Ouabain modulates epithelial cell tight junction

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Edited by Ramón Latorre, Centro de Neurociencias, Universidad de Valparaíso, Valparaíso, Chile, and approved April 23, 2010 (received for review January 13, 2010)

Epithelial cells treated with high concentrations of ouabain (e.g., 1 μM) retrieve molecules involved in cell contacts from the plasma membrane and detach from one another and their substrates. On the basis of this observation, we suggested that ouabain might also modulate cell contacts at low, nontoxic levels (10 or 50 nM). To test this possibility, we analyzed its effect on a particular type of cell–cell contact: the tight junction (TJ). We demonstrate that at concentrations that neither inhibit K^+ pumping nor disturb the K^+ balance of the cell, ouabain modulates the degree of sealing of the TJ as measured by transepithelial electrical resistance (TER) and the flux of neutral 3 kDa dextran (J_{DEX}). This modulation is accompanied by changes in the levels and distribution patterns of claudins 1, 2, and 4. Interestingly, changes in TER, J_{DEX}, and claudins behavior are mediated through signal pathways containing ERK1/2 and c-Src, which have distinct effects on each physiological parameter and claudin type. These observations support the theory that at low concentrations, ouabain acts as a modulator of cell–cell contacts.

claudins | c-Src | ERK1/2 | Madin-Darby canine kidney | occludin

Several lines of evidence, including the high affinity and specificity of ouabain for Na^+,K^+-ATPase, suggest that endogenous ouabain analogs might exist. In keeping with this possibility, Hamlyn et al. (1) demonstrated the presence of a substance in plasma that cannot be distinguished from ouabain even by specific antibodies and mass spectrometry (1–4). Endogenous ouabain levels are increased during exercise (5) and in pathological conditions such as arterial hypertension (6–9) and eclampsia (10), raising the possibility that ouabain functions as a hormone and prompting efforts to elucidate physiological role.

We have previously demonstrated that ouabain acts on cell-substrate and cell–cell contacts in Madin-Darby canine kidney (MDCK) cells. At 1 μM, ouabain binding to the Na^+,K^+-ATPase results in pump inhibition and disassembly of molecules from the tight, adherens, and focal junctions (i.e., a P→A mechanism from pump to adhesion) (11). Consistent with these results, Rajasekaran et al. (12) have observed that 0.5 μM ouabain acts on the tight junction (TJ), decreasing transepithelial electrical resistance (TER) and increasing mannitol and inulin permeability in cultures of human retinal pigment epithelial cells. Furthermore, we have shown that in cocultures of wild-type MDCK and ouabain-resistant MDCK cells, ouabain treatment increases the expression of connexin 32, but not 26 or 43, and increases cell–cell communication via gap junctions to rescue the wild-type cells (13). Although these effects indicate that toxic levels of ouabain affect the structure and function of cell–cell junctions, we do not know how lower levels of ouabain affect adhesive structures.

In the present work, we focus on this question and demonstrate that 10 nM ouabain affects neither Na^+,K^+-ATPase nor the K^+ balance of the cells and does not induce the toxic effects that occur at higher concentrations, such as cell detachment. Despite the absence of pump inhibition, 10 nM ouabain modifies TJ functions through signaling pathways involving c-Src and ERK1/2 and by modulating the expression of specific TJ components.

Results

Ouabain at 10 nM Does Not Interfere with K^+ Pumping. One of the central roles of Na^+,K^+-ATPase is to pump extracellular K^+ into the cells. To test whether ouabain affects this function at concentrations as low as 10 nM, we measured the unidirectional flux of K^+ (J_K) from the bathing medium into MDCK cells grown on filter supports (14, 15). Previous studies have demonstrated that given the high K^+ content of the cytoplasm and the low concentration in the bathing medium (5.3 mM), the initial uptake of tracer is a valid measurement of J_K (16). Fig. 1*A* shows the expected inhibition of J_K by the addition of 1 μM ouabain for 3 h (■), but 10 nM ouabain does not significantly inhibit this parameter even after 3 d of treatment (●). Fig. 1*B* shows that treatment with 1 μM ouabain for 1 d (■) markedly decreases intracellular K^+ content (K_c), calculated through the amount of ^{86}Rb accumulated to equilibrium in the cells. Cells incubated with 10 nM ouabain (●) maintain K_c values similar to those of control cells (○) for at least 3 d.

