J. Membrane Biol. 198, 147–158 (2004)
DOI: 10.1007/s00232-004-0670-2

The Journal of
Membrane
Biology

Ouabain Binding to Na^+,K^+-ATPase Relaxes Cell Attachment and Sends a Specific Signal (NACos) to the Nucleus

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Received: 29 November 2003/Revised: 1 March 2004

Abstract. In previous work we described a "P → A mechanism" that transduces occupancy of the pump (*P*) by ouabain into changes in phosphorylation, stimulation of mitogen-activated protein kinase (MAPK), and endocytosis of cell-cell- and cell-substrate-attaching molecules (*A*), thereby causing a release of the cell from the monolayer. In the present work we try to understand the mechanism of this effect; whether, in order to trigger the P → A mechanism, ouabain should block the pumping activity of Na^+,K^+-ATPase as pump, or whether it would suffice that the drug occupies this enzyme as a receptor. We assay a series of drugs known to act on the pump, such as ouabain, digoxin, digitoxin, palytoxin, oligomycin, strophanthidin, neothyoside-A, proscillaridin-A, etc. We gauge their ability to block the pump by measuring the K^+ content in the cells, and their ability to detach the cells from the monolayer by determining the amount of protein remaining in the culturing well. None of the drugs tested was able to cause detachment without stopping the pump. Ouabain also enhances phosphorylation, yet pump inhibition and signal transduction do not seem to be intimately associated in a causal chain, but to occur simultaneously. To investigate the response of the site of cell attachment, we analyze the position of β-catenin by fluorescence confocal microscopy, and find that this adherent junction-associated molecule is sent to the nucleus, where it is known to act as a transcriptional cofactor.

Key words: Na^+,K^+-ATPase — Ouabain — Ion balance — NACos — Adhesion — MAPK — Signaling to the nucleus

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Introduction

The membrane enzyme Na^+,K^+-ATPase uses ATP energy to pump 2 K^+ and 3 Na^+ in opposite directions, thereby creating a ionic asymmetry that is responsible for the electrochemical potential difference of Na^+ and K^+ across the cell membrane. In turn, this potential drives the net movement of substances such as glucose, amino acids, Ca^{2+}, H^+ and Cl^-. Both, pumping and enzymatic activity can be specifically inhibited by the cardiac glycoside ouabain (Schatzmann, 1953; Skou, 1960), that binds to an extracellular site of its α subunit with a high affinity ($K_m < 10^{-8}$ M). In a previous work (Contreras et al., 1999) we have found that ouabain binding to the pump (*P*) triggers a process that we refer to as "P → A mechanism", which releases the grip of attaching molecules (*A*) to neighboring cells and to the substrate, and causes cell detachment.

In the present work we pursue the characterization of the P → A mechanism because of the potential roles that it may play, considering that:

(*a*) Damaged epithelial cells endanger the permeability barrier between higher organisms and the environment. An injured cell would conceivably produce less ATP, the activity of the Na^+,K^+-ATPase would decrease, thus triggering the P → A mechanism that promotes the cell's detachment and replacement. In fact, competition for attachment is a very well known property of epithelial cells; thus, the apoptosis of a given cell in a cultured epithelial monolayer causes a typical rosette formed by surrounding healthy cells, which are in the process of competing for and invading the territory being released by the dying one (Peralta et al., 1996).

(*b*) Cells can assemble Na^+,K^+-ATPase with at least 4 different types of α subunits that differ in their sensitivity to ouabain (Blanco & Mercer, 1998; Jorgensen, 2001). This suggests that in a given tissue,

cells might have a wide range of susceptibilities to detachment, and that the P → A mechanism would influence the architecture of the tissue.

(*c*) Actually, the response to ouabain challenge depends on the type of cell involved. Thus it may cause cell death in wild-type MDCK cells from dogs, but not in R-MDCK, which do not bind the drug, nor in Ma104 from monkeys that do bind ouabain but fail to retrieve their attachment-associated molecules and remain bound to each other and to the support. R-MDCK and Ma104 cells may even protect the dog MDCK cells in co-cultures (Bolivar et al., 1987; Contreras et al., 1995a, 1995b; Pchejetski et al., 2003; Valente et al., 2003).

(*d*) Conceivably, detachment and removal of a given cell type may also be promoted by endogenous ouabain-like substances whose plasma levels are known to increase in some diseases (Schoner, 2002).

(*e*) In this respect, it may be worth taking into account that metastases start by a detachment of cancer cells from the mass of the main tumor. Therefore one can envisage a situation in which a patient with cancer and who is also affected with heart failure, would enhance his proclivity to develop metastases if treated with ouabain in the classical manner.

(*f*) Lowering the concentration of Ca^{2+} in the bathing solution relaxes the cell-cell grip between cells, and causes specific junction-associated molecules called ''NACos'' (nuclear attachment complexes) to shuttle to the nucleus (Balda & Matter, 2003), indicating that the P → A mechanism may play roles other than just dislodging cells, such as cell differentiation, proliferation, etc.

The specific aim of the present work is to determine whether, in order to produce detachment, ouabain must stop the pump, or whether it would suffice that it occupies this membrane enzyme and triggers second messengers as if it were a hormonal receptor, and whether in response the affected cell-attachment site reduces its tenacity, or transforms itself into a relay station that sends signals to the nucleus.

Materials and Methods

Cell Culture

Starter MDCK cell cultures (epithelial, dog kidney) were obtained from the American Type Culture Collection (MDCK, CCL-34). Upon arrival, cells were cloned and all experiments reported here were performed in cells of Clone 7, chosen because of its intense blistering activity when plated on impermeable supports. Cells were grown at 36.5°C in disposable plastic bottles (Costar 3250) in a 5% CO_2 atmosphere (Forma Scientific CO_2 incubator, Steri-Cult 200). We used Dulbecco's modified Eagle's medium (Gibco 430-1600), with 100 U/ml penicillin, 100 mg/ml of streptomycin (Gibco 600-5145), 0.8 U/ml insulin (Eli Lilly) and 10% fetal calf serum (Gibco 200-6170). This medium will be referred to as CDMEM. When we modify the concentration of K^+, cells were incubated in MEM (In Vitro, México, 020813, 020822) without serum. Cells were harvested with trypsin-EDTA and plated on dishes. Cells used for the experiments reported here were usually of the 60th–80th passage. They were also freed of potential contaminations with *Mycoplasma*, through a treatment with BM-Cyclin 1 and 2, according to manufacturer-recommended procedures (Boehringer, Cat. No. 799 050), followed by further cloning in the presence of 0.5 $\mu g \cdot ml^{-1}$ of *Mycoplasma*-removal agent (MRA, ICN, Cat. No. 30-500-44). Experimental manipulations were performed in media without antibiotics.

