration within the physiological range) modulates cell-cell contacts such as tight junctions and apical/basolateral polarity. In this study, we examined whether 10 nM ouabain affects another important cell-cell feature: gap junction communication (GJC).

Methods: We employed two different approaches: 1) analysis of the cell-to-cell diffusion of neurobiotin injected into a particular MDCK cell (epithelial cells from dog kidneys) in a confluent monolayer by counting the number of neighboring cells reached by the probe and 2) measurement of the electrical capacitance. **Results:** We found that 10 nM ouabain increase GJC by 475% within 1 hour. The Na⁺-K⁺-ATPase acts as a receptor of ouabain. In previous works we have shown that ouabain activates c-Src and ERK1/2 in 1 hour; in the present study we show that the inhibition of these proteins block the effect of ouabain on GJC. This increase in GJC does not require synthesis of new protein components, because the inhibitors cycloheximide and actinomycin D did not affect this phenomenon. Using silencing assays we also demonstrate that this ouabain-induced enhancement of GJC involves connexins 32 and 43. **Conclusion:** Ouabain 10 nM increases GJC in MDCK cells

Copyright © 2014 S. Karger AG, Basel

A. Ponce and I. Larre shared first authorship

Marcelino Cerejido

Lab 28, Edificio Fisiología I, Departamento de Fisiología Biofísica y Neurociencias. Centro de Investigación y Estudios Avanzados del IPN (CINVESTAV). Avenida Instituto Politécnico Nacional #2508. Colonia San Pedro Zacatenco. México City, AP 07300, (México)
Tel. +525 5747-3364, Fax +525 5747-3800, ext. 5713, E-Mail cerejido@fisio.cinvestav.mx

Introduction

The high affinity and specificity of ouabain for the Na⁺-K⁺-ATPase led to search for endogenous analogs, which were subsequently identified as ouabain itself and other cardiac steroids [1, 2]. The plasma levels of ouabain increase in response to muscular exercise, a high-sodium diet and pathological conditions, such as eclampsia, certain forms of arterial hypertension and myocardial infarction [2-7]. The available experimental evidence suggests that the endogenous cardiac steroids are hormones [8-10], raising the question of their physiological role.

High levels of ouabain (≥ 300 nM) activate the retrieval of cell-adhesion molecules that are associated with, or form part of the plasma membrane, and this process causes the cells to detach (e.g., MDCK cells) from the substrate as well as from one another [11, 12]. Therefore, we hypothesized that one physiological role of ouabain is the modulation of cell contacts [9, 13]. We have already shown that low, non-toxic concentrations of ouabain modulate the hermeticity and molecular composition of tight junctions (TJ) [14]. Furthermore, we found that 10 nM ouabain accelerates ciliogenesis, one of the final stages of cell polarity development [15]. Interestingly, TJ and apical/basolateral polarity (as measured by ciliogenesis) are the essential differentiating characteristics of transporting epithelia. Ouabain is capable of modulating both of these characteristics at a concentration that falls within the range that is observed in mammals [16, 17] and, therefore, neither inhibits the Na⁺-K⁺-ATPase from pumping K⁺ nor distorts the cellular K⁺ balance [18-20].

Gap junctions are another noteworthy type of cell-cell contact that enable communication between neighboring cells and are involved in cellular processes as fundamental and diverse as proliferation, differentiation, metabolic cooperation, synchronization, cancer, metastasis and many others [21-23]. Accordingly, in the present study we explored whether ouabain at physiological levels observed in resting dogs and humans [16, 17] exerts an effect on gap junctional communication (GJC). For this purpose, we evaluated GJC via two distinct experimental methods: 1) by micro-injecting individual cells in confluent MDCK monolayers with biotin ethylenediamine (neurobiotin, NB), a substance that can diffuse from cell to cell via gap junctions and 2) by measuring the membrane capacitance of cells coupled via GJC using whole-cell patch clamp assays [24, 25]. Additionally we made silencing assays showing that connexins 32 and 43 are involved in this response.