Ouabain at 10 nM Induces Neither Cell Detachment Nor Apoptosis. As described above, 1 μM ouabain induces the disassembly of cell adhesion–associated proteins from the plasma membrane, causing the cells to detach from the monolayer (Fig. S1*A*, *Center*), which can be determined by the amount of protein remaining in the well (11, 13, 17) (Fig. S1*B*, ■). In contrast, cells treated with 10 or 50 nM ouabain behave like control cells (Fig. S1 *A* and *B*). At these concentrations, ouabain produces a small increase in the protein content on day 2 (Fig. S1*B*, ●). Disassembly or assembly of membrane proteins is often reflected by changes in the surface area of the cell, generally owing to altered endo- or exocytosis (18, 19). Therefore, we estimated cell surface area through membrane capacitance using impaling glass microelectrodes and observed that 1 μM ouabain decreases this parameter by 26% by the 12th hour, consistent with the fact that ouabain at this concentration causes disassembly and retrieval of cell adhesion molecules (Fig. S1*B*, ■) (11). However, 10 and 50 nM ouabain-treated cells have cell surface areas similar to those of control cells (Fig. S1*C*, ●).

Cell detachment may induce apoptosis (20), and conversely, apoptosis usually results in cell detachment (21). In smooth muscle, HeLa, and neuroblastoma cells, ouabain induces apoptosis (22, 23). In contrast, ouabain has an antiapoptotic effect in LLC-PK1 and kidney cells (24), preventing apoptosis induced through serum deprivation in renal proximal tubular rat cells (24). These by no means constitute discrepancies, but rather suggest that the effects of ouabain likely depend on cell type, dose, time of

Author contributions: I.L., M.S.B., K.M., and M.C. designed research; I.L., A.L., R.G.C., M.S.B., C.F.-M., A.P., D.F.-B., R.R.-H., T.P.-B., A.C., L.S., and M.C. performed research; M.S.B. and K.M. contributed new reagents/analytic tools; I.L., R.G.C., C.F.-M., A.P., and M.C. analyzed data; and I.L. and M.C. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1000500107/-/DCSupplemental.

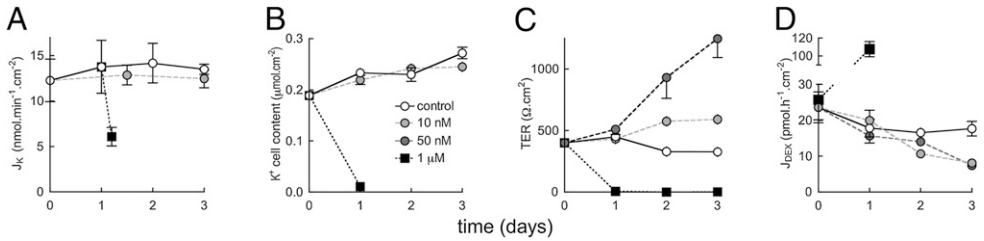

Fig. 1. Ouabain at 10 nM increases TER and decreases nonionic permeability in MDCK cells without blocking ion pumping. (*A*) J_K is the influx of K^+ as measured with ^{86}Rb in control cells (○). Cells were treated with 1 μM ouabain for 3 h added at day 1 (■) or 10 nM ouabain added at time 0 (●). Values are mean ± SEM, $n = 6$ for each time point at each condition. (*B*) Effect of 10 nM ouabain on K_c, the cellular content of K^+. Values are mean ± SEM, $n = 12$ for each experimental point. (*C*) TER as measured in monolayers incubated in the absence or presence of ouabain (10 or 50 nM and 1 μM, ● and ■, respectively) for 3 d. Values are mean ± SEM. For values at time 0, $n = 35$; for the other experimental time points, $n = 4$ for each time point at each condition. (*D*) Unidirectional flux of 3 kDa dextran (J_{DEX}) through the monolayer under control conditions or upon treatment with 10 or 50 nM or 1 μM ouabain. Values are mean ± SEM, $n = 9$–17 for each time point.

exposure, or other factors. Although 10 nM ouabain did not induce detachment (Fig. S1 *A* and *B*), we also tested whether ouabain may nevertheless induce low levels of apoptosis by measuring annexin V binding to the outer leaflet of the plasma membrane. Staurosporine at 1 μM induces apoptosis (Fig. S2*A*, ■), but 10 nM ouabain does not produce apoptosis or necrosis, even after 3 d of treatment (Fig. S2 *A* and *B*, ●). Furthermore, experiments measuring DNA degradation after 24 h of incubation with 10 nM of ouabain do not suggest stimulation of either apoptosis or necrosis [monolayer without ouabain 1.86 ± 0.24 ($n = 3$), monolayer with ouabain 1.98 ± 0.61 ($n = 3$)]. Ouabain at 3 μM increases caspase-3 in MDCK cells after 20 h (25). We observe that 10 nM ouabain does not increase the active form of caspase-3 within 2 d of treatment (Fig. S2*C*, ●) although staurosporine does (Fig. S2*A*, ■). In summary, at 10 nM, ouabain does not cause cell detachment, apoptosis, or necrosis and therefore does not have the toxic effects observed for 1 μM ouabain. This does not necessarily imply that this dose of ouabain does not affect cell–cell contacts such as the TJ. In the following section we analyze this aspect of ouabain activity.