Ouabain-tested Strains of MDCK Cells

Cultures of a given cell type can be contaminated with cells of a different type. A contamination of wild (W-MDCK) with ouabain-resistant ones (R-MDCK) or vice versa would seriously distort our results due to the phenomenon of cell-cell cooperation (Ledbetter & Lubin, 1979; Bolivar et al., 1987). Thus, a monolayer of reputedly R-MDCK cells might be in principle contaminated with up to 60% of W-MDCK ones, which resist high concentrations of ouabain because they are being rescued by the R-MDCK cells. Conversely, a monolayer of R-MDCK cells might appear to be vulnerable to relatively mild concentrations of the drug due to the presence of a sizable subpopulation of W-MDCK cells.

To insure that W-MDCK and R-MDCK cells are not contaminated with each other, we cloned them twice and plated the cells at a high dilution in 96-well multidishes with flat bottom, obtaining sister mini-monolayers. We took 40 monolayers derived from single W-MDCK cells, and divided them into two populations: 20 were treated overnight with 1.0 μM ouabain and the rest were left as control. If the twenty treated monolayers did not resist the drug and detached, the untreated counterparts were taken as truly W-MDCK. Conversely, if the 20 of the twice-cloned R-MDCK monolayers of cells resisted the drug, one of them was taken as parental batch of pure R-MDCK cells.

Cell Adhesion

To study the effect of ouabain and ouabain-like drugs on cell attachment, cells were plated on 24-well plates. At the end of this incubation period, cells were washed 3 times with ice-cold PBS to discard detached cells. The cells that remained attached were then extracted with 200 μl of 1% solution of sodium dodecyl sulfate (diluted in water), and the bottom of the well was scraped with a blue micropipette tip. The extract was then passed 10 times through a number-10 needle and proteins were measured on 2 μl samples with a Bicinchoninic Acid assay (BCA, Pierce, No. 23225) to quantify the cells attached to the well. The protein concentration is directly proportional to the amount of cells attached to the well, therefore, we express cell adhesion in protein concentration units.

Measurement of Ion Content

Monolayers of 2 cm^2, plated in Linbro 24-well chambers, were incubated overnight with medium containing ^{22}Na (Amersham, SKS1), or ^{36}Cl (Amersham, CLS3). This period is long enough to equilibrate the specific activity in the cells and in the bathing solutions (Cereijido et al., 1981). Monolayers were then washed five times with ice-cold 0.1 M $MgCl_2$, the last wash lasting 1.0 min, and extracted with 500 μl of 0.5 M NaOH for 2 h. ^{22}Na or ^{36}Cl radioactivity was determined in 400 μl samples added to 10 ml Aquasol (New England Nuclear) and counted in a beta counter. The protein concentration was measured by the BCA method.

K was analyzed in detergent extracts of monolayers plated in 24-well chambers. Each monolayer was extracted with 100 μl of 1%

SDS. After measuring protein concentration as indicated above, 80 μl of the extract were diluted with 1 ml of 1 N HNO_3, 1 ml of 10 mg · ml^{-1} $CsCl_2$ and water to complete a 3 ml final volume. These extracts were measured with an atomic emission spectrometer (Perkin-Elmer, No. 3100).

Ca^{2+} was measured with the calcium indicator fura-2 acetoxymethyl ester (fura-2/AM). MDCK cells were plated on a 60-mm tissue culture plate containing four or five glass cover slips (0.8 × 2.5 cm). After a 20-h incubation, cover slips with confluent monolayers were transferred to new culture plates with fresh medium containing 1.0 μM fura-2/AM previously dissolved in dimethyl sulfoxide, and incubated for 30 minutes at 37°C with continuous agitation in an orbital shaker. Monolayers were then washed seven times with a buffer containing (in mM): 140 NaCl, 5 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 20 Tris, 10 dextrose, 0.05 probenecid and 1 μM ouabain when needed. Cover slips were mounted in a Teflon stopper (Gonzalez-Mariscal et al., 1990) and placed inside plastic fluorometric cuvettes in a 30° angle with respect to the excitation source. The cuvette contained 3.0 ml of buffer with or without 1 μM ouabain, depending on the experiment. Fluorescence measurements were performed in a Perkin-Elmer LS-3B fluorometer. The excitation/emission wavelengths used to monitor dye fluorescence were 338/510 nm. Cytosolic Ca^{2+} was calculated according to the following general formula: $(Ca^{2+})_c = K_d\,(F - F_{min})/(F_{max} - F)$, where K_d is the dissociation constant for Ca^{2+} binding (224 nM for fura-2), F the initial fluorescence determination, F_{max} the maximal fluorescence obtained with 5.0 μM ionomycin, F_{min} the minimum fluorescence calculated after the addition of 2.0 mM $MnCl_2$ (F_{Mn}), according to the following equation: $(F_{min} = 1/6\ [F_{max} - F_{Mn}] + F_{Mn})$ (Gonzalez-Mariscal et al., 1990, Contreras et al., 1992).

Gauging of Extracellular Regulated Kinase ERK1/2 (MAPK)

Monolayers plated in 24 multiwell plates were washed three times with ice-cold phosphate-buffered saline solution (PBS, GIBCOBRL 21300-058), and then extracted for 30 minutes with 70 μl of cold lysis buffer, containing (in mM): 10 Tris, 1 Na_3VO_4, plus 1% SDS, pH 7.4. The extract was recovered with rubber policeman, forced 10 times through a 22-gauge syringe and centrifuged 20 minutes at 20,800 × g. The supernatant was obtained, its protein concentration measured, subsequently boiled in Laemmli sample buffer and resolved by SDS-polyacrylamide gel electrophoresis. SDS-PAGE-resolved proteins were transferred electrophoretically to PVDF sheets (Hybond, Amersham). These sheets were blocked with 3% BSA, and proteins of interest were detected with polyclonal antibodies against active MAPK and ERK1/2 (Promega cat. number V803A and V114A, respectively), followed by donkey anti-rabbit peroxidase-conjugated antibodies (Promega cat. Number V795A) and a chemiluminescent system for detection (ECL Amersham). The resolved bands were then quantitated with a densitometer (EDAS, Kodak).

Immunofluorescence

Glass coverslips containing cells cultured under the several experimental conditions described below were rinsed twice with PBS, fixed 20 min with 2% paraformaldehyde in ice-cold PBS and permeabilized with methanol at −20°C for 45 s, rinsed with PBS, incubated with 3% bovine serum albumin in PBS for 30 min, and treated for 1.0 h with a specific first antibody. Monolayers were then rinsed 3 times with PBS for 5 min each, incubated with a suitable FITC-labeled goat antibody for 30 min, rinsed as above, incubated 5 min with 10 μg/ml of propidium iodine in water, rinsed again, mounted in Fluorguard (Bio-Rad, Hercules, CA) and examined with a confocal microscope (Leica SP2, Leica, Hiena, Germany). Antibody against β-catenin was purchased from ZYMED (13-8400).