Materials and Methods

Cell culture, chemicals and antibodies

Starter MDCK-II cell cultures (MDCK and CCL-34) were obtained from the American Type Culture Collection. The cells were grown at 36.5°C in a 5% CO₂ atmosphere in Dulbecco's modified Eagle medium (DMEM; Life Technologies, Carlsbad CA, USA) supplemented with penicillin-streptomycin 10,000 U/ μ g/ml (In Vitro, Acayucan, Mexico) and 10% fetal bovine serum (GIBCO). This medium is hereafter referred to as CDMEM. The cells were harvested with trypsin-EDTA (In Vitro) and seeded on glass coverslips in 24-well multi-dishes (3524; Costar Corning, NY, USA) for immunofluorescence, biotin ethylenediamine, hydrobromide (Neurobiotin, A1593, Life Technologies) injection and electrical capacitance measurements or without glass coverslips for Western blot analysis. Cells were cultured at a saturating density of ~70%, maintained for one day in CDMEM, serum starved for 24 h in DMEM containing 1% fetal bovine serum and then treated with or without 10 nM ouabain (O-3125; Sigma-Aldrich, St Louis MO, USA). The cell monolayers were exposed to 25 μ M PD98059, an inhibitor of mitogen extracellular kinase-1 (MEK-1; 513000; Merk Millipore, Darmstadt GE) that impairs the activation of ERK1/2, or 10 μ M PP2, an inhibitor of c-Src kinase (529573; Merk Millipore). Actinomycin D (A9415; Sigma-Aldrich) was dissolved in DMSO (5 mg/ml) prior to use. Cycloheximide (C4859; Sigma-Aldrich) was obtained as a ready-made solution (100 mg/mL in DMSO). These inhibitors were added 1 h before the ouabain challenge was initiated.

Measurement of gap junctional communication by dye transfer assays

Glass micropipettes with a tip resistance of 5–10 MΩ were backfilled with a mixture of 2% of neurobiotin (NB, MW 0.37 kD), and 1% FITC-dextran (MW 20 kD, Fine Chemical/Amersham Pharmacia) in saline solution (120 mM KCl, 5 mM NaCl, 1 mM MgCl₂, and 5 mM HEPES, pH 7.4). After the pipettes were filled, they were attached to a holder mounted to a micromanipulator (PCS-750; Burleigh Instruments) for cell impalement. Monolayers grown on glass coverslips were deposited into a glass-bottom chamber filled with PBS containing 1.8 mM Ca²⁺ at room temperature. Then, the chamber was mounted on the stage of an inverted epifluorescence-equipped microscope (Diaphot 300; Nikon) to monitor impalement and injection. The cells were randomly selected for injection, and after impalement, they were injected with a pneumatic pulse using a microinjecting device (IM300; Narishige). Effective injection was verified by the diffusion of FITC-dextran throughout the cell. Fifteen minutes after injection, the cells were fixed for 15 min using cold methanol, rinsed with PBS, and incubated overnight at 4°C in TRITC-streptavidin (Zymed, catalog number 43-4314) at a dilution of 1:200. Finally, the samples were rinsed twice and mounted using VECTASHIELD® (H-1000; Vector Laboratories, Burlingame CA, USA). Eight-bit images of the fluorescent cells were acquired at room temperature using a Zeiss M200 inverted microscope equipped with a Plan-NeoFluar 63x N.A. 1.25 objective lens, an AxioCam MRm camera and Axovision 4.8 software. The captured images were imported into FIJI [Is Just ImageJ] software (release 2.8, NIH, Bethesda, MD, USA) to adjust the brightness and the contrast and GIMP (release 2.8.10, NIH) to compose the figures.

Measurement of gap junctional communication by electrical capacitance

GJC was estimated by recording the membrane capacitance of electrically coupled cells using the whole-cell patch-clamp technique [25–27]. Patch micropipettes were produced from borosilicate glass tubing (Kimax 51; Kimble) using a puller device (model p-87, Flaming/Brown micropipette puller; Sutter Instruments). The tip resistance of the micropipettes ranged from 2 to 5 MΩ after heat polishing. To generate gigaseals on the cells, the micropipettes were backfilled with saline solution (110 mM KCl, 5 mM NaCl, 5 mM EGTA-K, 1 mM MgCl₂, and 10 mM HEPES, pH 7.4) and attached via a pipette holder to a piezoelectric-driven micromanipulator (PCS-5000; Burleigh). The cells were bathed in external saline solution (115 mM NaCl, 5 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂, 10 mM HEPES, and 10 mM glucose, pH 7.4). The pipettes were mechanically manipulated under an inverted microscope (Diaphot 300; Nikon). A capacitive current (IC) was induced using a hyperpolarizing square voltage pulse from -80 to -90 mV and was recorded at 10 KHz. The membrane capacitance was estimated offline by integrating the area of the capacitive transient beginning at the onset of the pulse.