Ouabain at 10 nM Modulates TJ Permeability. The degree of tightness of the TJ can be tested by measuring the TER (26). We have already shown that toxic concentrations of ouabain open the TJ and reduce the TER to zero within 24 h (13, 17) (Fig. 1*C*, ■). In contrast, 10 or 50 nM ouabain causes a consistent increase in TER. The TER increases by 80% after 3 d of incubation with 10 nM ouabain and by 281% upon incubation with 50 nM ouabain (Fig. 1*C*, ●). TJs also control the nonionic permeability of the paracellular pathway, which can be measured via the diffusion of dextrans of different sizes (27). The unidirectional flux of 3 kDa dextran in the apical-to-basolateral direction through the whole monolayer is a measure of the restriction offered to neutral molecules by the TJ (J_{DEX}; Fig. 1*D*). The high value of J_{DEX} in the presence of 1 μM ouabain (■) further indicates that TJs are opened within 1 d. Interestingly, 10 and 50 nM ouabain reduce paracellular permeability to 3 kDa dextran at the second and third day of treatment (by 38.2% and 55.8%, respectively, for 10 nM; Fig. 1*D*, ●), indicating that it exerts an effect on TJs. However, this effect opposes the one elicited by ouabain at 1 μM (Fig. 1*D*, ■). Whereas J_{DEX} reflects paracellular permeability to neutral molecules, TER reflects the paracellular resistance to ions; often, but not necessarily, J_{DEX} and TER vary in opposite directions (28, 29). The independent regulation of the flux of ions (reflected by TER) and neutral substances (exemplified by J_{DEX}) is also stressed by the fact that the effect on TER is concentration dependent (Fig. 1*C*), but the effect on the flux of 3 kDa dextran is not (Fig. 1*D*).

Ouabain Acts on Molecular Components of the TJ. Some protein components of the TJ, such as occludin and claudins, traverse the whole plasma membrane and are involved in the regulation of paracellular permeability (28, 30). Therefore, we extended our study of TER by testing whether the effect of low concentrations of ouabain is mediated by changes in the expression of occludin and claudins at the protein level. Fig. 2*A* shows that the total claudin 1 (cln-1) content is increased at the first and second day but returns to control levels at the third day. Claudin 2 (cln-2) is increased at the second day and claudin 4 (cln-4) is only increased at the third day (Fig. 2*A*), but occludin expression is unchanged (Fig. 2*B*). Next we explored whether the effect of ouabain on the expression of claudins is accompanied by changes in their distribution. Confocal microscopy shows that ouabain affects the "chicken fence" pattern of the three claudins investigated (Fig. 2*C*) but does not modify occludin distribution (Fig. 2*D*). Cln-1 seems to become distributed more toward cell–cell junctions and the cytoplasm, whereas cln-2 and -4 become more noticeable at the cell–cell junction (Fig. 2*C*). Hence, besides modulating claudin expression, low concentrations of ouabain also affect claudin localization without affecting occludin (Fig. 2*D*).

We investigated whether ouabain modulates the transcription of cln-1. Fig. S3*A* shows that 10 nM ouabain activates the cln-1 promoter, as measured by a Luciferase reporter assay. Ouabain also increases the amount of cln-1 and -2 mRNA at 28 h (Fig. S3 *B* and *C*). These results indicate that low concentrations of ouabain increase the expression of three claudins (Fig. 2), an observation that correlates with the enhanced staining seen at cell–cell junctions. Additionally, at least for cln-1, the increased expression is correlated with an increase in mRNA levels as well as transcription (Fig. 3).

Signaling Pathways. Next we wanted to identify which of the several signaling pathways that have been linked to the regulation of paracellular permeability would be involved in the effects of low concentrations of ouabain (26–32). Fig. 3*A* [column 3 (throughout, references to column numbers are from left to right)] shows that ouabain increases TER when added from the basolateral but not from the apical side (Fig. 3*A*, column 2), suggesting that the ouabain receptor is on the basolateral surface.