Measurement of β-Catenin Tyrosine Phosphorylation

Cells were plated on Petri dishes (6 cm diameter), incubated as indicated in Results and Figure Legends, washed twice with ice-cold TBS (10 mM Tris-HCl pH 7.5,100 mM NaCl) and scraped with 500 μl of RIPA buffer (1% NP40, DOC 1%, SDS 0.1%, 150 mM NaCl, 10 mM Tris-HCl pH 7.4, 1 mM PMSF, 4 mM sodium orthovanadate, 40 mM β-glycerophosphate, 30 mM NaF, 30 mM benzamidin), supplemented with a protease inhibitor cocktail (Complete, Roche 1697498). The extracts were then forced 10 times through a 22-gauge syringe, centrifuged 20 min at 20,800 × g and the supernatants were pre-cleared by incubation with 15 μl of sepharose 4B beads coupled to goat anti-mouse antibodies (Zymed 62-6541) during 1.5 h at 4°C, with gentle agitation. Centrifugations and incubations were similarly performed in the following steps. The beads were removed by centrifugation and supernatants incubated overnight with 2.5 μg of a monoclonal anti-β-catenin antibody (Zymed 13-8400) and then 3 h with 15 μl of the sepharose beads. Precipitates were washed three times with washing buffer (50 mM Tris-HCl pH 8.5, 50 mM NaCl, 0.05% Na_3VO_4), re-suspended in Laemmli sample buffer to be separated by SDS-PAGE and blotted with monoclonal anti-phosphotyrosine antibody (Ptyr 100, Cell Signaling 9411) as indicated above.

Measurement of the Transepithelial Electrical Resistance

The degree of sealing of tight junctions (TJs) was assessed by measuring the transepithelial electrical resistance (TER) (Cereijido et al., 1978; Gonzalez-Mariscal et al., 1985). After incubation under a given condition, the filter with the monolayer was mounted as a flat sheet between two Lucite chambers with an exposed area of 0.69 cm^2. Current was delivered via Ag/AgCl electrodes placed at 2.0 cm from the monolayer; the voltage deflection elicited was measured with a second set of electrodes placed at 1.0 mm from the membrane. Values of TER reported were obtained by subtracting the contribution of the filter and the bathing solution. A given monolayer was used only for a single determination and discarded to avoid leaks due to edge damage.

Chemicals

As described below, we have tested ouabain (Sigma O-3125), and a series of ouabain-like substances: digitoxin (Sigma D-5878), digoxin (Sigma D-6003), strophanthidin (Sigma S-6626), proscillaridin A (Sigma P-2428), Neothyoside A (a generous gift from Dr. Rosalba Encarnación from the Marine Biology Department, University of Baja California (Encarnacion et al., 2004)). PST 2238 and PST 2744 are digitoxigenin derivatives selective for Na^+,K^+-ATPase. The first has strong anti-hypertensive activity in rat models (Quadri et al., 1997; Ferrari et al., 1998). We have also tested Oligomycin (ICN 1404-19-9), a macrolide antibiotic, which inhibits Na^+,K^+-ATPase by stabilizing Na^+ occlusion (Homareda et al., 2000), and palytoxin (Sigma P4954), which converts the Na^+,K^+-ATPase into a cation-selective channel (Hirsh & Wu, 1997).

Results are expressed as average ± standard error of the mean.

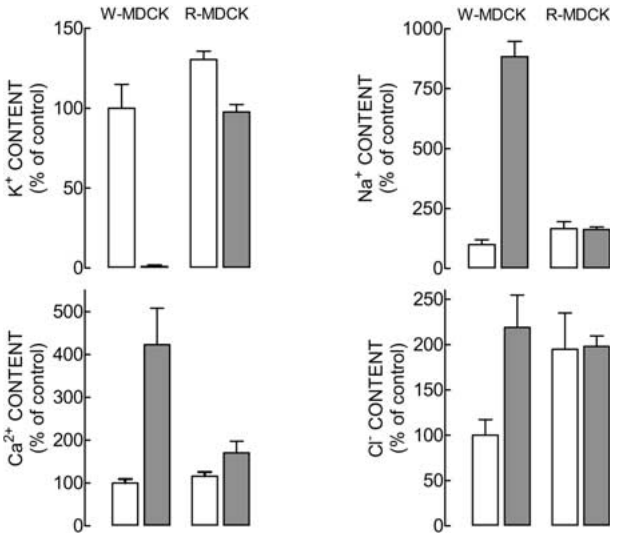

Fig. 1. Ouabain and the cellular content of major ions in wild (W-MDCK) and ouabain-resistant (R-MDCK) cells. Monolayers were exposed to ouabain for 6 h. Unless otherwise stated, the drug was used at 1.0 μM. K^+ was measured by atomic absorption, Na^+ and Cl^- through overnight pre-equilibration of the monolayers with a medium containing 0.2 μCi of ^{22}Na or ^{36}Cl per milliliter and counting the emission of β radiation, and Ca^{2+} with fluorometry using fura-2. In each pair of columns the white one refers to control monolayers and the shaded one to ouabain-treated cells, *n* of each column = 3–6.

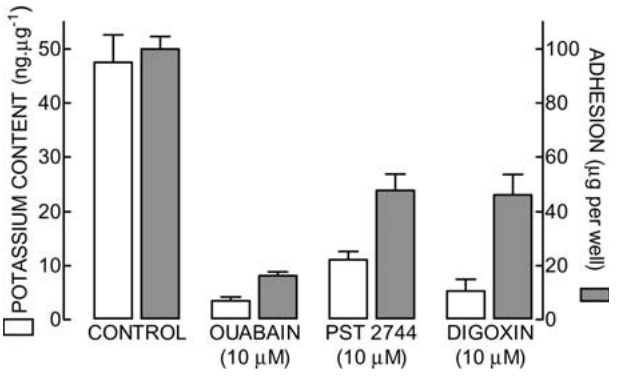

Fig. 2. K^+ content and cell attachment in cells treated with different inhibitors of the pump. Monolayers were incubated with ouabain, PST2744 or Digoxin for 8 hours. All drugs were used at 10 μM.

Results

Effect of Pump-blocking on the Content of Cellular Ions

We gauge the pumping ability of Na^+,K^+-ATPase by the cell content of K, but of course the inhibition of the enzyme distorts the content of other ions as well. Thus, Fig. 1 shows that the decrease of K^+ is also accompanied by the well known increase of Na^+,Ca^{2+} and Cl^-.

Can the "Pump-stopping" and the "P → A-Triggering" Effects of Ouabain Be Dissected?