The membrane capacitance (C_m) was calculated by dividing the integral of the IC by the amplitude of the pulse (-10 mV) according to the following equation:

$$C_m = \frac{\int_{t_0}^{\infty} IC \cdot \Delta t}{\Delta V},$$

where C_m is the membrane capacitance, and ΔV is the amplitude of the voltage pulse (-10 mV). The integral of the IC was calculated using the Clampfit module of the pCLAMP 8.0 software suite (Molecular Devices). The membrane surface area was estimated assuming a specific membrane capacitance of 0.01 pF/μm².

shRNA plasmids and cell transfection

Inhibitory shRNAs were designed against dog Cnx-32 (Acc. No. XM_538073 target sequence: 5'-GCCGTCTTCACTGTCTTCAT-3' and dog Cnx-43 (Acc. No. NM_00100295, target sequence: 3'-GGGAAGAGAACTGAACAAG-3') and cloned into the pLVX-shRNA2 vector (632179; Clontech Laboratories Inc.), which also expresses the green fluorescent marker ZsGreen1. MDCK cells were transiently transfected with the indicated plasmids using Lipofectamine® 2000 (Invitrogen); five hours after transfection, the cells were harvested and seeded at confluency on glass coverslips in 24-well dishes, maintained for one day in CDMEM and serum starved for 24h as described above.

Following incubation in 10 nM ouabain for 1 hour, dye was injected (on the same monolayer) into cells in which Cnx-32, Cnx-43 or both had been silenced (those cells expressing ZsGreen1) or not (those not expressing ZsGreen1).

Statistical analyses

Statistical analyses were performed using Prism 5 (GraphPad software). The results are expressed as the means $\pm$ standard error. Statistical significance was estimated via one-way analysis of variance (ANOVA) followed by Bonferroni's multiple comparison or Student's t-test and was denoted as follows: * $P < 0.05$, ** $P < 0.005$ and *** $P < 0.001$ and n is the number of observations obtained from at least 3 independent experiments.

Results

Ouabain (10 nM) promotes GJC in MDCK cells

Cells in a confluent monolayer were randomly injected with a mixture of FITC-dextran and NB; FITC-dextran was used to verify that only one cell had been injected, and NB was used to detect the number of cells that received NB via gap junctions. In the absence of ouabain, it was observed that in most cases, both FITC-dextran and NB remained only in the injected cell (Fig. 1A) indicating that GJC is rather infrequent. In contrast, in cell monolayers treated with 10 nM ouabain for 1 h, FITC-dextran was still observed only in the injected cell, but NB diffused to several neighboring cells (Fig. 1B *red*), demonstrating that ouabain enhances GJC (Fig. 1C *red circles*). This enhancement of GJC was statistically significant from as early as 10 min after the addition of ouabain, peaked at 1 h, and remained at a level that was significantly higher than that under control conditions (Fig. 1C, *open circles*) for at least 24 h. Fig. 1D shows that cycloheximide, an inhibitor of protein synthesis, and actinomycin D, an inhibitor of RNA synthesis, did not suppress the enhancing effect of ouabain on GJC, suggesting that the observed enhancement of GJC by ouabain does not involve the synthesis of new protein components.

To investigate this enhancement of GJC by ouabain using an entirely completely different method, we performed whole-cell patch clamp recording to determine the effect of ouabain on the electrical coupling of neighboring MDCK cells in monolayer, as estimated from capacitance measurements. We induced capacitive current transients (IC) (Fig. 1E, *above*) with voltage steps from -80 to -90 mV to estimate the membrane capacitance of coupled cells (*see methods*) [26, 27] and confirmed that 10 nM ouabain increased the area of the plasma membrane (Fig. 1E, *above and below, white vs. red capacitive transients and bars*), reflecting an increase in the number of electrically coupled cells. To determine whether this increase in area membrane is in effect due to enhanced GJC we tested the effect of octanol (Fig. 1E, OCT, *yellow bar*), an alcohol that is known to effectively uncouple gap junctions [28] and, as Fig. 1E (*orange bar*) shows, this treatment suppressed the observed increase in membrane surface. Therefore, these results were consistent with those obtained from observing the diffusion of NB. In summary, the two experimental approaches concur in demonstrating that 10 nM ouabain enhances GJC. Taken together, these observations strengthen our general hypothesis that ouabain modulates cell contacts [9]; as it is also in keeping with our previous observations of TJ [14] and ciliogenesis [15].

10 nM ouabain triggers GJC by acting on the Na⁺-K⁺-ATPase

The effects of ouabain rely on the receptor function of the Na⁺-K⁺-ATPase [29, 30]; in some cases, the Na⁺-K⁺-ATPase functions as a receptor regardless of the inhibition of pump activity and/or of the associated ionic imbalance [18-20]. Therefore, to determine whether the Na⁺-K⁺-ATPase mediates the GJC-enhancing effect of ouabain, we used R-MDCK, a mutant subclone resistant to high concentrations of ouabain ($> 1 \mu\text{M}$) [31], resulting from a substitution of cysteine by tyrosine or phenylalanine in the first transmembrane segment of the α -subunit of Na⁺,K⁺-ATPase [32]. Fig. 2A shows a control monolayer of wild-type MDCK cells (W-MDCK) injected with NB that displayed no communication between neighboring cells. The treatment of these cells with 10 nM ouabain for 24 h (Fig. 2B, and E *first pair of bars*) enabled NB to diffuse to several neighboring cells. Fig. 2C and 2D are analogous to the previous experiments, except that in this case, we used R-MDCK cells and detected no effect