Ouabain may affect several different signaling pathways, such as those involving c-Src and ERK1/2 (31–37). Therefore, we tested whether ouabain affects c-Src and ERK1/2 phosphorylation. As shown in Fig. 3 *B* and *C* (columns 1 and 2), ouabain was able to increase both. Furthermore, at 45 min, ERK1/2 phosphorylation depends on c-Src (Fig. 3*C*, columns 3 and 4). Therefore, we next studied whether the changes in expression of claudins induced by ouabain are affected by inhibition of c-Src and/or ERK1/2 using PP2 and PD98059 (commercially available inhibitors of c-Src and ERK1/2, respectively). Cln-1 expression is highly sensitive to the inhibition of c-Src and ERK1/2 (Fig. 4 *A* and *B*, columns 4 and 6), but cln-4 expression only depends on ERK1/2 (Fig. 4 *C* and *D*,

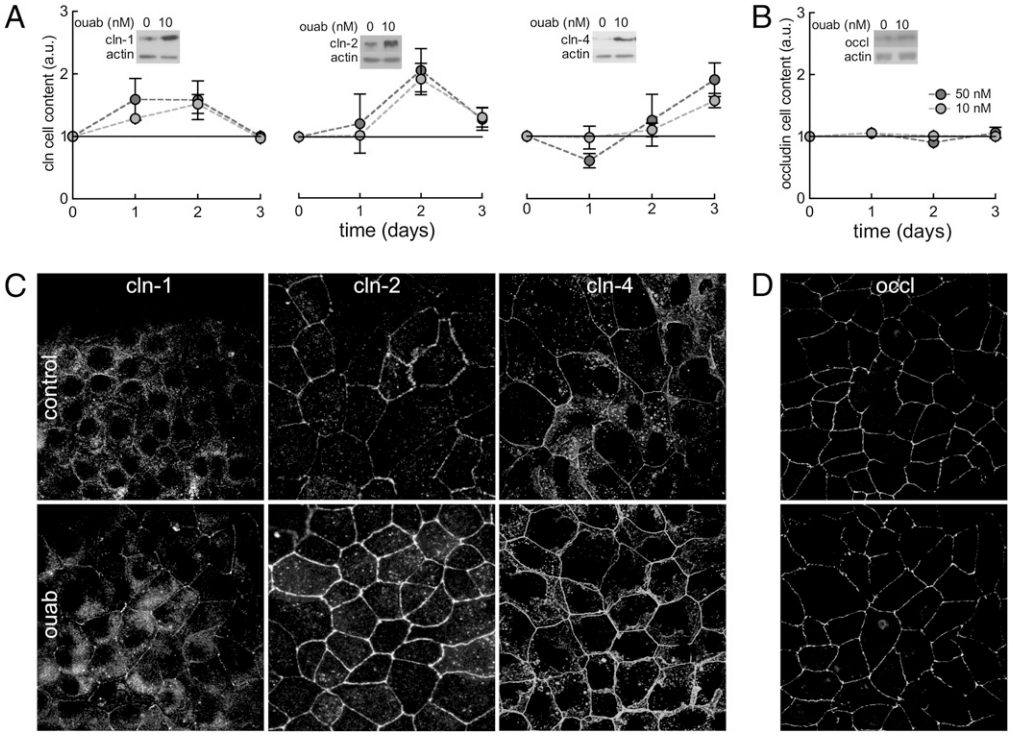

Fig. 2. Ouabain at 10 nM modifies the protein content and distribution of claudins. (*A*) Cell extracts from MDCK cells treated with or without ouabain for 1, 2, or 3 d were analyzed by Western blot. Values of the curves are mean ± SEM. For cln-1, $n = 4$, except for time = 0, where $n = 3$ for each condition and each point; for cln-2, $n = 3$–6; and for cln-4, $n = 5$, except for time = 0 for each time point at each condition. (*Insets*) Representative images of cln-1 and -2 as observed on the second day and cln-4 on the third day. This and the following images are composed by a set of upper bands corresponding to claudins, and lower ones to actin, which were obtained from the same membrane after stripping and blotting against this protein. (*B*) Expression of occludin over 3 d (blot image taken on the second day). Values are mean ± SEM, $n = 4$ for each time point at each condition. The representative image is a composite one, as in the case of claudins. (*C*) Representative images of claudins 1, 2, and 4, as well as (*D*) occludin, as observed by confocal microscopy on the second day after treatment with or without 10 nM ouabain.

columns 5 and 6). These results demonstrate that ouabain increases the level of cln-1 via c-Src and ERK1/2 activation, whereas the increase in cln-4 only depends on ERK1/2 activation. Taken together, these results indicate that ouabain modulates TJ in a very sensitive and complex manner, because each claudin isoform is controlled in a different way.