One of the aims of the present work is to study whether P → A is triggered by blockade of the pump and the ensuing perturbation of ion content, or whether it depends on the sole occupancy of the enzyme. We tested PST 2744, a digitalis analog derived from digitoxigenin, which is selective for the Na^+, K^+-ATPase, and digoxin, a cardenolide digitalis, and observed whether they exhibit pump-stopping as well as P → A-triggering abilities. Figure 2 shows that they produce a cell detachment that is invariably accompanied by a severe arrest of ion pumping (each dark column differs from control with a $P < 0.025$). Yet the relationship between the two types of activities varies from drug to drug, thus the degree of pump-blocking elicited by digoxin does not differ from the one achieved by ouabain, yet the extent of cell detachment is clearly more severe with ouabain than with digoxin ($P > 0.001$). This opens the possibility that, in order to detach, the ionic content should reach a certain minimum level, regardless of how it is achieved.

This led us to refine the strategy, and assay a variety of ouabain-like substances and Na^+,K^+-ATPase ligands but at different doses to produce an ample range of pump inhibition (K^+ loss), and study their effect on cell attachment. Each circle in Fig. 3 corresponds to measurements performed in a single monolayer; figures between parentheses indicate the number of monolayers tested. Although the line was drawn by eye and does not imply a specific mechanism, it can be seen, first, that detachment is always accompanied by a decrease of ion pumping; second, that the chemical nature of the substance itself is irrelevant, and third that, in spite of the large number of samples (overall $n = 206$), detachment is never observed until ion pumping decreases below a certain limit. These results suggest that P → A may be triggered simply by the inhibition of Na^+,K^+-ATPase pumping.

P → A Depends on Permanent Binding of Ouabain

A number of observations of the P → A mechanism found in a previous work (Contreras et al., 1999) and preliminary studies described below, may not be easily accommodated by the picture of a P → A triggered solely by blockade of the pump and proceeding through the ionic imbalance it provokes. Exploring these alternatives becomes important in view that ouabain is claimed to be a hormone (Schoner, 2002), with Na^+,K^+-ATPase acting as its specific receptor. In keeping with such possibility, we have previously found (Contreras et al., 1999) that,

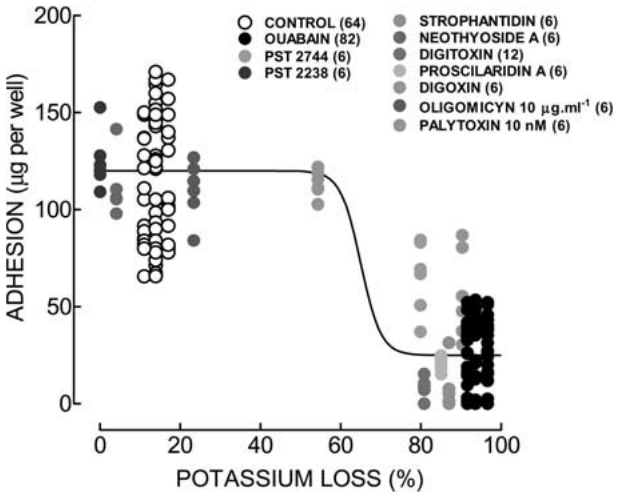

Fig. 3. Cell adhesion as a function of pump-blocking (K loss) elicited by a variety of substances used at several concentrations. Each circle corresponds to a different monolayer, and the number of monolayers tested is given in parentheses on the right-hand side. Since each drug was studied in runs that included two types of controls (untreated cells and those treated with ouabain) the number of observations of these controls is far larger. To ease the already crowded representation of these measurements control values are represented at slightly offset K-losses. Drugs whose concentration is not specified in the figure were tested at 10 μM. The curve was drawn by eye.

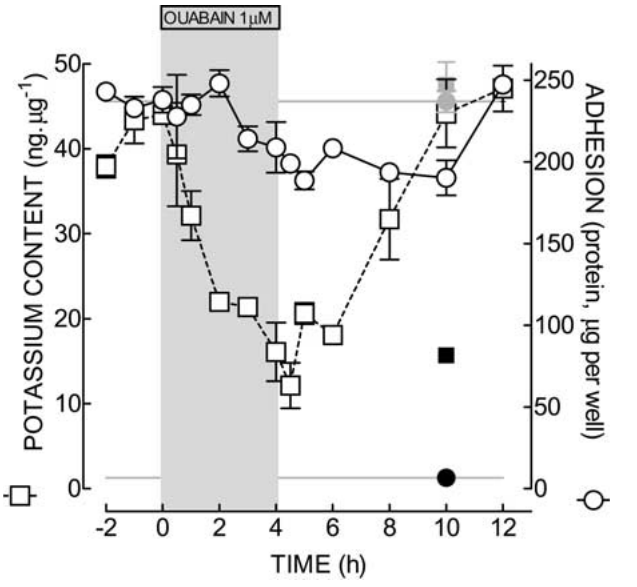

Fig. 4. Effect of a 4-h pulse (*shaded area*) of 1.0 μM ouabain on confluent monolayers of MDCK cells. Previous work from our laboratory has shown that at this concentration ouabain reaches a maximal specific binding and elicits a maximal blockade (Cereijido et al., 1981; Bolivar et al., 1987; Contreras et al., 1989). In four hours, K^+ content dropped by 63% and 20% of the cells detached (*empty circles*). Upon removal of the hormone at this time, $[K^+]$ starts to recover and detachment is halted. *Filled grey* and *black symbols* at the 10th hour correspond to the values of K^+ and adhesion of cells in control monolayers not exposed or continuously exposed to the hormone, respectively. Ouabain was added to the bathing medium in contact with the apical aspect. Yet, as demonstrated by Mullin et al. (1980), the drug readily crosses the monolayer and gains access to the pumps located in the basolateral domain. When the standard error of a given measurement is not shown, it means that it is smaller than the symbol.

once ouabain binds to Na^+,K^+-ATPase, it triggers a cascade of phosphorylations that results in the removal of membrane-attaching molecules, followed by exclusion of the cell from the monolayer. On this basis, it is conceivable that, once ouabain binds to this enzyme for a sufficient time, the triggered cascade will be selfsustaining in spite of the removal of the drug. To investigate this possibility we seeded MDCK cells at confluence in 24-well plates, and next day we administrated 1.0 μM ouabain in a pulse of 4 hours (Fig. 4, *shaded region*). It can be observed that, although the receptor is occupied by its specific ligand, and K^+ content is severely reduced, cells do not detach. This indicates that the process of detachment depends on signaling and/or ionic perturbation in a continuous manner, and suggests that some process downstream is required. There are several observations reported in previous articles that would agree with this view: (*i*) the pump can be inhibited with strophanthidin instead of ouabain, but the phenomenon is reversed with a faster kinetics, due to the weakness of the attachment of strophanthidin to the pump (Contreras et al., 1995b); (*ii*) No apoptosis was detected either before detachment, or in cells collected and spun after detachment (Contreras et al., 1999; Valente et al., 2003); (*iii*) As shown in Fig. 10 *A*, cells detached with ouabain as high as 10 μM can be washed, reseeded and they will grow normally.