Fig. 1. Ouabain (10 nM) promotes GJC between MDCK cells. Confluent monolayers were either left as control (A) or else challenged with 10 nM ouabain for 1 h (B). Under both conditions, a single cell was injected with a mixture of FITC-dextran and NB. The injected cell appeared yellow due to the merging of the green FITC-dextran and the red TRITC-streptavidin, which was used to reveal NB. (C) Statistical analysis of the number of stained cells as a function of time under control conditions (empty circles) and upon treatment with 10 nM ouabain (red circles). (D) Statistical comparison of the number of cells stained after dye injection in control monolayers (white bars), ouabain-treated (red bars), or in monolayers pre-treated with cycloheximide (10 $\mu\text{g}/\text{ml}$) or actinomycin (5 $\mu\text{g}/\text{ml}$). (E) Above: Representative capacitive current traces that were used to estimate the membrane surface of the electrically coupled cells. These traces were obtained via whole-cell patch clamp recording of cells in monolayers under either of the following treatments: control (white), incubated in 10 nM ouabain for 1 h (red), control preincubated in octanol (OCT; a de-coupler of gap junctions) for 1 h prior to the zero time point (yellow), or preincubated in octanol and then treated with 10 nM ouabain for 1 h (orange). Below: The corresponding statistical analyses. White bar: control value; red bar: the area of the communicating cells in monolayers stimulated with 10 nM ouabain; yellow bar: control monolayers pre-treated with octanol; and orange bar: ouabain-treated monolayers pre-exposed to octanol. The numbers above symbols and bars of figures 1C to E indicate the number of total trials, from three distinct assays.

of ouabain (Fig. 2E, second pair of bars). These results indicate that the receptor by which ouabain increases GJC is the $\text{Na}^+\text{-K}^+\text{-ATPase}$.

Involvement of c-Src and ERK1/2 in GJC induced by 10 nM ouabain

The $\text{Na}^+\text{-K}^+\text{-ATPase}$ forms a receptor complex with c-Src and $\text{IP}_3\text{-Receptor}$, both of which activate signaling pathways (such as ERK1/2) and stimulate Ca^{2+} waves [33, 34]. In recent studies, we found that 10 nM ouabain provokes a single wave of c-Src and ERK1/2 phosphorylation that peaks within the first hour of application, coinciding with the peak of GJC. Furthermore, incubation in PP2 inhibits c-Src, and incubation in PD98059 inhibits MEK kinase at least for 8 h [12]. These signaling proteins also modulate the effect of ouabain on TJ [14] and ciliogenesis [15]. In keeping with these evidences, we now show the effect of the inhibitors PP2 and PD98059 on the GJC-enhancing effect of 10 nM ouabain after 1 h of

Fig. 2. Ouabain elicits its GJC-enhancing effect by acting on the $\text{Na}^+\text{-K}^+\text{-ATPase}$. Representative examples of NB injection trials in W-MDCK (A and B) or R-MDCK cells (C and D) in monolayers that were either non-treated (A and C) or treated with 10 nM ouabain (B and D). (E) The corresponding statistical analyses of the monolayers of W-MDCK and R-MDCK cells under control or 10 nM ouabain challenge conditions. The numbers above bars of figure 2B indicate the number of total trials, from three distinct assays.

Fig. 3. Ouabain modulates GJC via a signaling pathway that involves c-Src and ERK1/2. Nuclei are stained in blue, and NB in red. (A) A control monolayer; (B) a monolayer treated with 10 nM ouabain for 1 h. C and D are homologous to A and B, respectively, except that these monolayers were pre-treated for 1 h and throughout the experimental test with the c-Src inhibitor PP2. (E and F) are homologous to A and B, except that these monolayers were pretreated 1 h, and throughout the experimental test with PD98059 (PD), a substance that inhibits MEK kinase and, therefore, impairs the activation of ERK1/2. (G) Corresponding statistical analyses. White bar: control monolayers; red bar: monolayers treated with 10 nM ouabain; light blue bar: monolayers pretreated with PP2; dark blue bar: monolayer pretreated with PP2 for 1 h before adding ouabain; light green bar: monolayer pretreated with PD98059; and dark green bar: monolayer pretreated with PD98059 before adding ouabain.