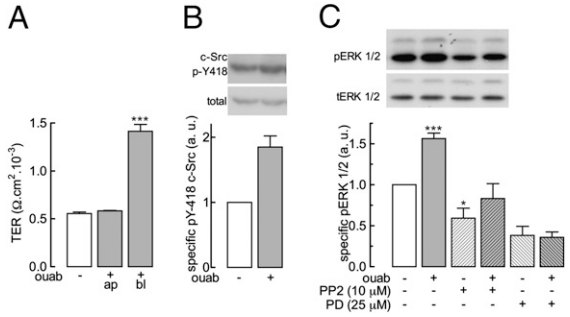

Fig. 3. Ouabain at 10 nM stimulates tyrosine phosphorylation of c-Src and ERK1/2. (*A*) Ouabain was added from the apical (ap, second column) or basolateral (bl, third column) side of the monolayer, and TER was measured on the second day. Values are mean ± SEM, $n = 3$ for each time point at each condition. (*B*) Representative images of Western blots for c-Src phosphorylated at Y-418 (p-Y418) and total c-Src in the presence or absence of ouabain for 3 min. Values are mean ± SEM, $n = 6$ for each condition. This and the following images are composed by a set of upper bands corresponding to c-Src Y418, and lower ones to total c-Src, which were obtained from the same membrane after stripping and blotting against this protein. (*C*) Western blots and statistical analysis of ERK1/2 phosphorylation (pERK1/2) in the presence and absence of ouabain ($n = 14$) and in the presence of PP2 ($n = 7$) or PD98059 ($n = 3$). Values are mean ± SEM. This and following images are composed as explained in *C*, except that upper bands correspond to phosphorylated ERK1/2, and lower ones to total ERK1/2, which were obtained from the same membrane after stripping and blotting against this protein.

Once we observed changes in the level of claudin expression in the presence and absence of inhibitors PP2 and PD98059, we next explored the participation of c-Src and ERK1/2 signaling in the regulation of ionic and nonionic permeability (reflected by TER and dextran flux, respectively). Inhibition of c-Src with PP2 decreases TER and reverses the increase in TER induced by ouabain (Fig. 5*A*, columns 3 and 4). ERK1/2 inhibition with PD98059 does not affect basal TER but partially suppresses the stimulation of TER induced by ouabain (Fig. 5*A*, columns 5 and 6). These results indicate that basal ionic paracellular permeability is regulated by c-Src, whereas the increase in TER induced by ouabain depends on both c-Src and partially on ERK1/2 activation. The nonionic permeability reflected by J_{DEX} is regulated instead by ERK1/2 (Fig. 5*A*, columns 5 and 6) but not c-Src activation (Fig. 5*A*, columns 3 and 4). These results further indicate that ouabain modulates the passage of ions and nonionic molecules in different manners.

Discussion

Endogenous ouabain was found to be synthesized and stored in the adrenal cortex (38) and the hypothalamus (39, 40). Its concentration is increased under physiological conditions, for example during exercise (5), and under pathological conditions such as hypertension (6–9) and eclampsia (10). This led some researchers to propose that it may act as a hormone (41). Thus, it is necessary to investigate its physiological role and molecular mechanism of action. In previous work we found that high concentrations of ouabain (e.g., 1 μM) trigger cell detachment (11) and hypothesized that