Degree and Time Course of Pump Stopping and Cell Detachment

Ouabain inhibits pumping in a concentration-dependent manner (Schatzmann, 1953; Goto & Yamada, 2000), a phenomenon reflected in the time course and extent of K^+ loss (Fig. 5*A*). The effect of ouabain on detachment follows roughly the same pattern (Fig. 5*B*). Interestingly, there is a considerable delay between the blockade of pumping and cell detachment. Thus, at the highest dose of ouabain assayed (*triangles*) the loss of K^+ has virtually reached a steady level by the 3rd hour, yet at this time cells remain attached, and a period 4 times as long (>12 h) is required to completely detach them (Fig. 5*B*). The case of 0.1 μM ouabain is even clearer (*squares*) because the effect on pumping is essentially complete around the 6th hour but detachment is only observed after 24 h.

Since both, as an ATP-hydrolyzing enzyme and as a pump, Na^+,K^+-ATPase requires K^+ on the extracellular side, in principle it can be equally stop-

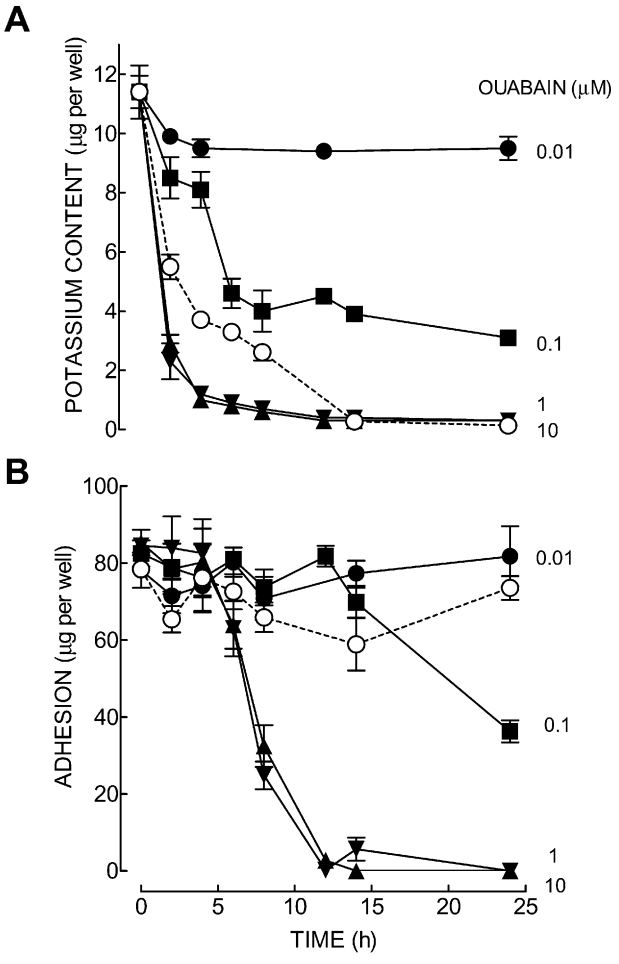

Fig. 5. (*A*) Time course of K^+ loss as a function of ouabain concentration. (*B*) Cells remaining adhered in the plate as a function of ouabain treatment. Empty circles correspond to monolayers that were not treated with ouabain, but in which pumps were inhibited by incubation in a medium containing only 0.1 mM K.

ped by ouabain or by K^+ removal from the bathing solution. Figure 5*A* (*empty circles*) shows that this is the case: bathing the monolayers in low-K^+ medium mimics the effect of ouabain. Yet to our surprise, the sharp decrease in cell K^+ does not detach the cells (Fig. 5*B*, *empty circles*). This emphasizes the suggestion made above that P → A is not a straightforward consequence of ionic perturbation.

The Role of K^+ Depletion in Ouabain-resistant MDCK Cells (R-MDCK)

The conclusion reached in the previous paragraph can be tested with an entirely different approach. Thus, R-MDCK cells are known to withstand high concentrations of ouabain due to the low affinity of their Na^+,K^+-ATPases for the drug (Soderberg et al., 1983; Bolivar et al., 1987) (Fig. 1). Since in order to compare ouabain vs K-removal effects we had to manipulate the concentration of K^+ in the bathing solution, we incubated the cells in a special medium, manufactured by In Vitro, without this ion, but to which it can be added at the desired concentration. At the high concentration of ouabain used in the present study (1.0 μM), which is some three orders of magnitude higher than the K_m, R-MDCK cells do decrease their K^+ content (Fig. 6: columns *3* vs *1*) but to a degree that does not elicit cell detachment (columns *4* vs *2*). By the way, this degree of K^+ loss in R-MDCK cells does not produce detachment of W-MDCK cells either (Fig. 3). When the bathing medium contains only 0.1 mM K^+, the content of this ion drops sharply (columns *5* and *7* vs 1; $P < 0.001$), and cells do experience a detachment, albeit not as drastic as this level of K^+ would cause in W-MDCK cells (compare with Fig. 5*A* and *B*). In keeping with the low affinity for the Na^+,K^+-ATPase of R-MDCK cells, the drug cannot trigger the P → A mechanism in this cell type (Contreras et al., 1999). Under these conditions, ouabain does not provoke further detachment of the cells beyond the one elicited by the decrease in K^+ (columns *8* vs *6*).

The Relationship between K^+ Content and the Cascade of Phosphorylations

As discussed in the comparison of Figs. 5*A* and 5*B*, the considerable difference in time courses between K^+ loss and detachment suggests that the effect of this ion is being mediated by some other cellular process. This agrees with the observation that, once ouabain triggers P → A, it is followed by a cascade of phosphorylations and retrieval from the membrane of proteins involved in cell-cell and cell-substrate attachment (occludin, ZO-1, desmoplakin, cytokeratin, α-actinin, vinculin and actin) (Contreras et al., 1999). As part of this cascade, mitogen-activated protein kinase (MAPK) enhances its activity (Contreras et al., 1999). When these variables are studied as a function of time or as functions of the concentration of ouabain, K^+ loss is closely accompanied by an activation of MAPK (Fig. 7). Furthermore, the activation of MAPK shows a strong correlation with detachment (Fig. 8). Yet, in spite of their parallelism, K^+ loss and MAPK activity may not necessarily be cause-related, as the enzyme exhibits a weak and scattered correlation with ion content (Fig. 9).