exposure (see also Fig. 3G, white bar vs. red bar). Fig. 3C and D shows the effects of 1 h of pre-treatment with PP2 (c-Src-inhibitor) in the absence and presence of treatment with 10 nM ouabain, respectively. Pre-treatment with inhibitor PP2 in the absence of ouabain (Fig. 3C

Fig. 4. Transfection of MDCK cells with shRNA designed to silence Cnx-32 and/or Cnx-43 markedly decreased ouabain-induced GJC. MDCK cells were transiently transfected with plasmids expressing a green fluorescent protein (ZsGreen1) and a shRNA sequence designed to silence Cnx-32 or Cnx-43. After 48 hrs of transfection all monolayers were incubated for 1 h in 10 nM ouabain before dye transfer assays. (A) A representative example of ouabain-mediated stimulation of GJC in a cell that did not express ZsGreen1 or shRNA (arrow); (B) a cell expressing ZsGreen1 and Cnx 43 shRNA; and (C) a cell transfected with ZsGreen1 and a scrambled shRNA sequence. (D) The bars correspond to the mean $\pm$ SE number of cells stained after NB injection into a cell that either did not express ZsGreen1 (white) or expressed ZsGreen1 (green) in the presence of ouabain. The numbers above bars of figure 4D indicate the number of total trials, from three distinct assays.

and 3G, *light blue bar*) increases cell-to-cell communication but completely blocks the effect of 10 nM ouabain (Fig. 3D and 3G, *blue bar*). Figs. 3E and F show an analogous experiment using the ERK1/2 inhibitor PD98059 prevents the effect of ouabain on GJC. Therefore, c-Src and ERK1/2 participate in the ouabain-mediated GJC enhancement at 1 h.

Participation of Cnx-43 and Cnx-32 in the 10 nM ouabain-mediated induction of GJC

GJC occurs via connexons, whose primary protein components are connexins (Cnxs) [21-23]. Given that in previous studies using different experimental conditions and 1 μ M ouabain it has been observed that ouabain modulate Cnx-32 in MDCK cells [13, 35], we examined whether these two Cnxs participate in the ouabain-mediated enhancement of GJC. For this purpose, we made plasmids that contained a shRNA aimed to silence Cnx-32 or Cnx-43, along with ZsGreen1 as a reporter of expression, then we made transient transfections of MDCK cells with either one of those two plasmids as well as co-transfection of both. In order to start the dye transfer assays, we waited 48 h to insure that the analyzed connexin has sufficient time to reduce its expression in the transfected cell. In the same monolayer, we made dye transfer assays on cells expressing ZsGreen1 (i.e., contained shRNAs) or not to compare the number of communicating cells. As shown in Fig. 4 A, B, and C, dye transfer assays were performed on cells incubated for 1 h in 10 nM ouabain. Fig. 4A demonstrates that in a cell not expressing ZsGreen1 (shown by the *arrow*), cell-to-cell communication occurred similarly to that in Fig. 1A. Alternatively, in a cell expressing ZsGreen1 (i.e., containing an shRNA targeting Cnx-43, *yellow*), cell-to-cell communication was blocked (Fig. 4B). Fig. 4C (*yellow*) shows a cell that was transfected with a scrambled shRNA sequence, demonstrating that the passage of NB was not inhibited and that the blockade elicited by the targeted shRNA was specific, as shown in panel B (*yellow*). Fig. 4D contains the statistical analysis of ouabain-induced GJC in

cells expressing the targeted shRNA (*green bars*), in cells not expressing shRNA (*white bars*) and in cells transfected with a scrambled shRNA (last *green bar*) as a negative control. As shown in the first two columns, the number of communicating cells when the cell that was injected with NB did not express shCnx32 (*white bar*) was compared to that when the cell that was injected with NB did express shCnx32 (*green bar*). This significant decrease in cell-to-cell communication demonstrates that Cnx-32 participates in the response to ouabain. The second pair of columns is analogous, but the Cnx evaluated was Cnx-43; these results indicate that Cnx-43 also participates in ouabain-induced GJC. The third pair of columns demonstrates that the co-transfection of cells with both targeted shRNAs further decreased GJC. The final pair of columns shows that this effect was effectively due to a specific silencing of the target sequence, since transfection with a scrambled sequence did not produced a significantly change. These results indicate that the enhancement of GJC by ouabain requires the presence of both Cnx-32 and Cnx-43, although the participation of other Cnx isoforms cannot be excluded.

Discussion

Previous findings led us to hypothesize that one possible function of ouabain is the modulation of cell-cell contacts [13]. To explore this possibility, we first examined the effect of 10 nM ouabain on TJ and found that ouabain modifies the molecular composition and distribution pattern of specific claudins and enhances sealing of this junction (as revealed by the paracellular permeability to electrically neutral molecules and transepithelial electrical resistance, TER) [14]. Another clear demonstration of the effect of ouabain on cell-cell contacts is its modulation of ciliogenesis [15], which occurs at the culmination of the process of apical/basolateral polarization [36, 37].