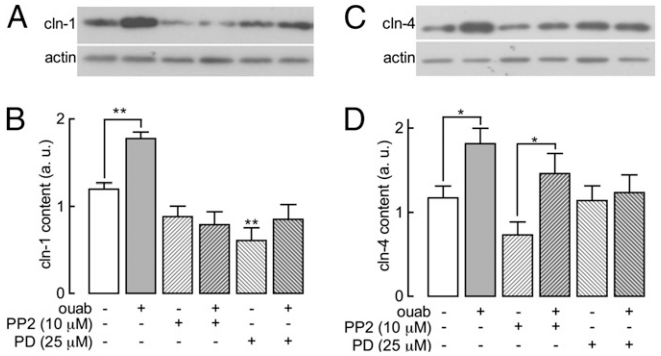

Fig. 4. c-Src and ERK1/2 modulate the effect of ouabain on claudin expression. (*A*) Representative image of a Western blot of cln-1 in extracts from cells incubated with or without ouabain for 2 d in the presence or absence of PP2 or PD98059. This and the following images are composed by a set of upper bands corresponding to claudins, and lower ones to actin, which were obtained from the same membrane after stripping and blotting against this protein. (*B*) Statistical analysis of Western blots for cln-1. Values are mean ± SEM, $n = 6$ for each condition. (*C*) Western blot of cln-4 of cells incubated under control conditions or in the presence of ouabain for 3 d, with or without PP2 or PD98059. (*D*) Statistical analysis of Western blots for cln-4. Values are mean ± SEM. $n = 8$ for control condition (with or without ouabain); $n = 8$ for PP2; $n = 6$ for PD98059.

lower concentrations of ouabain may also act to modulate cell–cell contacts. In the present work, we have tested this possibility, focusing on the TJ, a special type of cell–cell contact. We found that 10 nM ouabain does not inhibit the pump, disrupt cellular ionic content, or detach the cell from its neighbors or substrate (Fig. 1 *A* and *B* and Fig. S1). Ouabain at 10–50 nM produces an increase in TER that lasts for at least 3 d (Fig. 1*C*). This tightening of the TJ is also reflected by a decrease in the flux of 3 kDa dextran through the paracellular permeation pathway (Fig. 1*D*).

The ionic permeability of the TJ has been correlated with claudin expression (42). Similarly, the expression of different claudin isoforms has been shown to affect TER in MDCK cells (43). Overexpression of cln-1 and -4 increases TER (44, 45), whereas cln-2 overexpression decreases TER (46). Our results show that 10 nM or 50 nM of ouabain increases TER as well as the expression of cln-1, -2, and -4 but not of occludin (Figs. 1 and 2), suggesting that TER is not only modulated by the absolute levels of specific claudins but also by their relative abundances (i.e., ratios to one another). It is important to note that different clones of MDCK cells produce monolayers with different TER values, owing to the expression of different claudin isoforms (43). In the present study, we used a batch of cells that produce monolayers with relative low TER (≈450 Ω·cm^2), and it is possible that 10 or 50 nM ouabain may have different effects on monolayers with higher initial TER values. However, we expect that ouabain would modulate the cell–cell contacts made by the TJ regardless of TER value.

We also observe an increase of Luciferase transcription driven by the cln-1 promoter, indicating that ouabain can regulates transcription factors. Cln-1 transcription has been shown to be regulated by several signaling pathways, including β-catenin-Tcf/LEF, Snail, SP1 and SP3, p63, and RUNX3, in different cellular contexts (47–50). Therefore, future studies should address which transcription factors mediate the effects of ouabain on cln-1 as well as examine changes in the expression of cln-2 and cln-4.

The fact that ouabain's effect is elicited when it is added to the basolateral side of the epithelial layer (Fig. 3*A*) suggests that its receptor is accessible on this surface. Therefore, it is likely that the effects of ouabain were mediated by the Na^+,K^+-ATPase, which is located on the basolateral side (51). Ouabain is also known to activate multiple signaling pathways, including c-Src and ERK1/2 (31–34, 36, 37). Coincidentally, similar signaling pathways are known to regulate TJ function. For example, increases in ERK1/2 activity enhance the expression of cln-1 and the value of TER (52–54). Overexpression of the oncogene Raf-1 in Pa-4 cells (rat salivary gland epithelial cells) increases ERK1/2 phosphorylation but down-regulates the expression of cln-1 and disrupts the TJ (55). In MDCK cells, inhibition of ERK1/2 with PD98059 recruits cln-1 and other TJ proteins to cell–cell adhesion sites and favors the assembly of the TJ (56). Furthermore, we have also observed that EGF modulates cln-4 expression through ERK1/2 (52). It has also been observed that c-Src can regulate the expression of claudins. For example, inhibition of c-Src with PP2 decreases cln-4 protein content in acinar cells (57). Here, we show that in MDCK cells PP2 also reduces the cellular content of cln-4 per se, in the absence of ouabain (52). Furthermore, we found that the presence of PP2 and PD98059 is able to inhibit the effect of ouabain on cln-1 expression (Fig. 4*A*), whereas only PD98059 inhibits the effect of ouabain on cln-4 (Fig. 4*B*). Thus, we have shown that ouabain can activate multiple signaling pathways that converge on ERK1/2 to regulate cln-1 and -4 expression (Fig. 6). The effect of ouabain on ionic permeability is regulated by PP2 and partially by PD98059 (Fig. 5*A*). In the case of paracellular permeability to noncharged molecules (dextran), the inhibition of ERK1/2 is sufficient to inhibit the effect of ouabain (Fig. 5*B*), indicating that c-Src is not involved in the regulation of nonionic permeability. Taken together, these results show that ouabain is able to independently regulate the flux of ions and noncharged substances, emphasizing the fact that ouabain modulates several properties of cell–cell contacts. Furthermore, we have linked the effect of low concentrations of ouabain to TJ protein expression and function, as well as to c-Src and ERK1/2 signaling pathways, suggesting that the activation of ERK1/2 by ouabain involves c-Src–dependent and -independent routes. These as-yet-unknown upstream signaling proteins need to be identified in future work.