NACos: Further Signaling from Cell-attachment Sites

As we see, at the ouabain concentrations used to characterize the P → A mechanism, the final outcomes are mainly imbalance of K^+, Na^+, Ca^{2+} and Cl^-, as well as diverse types of phosphorylation, cell detachment, and cell death. Yet it cannot be assumed that P → A is an all-or-nothing response. In the

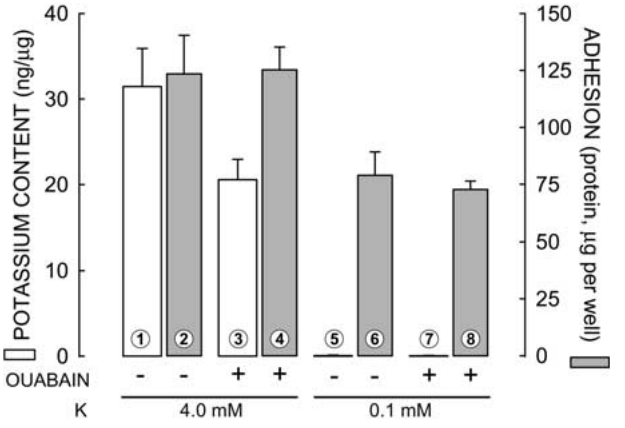

Fig. 6. Effect of ouabain on K^+ content and cell attachment in ouabain-resistant cells (R-MDCK). Comparison of column 1 with 3, and 2 with 4 indicates that in this medium ouabain does not elicit the expected P → A effect. Under these conditions, a reduction of the concentration of K^+ in the incubating medium from 4.0 to 0.1 mM causes a drastic reduction of K^+ content (column *5*) as well as a significant but relatively milder reduction in cell attachment (column *6*; $P < 0.001$). Ouabain does not enhance detachment (columns *8* and *6*); $n = 6$ in each column.

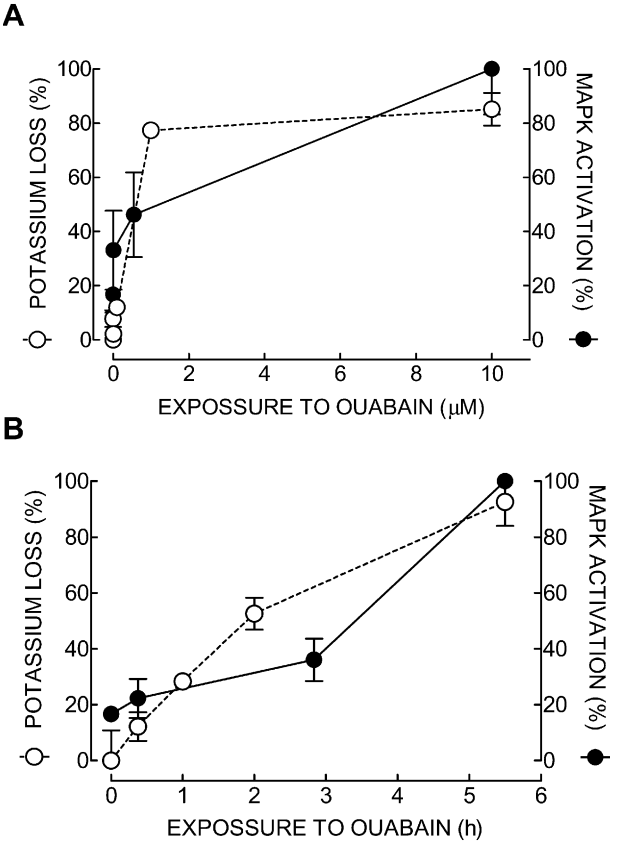

Fig. 7. Activation of mitogen-activated protein kinase (MAPK) and K^+ loss as functions of (*A*) the concentration of ouabain and (*B*) the time of exposure to 10 μM ouabain. Curves were drawn by eye.

present study we have used ouabain at suitable concentrations to analyze the mechanisms involved. But P → A may conceivably play other roles at subtler degrees of detachment. Figure 10*A* shows that cells detached by 10 μM ouabain in 5 h can be reseeded and resume proliferation, indicating that ouabain may stop the pump without causing irreversible damage. Figure 10*B* supports the idea that the detachment brought about by ouabain may not be an all-or-nothing response, as 300 nM ouabain causes a significant ($P < 0.001$) drop of TER without causing a release of cells from the monolayer. Moreover, there is a family of proteins (NACos) pertaining to one of the several types of cell-cell and cell-substrate junctions, that shuttle to the nucleus in response to changes in the strength of cell contacts. Once arrived at the nucleus, NACos regulate gene expression, thereby influencing cell proliferation and differentiation (Balda & Matter, 2003). One of them is β-catenin, an 85 kDa protein belonging both to adherens junctions and to the Wnt signaling pathway. In confluent monolayers of MDCK, β-catenin localizes to the vicinity of the plasma membrane (Fig. 10 *C*, *control*). Enhanced phosphorylation of tyrosine residues on β-catenin is almost invariably associated with loss of the cadherin-actin connection concomitant with loss of adhesive function (Lilien et al., 2002; Wheelock & Johnson, 2003). As shown in Fig. 10*D*, β-catenin undergoes tyrosine phosphorylation in response to ouabain treatment. Therefore, we have chosen to study whether the ouabain-induced tyrosine phosphorylation through P → A is accompanied by a shuttle to the nucleus of this NACo. Figure 10*C* (*ouabain*) shows that, in fact, ouabain (100 nM) causes β-catenin to travel to the nucleus and accumulate in discrete spots and show a diffuse staining, except at the nucleoli. It may be noticed that this effect is observed with ouabain concentrations that do not detach the cells in 9, 24 and 48 h (Figs. 5, 6).

Discussion

The beneficial pharmacological action of cardiac glycosides in heart failure has been known for centuries (Miura & Biedert, 1985), yet the existence of vectorial ion transport affected by this drug is still marked by breath-taking surprises and amazing connections: (1) vectorial movement of Na^+ has been once discarded as in violation of the laws of thermodynamics (Cereijido et al., 1993; Cereijido, Shoshani & Contreras, 2001). (2) The possibility that a scalar phenomenon could drive a vectorial one was also ruled out. (3) Nevertheless, a Na^+,K^+-ATPase that operates such flux has been found, and recognized as the star in the movement and distribution of substances, ranging from ions to sugars and aminoacids (Philpott et al., 1992; Blaustein,

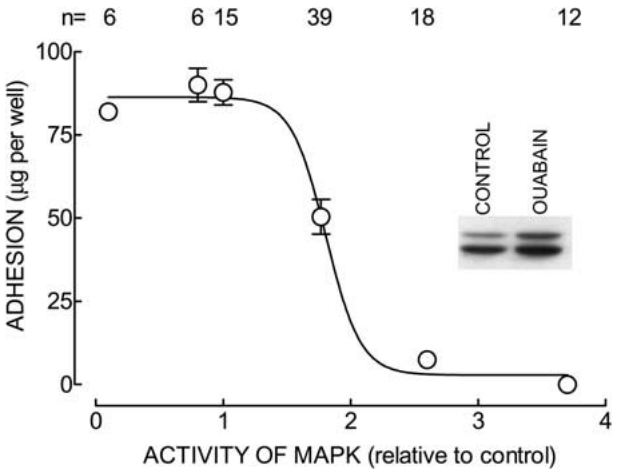

Fig. 8. Cell adhesion as a function of MAPK activity. A variety of pump-blocking agents was used at several concentrations, as in Fig. 3. Proteins extracted from the different monolayers were separated by electrophoresis, and the gels were blotted with an antibody against phosphorylated MAPK (*Inset*). Optical density of the 42 KDa band in monolayers treated with a given drug was normalized as percentage of the intensity in control monolayers in the same run. The curve represents the non-linear adjustment of the experimental data to the Boltzmann sigmoidal equation.