To further explore the hypothesis that nanomolar concentrations of ouabain modulate cell-cell contacts, we determined whether 10 nM ouabain acts on another type of cell-cell contact, the gap junction. We found that in fact, ouabain significantly enhances GJC in as early as 10 min (Fig. 1C), peaks in an hour, and lasts for at least 24 h. On these basis we decided to explore the effect of ouabain in the first hour. The observation that inhibitors of transcription and of translation do not affect the induction of GJC by ouabain, indicates that cells express a sufficient level of connexins to account for the observed enhancement of GJC.

Early studies performed with MDCK cells seeded at confluence, and explored with Lucifer Yellow as cell-cell diffusion marker and freeze fracture microscopy to observe the image of gap junctions, have shown that GJC shows an increase between the 9-15 h after plating but disappear thereafter [38], a well known phenomenon in fetal tissues. In keeping with that observation, Berthoud and coworkers [35] found that Cnx-43 expression is relatively high during proliferation, and drastically decreased after MDCK cells reach confluence. This phenomenon may not perturb the results of the present study, as our assays were performed using monolayers that had been confluent for 48 h and that displayed a TER of several hundred ohms per square centimeter.

We found that 10 nM ouabain acts on the Na⁺-K⁺-ATPase, and c-Src and ERK1/2 are necessary to modulates GJC. This modulation was detected after incubation with ouabain for just minutes, which is considerably shorter than the period of incubation required to induce modifications in TJ (hours) and is markedly shorter than the process of ciliogenesis (days), which we explored in previous studies [14, 15]. Thus, ouabain regulates both TJ and GJC. However, the mechanisms by which these junctions are regulated are distinct; the inhibition of c-Src and ERK1/2 completely blocks the effect of ouabain on GJC, but its effect on TJ also depends on other signaling pathways. Our results are consistent with those of Wang et al. [33] and Zhang et al. [34], who found that Na⁺-K⁺-ATPase functions as a receptor of ouabain. Taken together, these results demonstrate that ouabain acts on this transmembrane enzyme to independently modulate various types of cell-cell contacts in the following sequence: gap junctions (within approximately 10 min), adherens junctions (8-12 h), tight junctions (24-48 h) and the cell-cell contacts that are involved in ciliogenesis (48-72 h).

Implications of the GJC-enhancing effect of 10 nM ouabain for physiological processes

When ouabain binds to the Na⁺-K⁺-ATPase in renal epithelial cells, it activates a cytosolic Ca²⁺ oscillatory wave that activates NF-κB and protects cells from serum deprivation-induced apoptosis [29]. In rats that are exposed to malnutrition and serum-starved kidney explants, ouabain activates the signaling of the calcium-dependent transcription factor NF-κB, which protects kidney development from the adverse effects of malnutrition [39, 40]. It is conceivable that if cytosolic Ca²⁺ oscillatory waves diffuse across gap junctions, the enhancement of GJC by ouabain described in the present study may aid in the prevention of adverse events during kidney development.

Acknowledgments

We thank Elizabeth del Oso, E. Méndez, and J. Soriano for their technical and administrative assistance. We acknowledge Dr. K. Soderberg for donating the R-MDCK cells.

Grants: This work was supported by the CONACYT (Mexican Research Council 127329) and ICyTDF (México City Research Council). Isabel Larre was supported by a postdoctoral research fellowship from Becas Mujeres ICyTDF (2012) and financed by Cinvestav, and Adrian Romero and Jacqueline Martínez-Rendón were supported by CONACYT research fellowships.