Should ouabain in fact act as a hormone, its modulating effect on cell–cell contacts would indeed play a very important physiological role.

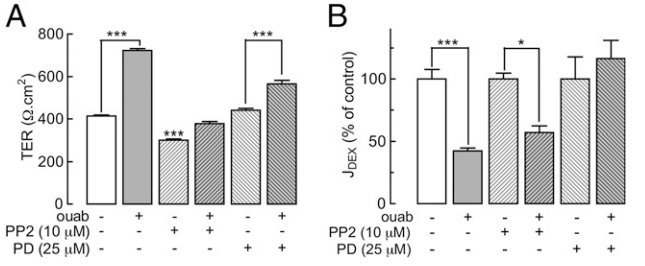

Fig. 5. c-Src and ERK1/2 modulate the effect of ouabain on TER and J_{DEX}. (*A*) TER across monolayers of MDCK cells incubated with or without ouabain ($n = 23$), in the absence (columns 1 and 2) or presence of PP2 ($n = 18$, columns 3 and 4) or PD98059 ($n = 17$ to 18, columns 5 and 6). Values are mean ± SEM. (*B*) J_{DEX} was determined under analogous conditions. For control conditions or in the presence of ouabain, $n = 14$–16; for PP2, $n = 8$–9; for PD98059, $n = 8$–10. Values are mean ± SEM.

Materials and Methods

Cell Culture. Starter MDCK-II cultures were obtained from the American Type Culture Collection (MDCK, CCL-34) and maintained in DMEM supplemented (CDMEM) with 10% bovine serum and 10,000 U/mg per milliliter penicillin-streptomycin (in vitro). Cells were harvested with trypsin-EDTA and plated on Transwell permeable supports (≈2×10^5 cells/cm^2; Corning Costar) and maintained for 3 d in CDMEM. The concentration of serum was reduced from 10% to 1% for 24 h, and the monolayer was then challenged with or without ouabain. Inhibitors were added 1 h before ouabain and renewed daily, remaining present throughout. Their effect was also checked by Western blot analysis of the target protein.

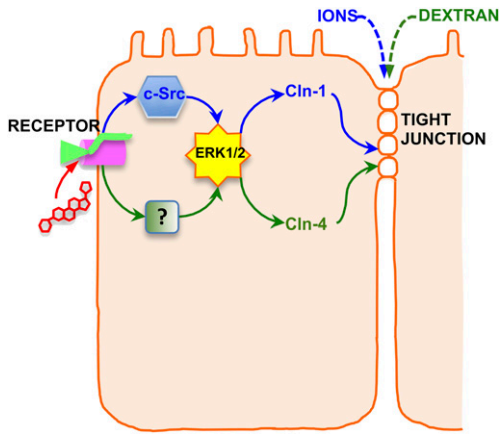

Fig. 6. Schematic representation of the signaling pathways affected by low concentration of ouabain to regulate TJ protein expression and function. Ouabain (red silhouette) activates ERK1/2 via c-Src–dependent and -independent (green square) pathways, which in turn regulate the expression and localization of cln-1 and -4. The ouabain receptor is likely to be Na^+, K^+-ATPase located on the basolateral surface. Regulation of cln-1 depends on c-Src and ERK1/2. In turn, regulation of ionic permeability or TER depends on c-Src and partially on ERK1/2. The c-Src–independent activation of ERK1/2 leads to the regulation of cln-4 and nonionic permeability or J_{DEX}.

K^+ Uptake. MDCK cells were plated in 24-well chambers as indicated above and incubated with or without ouabain (10 nM and 1 μM) for 3 h. At the end of this period they were incubated for 3 min in similar media containing ^{86}Rb (1 mCi/mL; Perkin Elmer Life and Analytical Science, NEZ072) and processed as described previously (14–16).