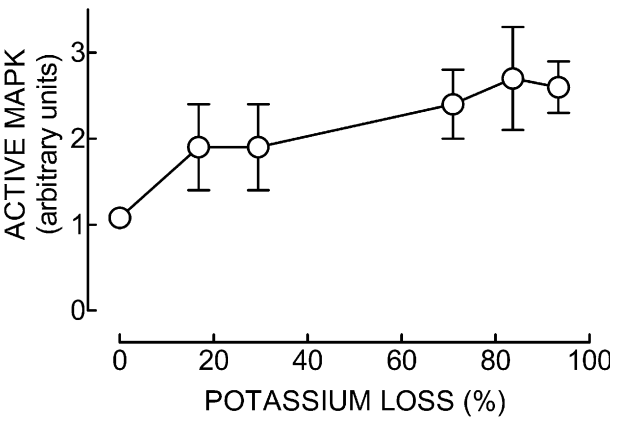

Fig. 9. Activation of MAPK as a function of K^+ loss elicited by different pump-arresting drugs. Ouabain concentration was 10 μM.

1996), and (4) there is at present a debate over endogenous ouabain-like substances and ion pumps that would act as hormonal receptors (Schoner, 2002), and that may even be implicated in arterial hypertension and several other pathological processes (Blaustein, 1996; Balzan et al., 2001). In this context we have found that (5) the Na^+,K^+-ATPase is involved in a mechanism $P \rightarrow A$, which causes detachment of the cell (Contreras et al., 1999). The present work is part of an effort to understand the mechanisms of this process.

Several observations reported in the present work support the disruption of ion balance as a cause of detachment: (*a*) ouabain causes detachment of W-MDCK cells, but not of R-MDCK ones because these have extremely low affinity for the drug (Soderberg et al., 1983; Bolivar et al., 1987) and only exhibit a relatively small ion perturbation (Fig. 6); (*b*) detachment exhibits a close correlation with K^+ loss (Fig. 5*A* and *B*); (*c*) ouabain-like substances provoke detachment with an efficiency that parallels their ability to decrease K^+ (Fig. 3). Yet a number of findings suggest that cellular processes other than K^+ loss are also playing a significant role: (*i*) in the absence of ouabain, K^+ loss only causes a much smaller degree of detachment of R-MDCK cells (Fig. 6); (*ii*) by the time K^+ is markedly decreased, W-MDCK cells do not detach (Fig. 5*A* and *B*); (*iii*) if ouabain is removed once it has caused a considerable K^+ loss, cells remain attached (Fig. 4); (*iv*) K^+ loss is closely associated with activation of MAPK both, as a function of time and as a function of concentration of ouabain (Fig. 7 and 8); (*v*) detachment has a clear correlation with the activity of MAPK (Fig. 9); (*vi*) retrieval of molecules participating in cell attachment—an unavoidable requirement for cell detachment—involves phosphorylation (Citi, 1992; Perez-Moreno et al., 1998; Morgado-Diaz & de Souza, 2001); (*vii*) ouabain does not detach the cells in the presence of Genistein and UO126, which inhibit protein tyrosine kinase and MAPK, respectively (Contreras et al., 1999). But it does not necessarily follow from this that K^+ loss ignites the cascade of phosphorylations, as MAPK activation keeps a loose correlation with K^+ content (Fig. 9). It should be stressed that this parameter is used here as a sensible way of gauging ion pumping, but it does not necessarily mean that $P \rightarrow A$ is triggered by the specific decrease of K. As shown in Fig. 1, ouabain causes an increase of Ca^{2+} content, and this ion is known to spark a variety of phosphorylations (Deisseroth & Tsien, 2002; Frank et al., 2003).

In a previous work (Contreras et al., 1999) we explored possible signal transduction pathways of the $P \rightarrow A$ mechanism, such as those involving changes in cytosolic Ca^{2+}, PTK, $p190^{Rho\text{-}GAP}$. Other groups (Valente et al., 2003; Xie, 2003) found that ouabain induces the production of reactive oxygen species (ROS). Xie (2003) and Haas, Askari and Xie (2000) studied the routes activated by ouabain in cardiocytes that involve c-src, $p190^{Rho\text{-}GAP}$, and ERK1/2. These signaling pathways are known to regulate cell adhesion through specific attachment molecules (Noren et al., 2000; Izawa et al., 2002; Benais-Pont et al., 2003) and to modify the interaction with the extracellular matrix (Noren et al., 2000). The physiological role of the $P \rightarrow A$ mechanism could be related to cell differentiation and cell-cycle regulation, as recent works have shown that integrin signaling in combination with RPTKs are involved in cell-cycle regulation and that they exert their function via RhoA-, JNK- and MAPK-signaling pathways (Assoian & Schwartz, 2001; Gout et al., 2001).