References

- 1 Hamlyn JM, Blaustein MP, Bova S, DuCharme DW, Harris DW, Mandel F, Mathews WR, Ludens JH: Identification and characterization of a ouabain-like compound from human plasma. *Proc Natl Acad Sci U S A* 1991;88:6259-6263.
- 2 Bagrov AY, Shapiro JI, Fedorova OV: Endogenous cardiotonic steroids: physiology, pharmacology, and novel therapeutic targets. *Pharmacol Rev* 2009;61:9-38.
- 3 Hamlyn JM, Ringel R, Schaeffer J, Levinson PD, Hamilton BP, Kowarski AA, Blaustein MP: A circulating inhibitor of (Na⁺ + K⁺)ATPase associated with essential hypertension. *Nature* 1982;300:650-652.
- 4 Komiyama Y, Dong XH, Nishimura N, Masaki H, Yoshika M, Masuda M, Takahashi H: A novel endogenous digitalis, telocinobufagin, exhibits elevated plasma levels in patients with terminal renal failure. *Clin Biochem* 2005;38:36-45.
- 5 Liu ZQ, Ma AQ, Zhang L, Yang DY: Intra-cellular electrolyte changes and levels of endogenous digoxin-like substance within the plasma in patients with congestive heart failure. *Int J Cardiol* 1990;27:47-53.
- 6 Lopatin DA, Ailamazian EK, Dmitrieva RI, Shpen VM, Fedorova OV, Doris PA, Bagrov AY: Circulating bufodienolide and cardenolide sodium pump inhibitors in preeclampsia. *J Hypertens*. 1999 Aug;17(8):1179-87.
- 7 Manunta P, Hamilton BP, Hamlyn JM: Salt intake and depletion increase circulating levels of endogenous ouabain in normal men. *Am J Physiol Regul Integr Comp Physiol* 2006;290:R553-559.
- 8 Aperia A: New roles for an old enzyme: Na,K-ATPase emerges as an interesting drug target. *J Intern Med*. 2007;261:44-52.
- 9 Cereijido M, Contreras RG, Shoshani L, Larre I: The Na⁺-K⁺-ATPase as self-adhesion molecule and hormone receptor. *Am J Physiol Cell Physiol* 2012;302:C473-481.
- 10 Schoner W, Bauer N, Müller-Ehmsen J, Krämer U, Hambarchian N, Schwinger R, Moeller H, Kost H, Weitkamp C, Schweitzer T, Kirch U, Neu H, Grünbaum E-G: Ouabain as a mammalian hormone. *Ann N Y Acad Sci* 2003;986:678-684.
- 11 Contreras RG, Shoshani L, Flores-Maldonado C, Lázaro A, Cereijido M: Relationship between Na(+),K(+)-ATPase and cell attachment. *J Cell Sci* 1999;112:4223-4232.
- 12 Rincon-Heredia R, Flores-Benitez D, Flores-Maldonado C, Bonilla-Delgado J, García-Hernández V, Verdejo-Torres O, Castillo AM, Larre I, Poot-Hernández AC, Franco M, Gariglio P, Reyes JL, Contreras RG: Ouabain induces endocytosis and degradation of tight junction proteins through ERK1/2-dependent pathways. *Exp Cell Res* 2014;320:108-118.
- 13 Larre I, Ponce A, Fiorentino R, Shoshani L, Contreras RG, Cereijido M: Contacts and cooperation between cells depend on the hormone ouabain. *Proc Natl Acad Sci U S A* 2006;103:10911-10916.
- 14 Larre I, Lázaro A, Contreras RG, Balda MS, Matter K, Flores-Maldonado C, Ponce A, Flores-Benitez D, Rincon-Heredia R, Padilla-Benavides T, Castillo A, Shoshani L, Cereijido M: Ouabain modulates epithelial cell tight junction. *Proc Natl Acad Sci USA* 2010;107:11387-11392.