Intracellular K Content. The amount of potassium in the cell compartment (in micromoles) is equal to the amount of tracer accumulated at equilibrium (cpm = cpm) divided by the specific activity of the tracer (cpm/μmole). In previous experiments, we have shown that ^{86}Rb is a valid tracer for K, and its specific activity equilibrates with the tracer added to the bathing solution in a few hours (this process requires less than 3 h in MDCK cells). Furthermore, at equilibrium the specific activity of ^{86}Rb in the cells is equal to that in the bathing solution. Therefore, to measure K content in MDCK monolayers, these cells were incubated overnight with a medium containing ^{86}Rb before samples were taken. At the end of this period, monolayers were rinsed with ice-cold PBS, blotted, and subjected to ^{86}Rb counts; the bathing solution was also sampled to count ^{86}Rb. These correlations are valid and independent of experimental conditions (for details and examples see refs. 14, 16, and 58).

Transepithelial Electrical Resistance. The degree of TJ permeability to ionic solutes was assessed by measuring the TER of the cells grown on Transwell permeable supports using an EVOM (Epithelial Volt Hom Meter; World Precision Instruments). TER was measured before and after 1, 2, and 3 d of ouabain treatment. Final values were obtained by subtracting the resistance of the bathing solution and an empty support. Results are expressed as ohms per square centimeter ($\Omega \cdot cm^2$) (26, 52).

Transepithelial Flux of Dextran (J_{DEX}). Paracellular permeability was assessed by FITC-dextran (3 kDa) flux through confluent monolayers treated for 1, 2, and 3 d with ouabain. After TER measurement, upper and lower Transwell compartments were washed twice with P buffer [10 mM hepes (pH 7.4), 1 mM sodium pyruvate, 10 mM glucose, 3 mM $CaCl_2$, and 145 mM $NaCl_2$]. A freshly prepared solution containing 10 μg/mL of FITC-dextran dissolved in P buffer was added to the apical compartment. The bottom chamber only contained P buffer with or without ouabain. After 1 h incubation at 37 °C, the basal medium was collected and the fluorescence of the transported FITC-dextran was measured with an LS-3B fluorescence spectrometer (Perkin-Elmer) at 492 nm (excitation) and 520 nm (emission). The quantity of FITC was calculated by comparison with a standard curve. The unidirectional flux of dextran in the apical to basolateral direction (J_{DEX} in $ng \cdot cm^{-2} \cdot h^{-1}$) was calculated by dividing the fluorescence intensity of a given sample of the bottom solution (arbitrary units) by the corresponding value of the upper solution (at convenient dilutions).

Generally TER and permeability vary in opposite directions. Nevertheless, variations in the same directions are possible and have been observed in MDCK monolayers (28, 29).

Reporter Assays. MDCK cells were plated into 48-well dishes at low (2,000 cells per well) concentrations 1 d before transfection. To measure cln-1 promoter activity, two plasmids were cotransfected using the calcium phosphate method: one with a cln-1 promoter (−748 to +252) in a pGL3 vector driving Luciferase expression, kindly provided by Dr. Amparo Cano (59), and the other a pRL-CMV plasmid driving Renilla expression to normalize transfection (60). Total DNA concentrations were kept constant with empty vectors. Once cells were transfected, they were treated with or without ouabain for 10–12 h. This medium was removed, and cells were scraped using the Dual-Luciferase kit according to the manufacturer's directions (Promega). Luminescence analysis was carried out using the Fluoroskan Ascent FL (Thermo Scientific). The relative luciferase activity was calculated as amount of Luciferase/amount of Renilla. The control value was set to 1.

Statistical Analysis. All statistical analyses were performed with GraphPad Prism 4 software. The results are expressed as mean ± SE. Statistical significance in a one-way ANOVA followed by a Bonferroni multiple or selected pair comparisons tests is indicated as follows: $*P < 0.05$, $**P < 0.005$, and $***P < 0.001$. n = number of observations of a given monolayer. To compare effects obtained in different monolayers on different days, we normalized expression data according to the actin content simultaneously measured in the same sample.

SI Materials and Methods provides descriptions of antibodies, reagents, immunofluorescence, immunoblot, caspase-3 activity, flow cytometry (annexin V and TUNEL assay), plasma membrane surface measurement, and mRNA.

ACKNOWLEDGMENTS. We thank Elizabeth del Oso, Eduardo Méndez, and Oscar Medina for their contributions on this project. I.L., D.F.-B., R.R.-H., and T.P.-B. are Fellows of the National Research Council of México and the Instituto de Ciencia y Tecnología del Distrito Federal, which provide financial support for their work.

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