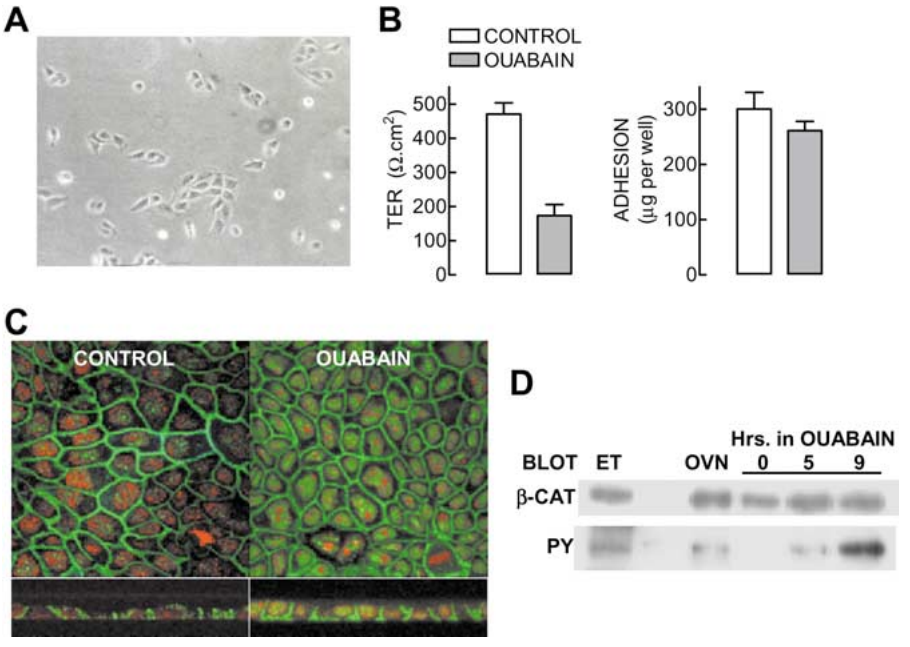

Fig. 10. The P → A mechanism may not necessarily trigger an all-or-nothing response that results in the removal of a cell from the monolayer, but may play other physiological roles in which cell-cell contacts are involved. (*A*) MDCK cells that have been detached with 10 μM ouabain can be plated again and resume proliferation. (*B*) At 300 nM, ouabain causes a significant ($P < 0.001$) drop of TER in 24 h, presumably due to relaxation of the tight junctions, without causing a release of cells from the monolayer. (*C*) Control monolayers of MDCK cells showing that β-catenin (*green*) distributes mainly in the periphery, and that its accumulation in the nuclei is comparatively scarce. Spots of β-catenin in the nucleus increase upon 9 h treatment with 100 nM ouabain. (*D*) β-Catenin not only shuttles to the nucleus in response to ouabain, but also undergoes tyrosine phosphorylation (*PY*) without clearly increasing its amount in the cell (β-*CAT*) in the same 9 h. β-Catenin was immonoprecipitated and blotted with anti-phosphorylated tyrosine antibody, then stripped and blotted again with anti β-catenin antibody. *ET*: total extract, *OVN*: Sodium orthovanadate-treated cell extract as positive control.

The mitogen-activated protein kinase (MAPK or ERK) pathway commonly starts with the activation of a protein tyrosine kinase receptor (RPTK) by a variety of stimuli like cytokines or growth factors, or by mechanical stress and cell swelling. Activated RPTK provides the scaffolding to recruit adaptor proteins that promote the activation of the basic MAPK module, composed of small GTP-binding proteins (ras and raf), MAPK kinase and MAPK (Widmann et al., 1999). In cardiac myocytes, ouabain sequentially activates Src, the epithelial growth factor, and the MAPK module to induce hypertrophy, while in epithelial cells, ouabain (Contreras et al., 1999; Dmitrieva & Doris, 2003) and hepatocyte growth factor (Paumelle et al., 2000) require MAPK pathway activation to detach. Moreover, the inhibition of MAPK induces the formation of tight and adherens junctions in MDCK cells transformed with the *ras* gene (Chen et al., 2000). Thus, activation of MAPK pathways may be a common feature of the signal-transducing function of Na^+,K^+-ATPase in most cells (Haas et al., 2000). Nevertheless, ouabain effects may be exerted or regulated through an extensive crosstalk between different pathways, as is known for integrin signaling during cell migration, differentiation and death (Miranti & Brugge, 2002).

The fact that the P → A mechanism is reversible (Fig. 4) and that cells detach without a detectable process of apoptosis, either before or after detachment (Contreras et al., 1999), implies that this mechanism may constitute a truly physiological attribute, in particular because there are several characteristics of Na^+,K^+-ATPase that await plausible explanations. Among these characteristics we can mention: (*i*) the pump can be assembled with not less than four different α subunits (Blanco & Mercer, 1998; Jorgensen, 2001). (*ii*) The expression of a given type is distinctive of specific tissues and development stage (Lopez, Quintas & Noel, 2002). (*iii*) The expression of different subunits of the Na^+,K^+-ATPase of a given cell type does not remain constant throughout the cell cycle, but varies according to the phase (Jones, Daries & Kidder, 1997; Kometiani et al., 2001). (*iv*) Isoforms of the α subunit have different sensitivities to ouabain. (*v*) Interaction of ouabain with Na^+,K^+-ATPase turns on and off a variety of genes (Peng et al., 1996). (*vi*) Although it is still a controversial matter (*see*, for instance, Doris, Jenkins & Stocco, 1994; Hansen, 2003), there seem to be endogenous ouabains (Goto et al., 1992) that may play a role in human diseases (Balzan et al., 2001; Cusi, 2002; Fridman et al., 2002), protein phosphorylation

(Lago et al., 2001), and cell growth (Dmitrieva & Doris, 2003). (*vii*) Ward, Hamilton and Hamlyn (2002) have recently described the union of ouabain to a receptor different from Na^+,K^+-ATPase whose function is not yet elucidated, suggesting that, eventually, the P → A mechanism may be found to be triggered through a receptor other than the α subunit of the Na^+,K^+-ATPase.

The Wnt signaling pathway is involved in a variety of mammalian developmental processes, including cell proliferation, differentiation, and epithelial-mesenchymal transition, through which they contribute to the development of tissues and organs. The Wnt signaling pathway operates through the cytosolic stabilization of the transcriptional cofactor β-catenin (Smalley & Dale, 1999). We further show that relatively low concentrations of ouabain, which do not cause appreciable disturbance of ion contents, shuttle a signal (β-catenin) from the membrane to the nucleus (Fig. 10). This protein is known to be phosphorylated by several protein kinases, so it would not be surprising if it were demonstrated to participate in the cascade of phosphorylation triggered by ouabain (Contreras et al., 1999). Taken together these observations suggest that the present study of the P → A mechanism may open some new avenues to inquiry.

In summary, in the Introduction we posed the question of whether ouabain starts the P → A mechanism by simply blocking the pump, or through a cascade of phosphorylations. On the basis of the present results we can discard neither. On the contrary both, ionic perturbation and phosphorylations seem to act rather independently, a suggestion that does not exclude the possibility of cross-talk. Furthermore, ouabain, acting as a hormone, elicits a third process: signaling. Given that NACos signaling is involved in differentiation and proliferation, the present work indicates that the ion pump, through the P → A mechanism, plays a novel and important role.

We thank Drs. G. Bianchi and Patrizia Ferrari from the Prassis Research Institute (Milano, Italy), who have generously provided the PST 2238 and PST 2744 that they synthesized; Dr. Rosalba Encarnación from the Marine Biological Department (University of Baja California, México) for providing neothyoside A, and Dr. Luz María del Razo for allowing us to use her atomic emission spectrometer to measure K, and helping us with the procedures. We also thank Mrs. E. del Oso and Y. de Lorenz for administrative help. This work was performed with the economic support of the National Research Council of México (CONACYT).

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