- 15 Larre I, Castillo A, Flores-Maldonado C, Contreras RG, Galvan I, Muñoz-Estrada J, Cereijido M: Ouabain modulates ciliogenesis in epithelial cells. *Proc Natl Acad Sci U S A* 2010;107:11387-11392.
- 16 Bauer N, Müller-Ehmsen J, Krämer U, Hambarchian N, Zobel C, Schwinger RHG, Neu H, Kirch U, Grünbaum E-G, Schoner W: Ouabain-like compound changes rapidly on physical exercise in humans and dogs: effects of beta-blockade and angiotensin-converting enzyme inhibition. *Hypertension* 2005;45:1024-1028.
- 17 Dvela M, Rosen H, Ben-Ami HC, Lichtstein D: Endogenous ouabain regulates cell viability. *Am J Physiol Cell Physiol* 2012;302:C442-452.
- 18 Akimova OA, Hamet P, Orlov SN: $[Na^+]/[K^+]_i$ -independent death of ouabain-treated renal epithelial cells is not mediated by Na^+, K^+ -ATPase internalization and de novo gene expression. *Pflugers Arch* 2008;455:711-719.
- 19 Contreras RG, Flores-Maldonado C, Lázaro A, Shoshani L, Flores-Benitez D, Larre I, Cereijido M: Ouabain binding to Na^+, K^+ -ATPase relaxes cell attachment and sends a specific signal (NACos) to the nucleus. *J Membr Biol* 2004;198:147-158.
- 20 Lai F, Madan N, Ye Q, Duan Q, Li Z, Wang S, Si S, Xie Z: Identification of a Mutant $\alpha 1$ Na/K-ATPase That Pumps but is Defective in Signal Transduction. *J Biol Chem* 2013;288:13295-13304.
- 21 Oyamada M, Oyamada Y, Takamatsu T: Regulation of connexin expression. *Biochim Biophys Acta* 2005;1719:6-23.
- 22 Saez JC, Berthoud VM, Branes MC, Martinez AD, Beyer EC: Plasma membrane channels formed by connexins: their regulation and functions. *Physiol Rev* 2003;83:1359-1400.
- 23 Solan JL, Lampe PD: Connexin43 phosphorylation: structural changes and biological effects. *Biochem J* 2009;419:261-272.
- 24 Cereijido M, Robbins E, Sabatini DD, Stefani E: Cell-to-cell communication in monolayers of epithelioid cells (MDCK) as a function of the age of the monolayer. *J Membr Biol* 1984;81:41-48.
- 25 de Roos AD, van Zoelen EJ, Theuvsen AP: Determination of gap junctional intercellular communication by capacitance measurements. *Pflugers Arch* 1996;431:556-563.
- 26 Ponce A, Bolívar JJ, Vega J, Cereijido M: Synthesis of plasma membrane and potassium channels in epithelial MDCK cells. *Cell Physiol Biochem* 1991;1:195-204.
- 27 Talavera D, Ponce A, Fiorentino R, Gonzalez-Mariscal L, Contreras RG, Sánchez SH, García-Villegas MR, Valdés J, Cereijido M: Expression of potassium channels in epithelial cells depends on calcium-activated cell-cell contacts. *J Membr Biol* 1995; 43:219-226.
- 28 Spray DC, White RL, Mazet F, Bennett MV: Regulation of gap junctional conductance. *Am J Physiol* 1985;248:H753-764.
- 29 Aizman O, Uhlén P, Lal M, Brismar H, Aperia A: Ouabain, a steroid hormone that signals with slow calcium oscillations. *Proc Natl Acad Sci USA* 2001;98:13420-13424.
- 30 Liu J, Tian J, Haas M, Shapiro JI, Askari A, Xie Z: Ouabain interaction with cardiac Na^+-K^+ -ATPase initiates signal cascades independent of changes in intracellular Na^+ and Ca^{2+} concentrations. *J Biol Chem* 2000;275:27838-27844.
- 31 Soderberg K, Rossi B, Lazdunski M, Louvard D: Characterization of ouabain-resistant mutants of a canine kidney cell line, MDCK. *J Biol Chem* 1983;258:12300-12307.
- 32 Canessa CM, Horisberger JD, Louvard D, Rossier BC: Mutation of a cysteine in the first transmembrane segment of Na,K-ATPase alpha subunit confers ouabain resistance. *EMBO J* 1992;11:1681-1687.
- 33 Wang H, Haas M, Liang M, Cai T, Tian J, Li S, Xie Z: Ouabain assembles signaling cascades through the caveolar Na^+-K^+ -ATPase. *J Biol Chem* 2004;279: 7250-7259.
- 34 Zhang S, Malmersjö S, Li J, Ando H, Aizman O, Uhlén P, Mikoshiba K, Aperia A: Distinct role of the N-terminal tail of the Na,K-ATPase catalytic subunit as a signal transducer. *J Biol Chem* 2006;281:21954-21962.
- 35 Berthoud VM, Ledbetter ML, Hertzberg EL, Sáez JC: Connexin43 in MDCK cells: regulation by a tumor-promoting phorbol ester and Ca^{2+} . *Eur J Cell Biol* 1992;57:40-50.
- 36 Philp N, Shoshani L, Cereijido M, Rodriguez-Boulton E: Epithelial Domains, in Nabi IR (ed): Cellular Domains. John Wiley & Sons, Inc., Hoboken, NJ, USA, 2011.
- 37 Smith EF, Rohatgi R: Cilia 2010: the surprise organelle of the decade. *Sci Signal* 2011;4(155): doi: 10.1126/scisignal.4155mr1.
- 38 Cereijido M, Robbins E, Sabatini DD, Stefani E: Cell-to-cell communication in monolayers of epithelioid cells (MDCK) as a function of the age of the monolayer. *J Membr Biol* 1984;81:41-48.
- 39 Khodus GR, Kruusmägi M, Li J, Liu XL, Aperia A: Calcium signaling triggered by ouabain protects the embryonic kidney from adverse developmental programming. *Pediatr Nephrol (Berlin, Germany)* 2011;26:1479-1482.
- 40 Li J, Khodus GR, Kruusmägi M, Kamali-Zare P, Liu XL, Eklöf A-C, Zelenin S, Brismar H, Aperia A: Ouabain protects against adverse developmental programming of the kidney. *Nat Commun* 2010;1:1-7.