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Ouabain Resistance of the Epithelial Cell Line (Ma104) is not Due to Lack of Affinity of its Pumps for the Drug

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Abstract. Na^+, K^+-pumps of most eukaryotic animal cells bind ouabain with high affinity, stop pumping, and consequently loose K^+, detach from each other and from the substrate, and die. Lack of affinity for the drug results in ouabain resistance. In this work, we report that Ma104 cells (epithelial from Rhesus monkey kidney) have a novel form of ouabain-resistance: they bind the drug with high affinity (Km about 4×10^{-8} M), they loose their K^+ and stop proliferating but, in spite of these, up to 100% of the cells remain attached in 1.0 μM ouabain, and 53% in 1.0 mM. When 4 days later ouabain is removed from the culture medium, cells regain K^+ and resume proliferation. Strophanthidin, a drug that attaches less firmly than ouabain, produces a similar phenomenon, but allows a considerably faster recovery. This reversal may be associated to the fact that, while in ouabain-sensitive MDCK cells Na^+, K^+-ATPases blocked by the drug are retrieved from the plasma membrane, those in Ma104 cells remain at the cell-cell border, as if they were cell-cell attaching molecules. Cycloheximide (10 μg/ml) and chloroquine (10 μM) impair this recovery, suggesting that it also depends on the synthesis and insertion of a crucial protein component, that may be different from the pump itself. Therefore ouabain resistance of Ma104 cells is not due to a lack of affinity for the drug, but to a failure of its Na^+, K^+-ATPases to detach from the plasma membrane in spite of being blocked by ouabain.

Key words: Na^+, K^+-pump — Na^+, K^+-ATPase — K^+ content — Epithelial cells — Proliferation — Attachment — Ouabain — Chloroquine — Strophanthidin

Introduction

The cardiac glycoside ouabain binds to the Na^+, K^+-ATPase of most cell types with high affinity and blocks its ability to function as ionic pump, with the consequent impairment of the viability of the cell (Skou, 1960; Post et al., 1960). However, some cell types are able to live in the presence of relatively high concentrations of the drug (Repke, East & Portius, 1965). This property, termed "resistance to ouabain," is generally due to a low affinity of the Na^+, K^+-pumps for the drug (Baker, 1976; Sweadner, 1979). In this article we present evidence that Ma104 cells, a line derived from the kidney of Rhesus monkey, exhibit a novel type of ouabain resistance. As shown below, these cells bind ouabain with a relatively high affinity and, accordingly, lose most of their K^+ and stop proliferating, but do not detach from each other nor from the substrate as ouabain-sensitive MDCK cells do. Furthermore, when several days later Ma104 cells are transferred to medium without the drug, they regain K^+ and resume proliferation in a few hours, a property that depends on the synthesis of proteins and on their incorporation to the plasma membrane through exocytic fusion.

Materials and Methods

Cell Culture

Ma104 cells (epithelial line derived from Rhesus monkey kidney) were a generous gift of Dr. E. Rodríguez-Boulán (Cornell U., NY). MDCK (epithelial line derived from dog kidney) were purchased from the American Type Tissue Collection (CCL-34, Rockville, MD). Upon arrival cells were cloned and grown at 36.5°C in disposable plastic bottles (Costar 3250, Cambridge, MA.) with an air-5% CO_2 atmosphere (VIP CO_2 incubator 417, Lab Line Instruments, New Brunswick, NY) and 20 ml of Dulbecco's modified Eagle's medium DMEM (Grand Island Biological, GIBCO 430–1600, Grand Island, NY) with 100 U/ml of penicillin, 100 μg/ml of streptomycin (GIBCO 600–5145), 0.8 U/ml of insulin (Eli Lilly, Mexico, DF), and 10% foetal calf serum (GIBCO 200–6170). In the following text this complete medium is referred to as CDMEM. Cells were harvested with trypsin-EDTA (In

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Vitro, México, D.F.) and plated on 24-well chambers (Linbro 76-033-05, Flow Lab., VA).

Cell Growth and Cell Number

Cells were plated at low densities (10^4/dish) in a set of 30-mm Petri dishes. Three dishes were counted daily by detaching the cells with Ca-free PBS and trypsin-EDTA. Cell number was measured with a Coulter Counter (Coulter Electronics, Hialeah, FL). Alternatively cells were labeled with 2.0 mCi of ^{35}S-methionine (New England Nuclear) per milliliter of culture media added to confluent cell monolayers growing in 75-cm^2 Costar bottles. 24 hr later cells were thoroughly washed and left for 24 hr in media with 2.0 mM cold methionine (Sigma Chemical, St. Louis, MO). They were then harvested and plated in a multiwell plate. 24 hr later, cells were thoroughly washed with PBS and extracted with 2% SDS and precipitated with 10% trichloroacetic acid for ^{35}S counting in the ^{14}C channel of a Packard 2000B Counter. Further details on the reliability of this procedure is given by Bolivar et al. (1987).

[^{3}H]-Ouabain Binding

Monolayers were washed three times with a Ringer solution containing (mM): 140 NaCl; 1.8 $CaCl_2$; 5.0 dextrose and 10 Tris-HCl at pH 7.4 at room temperature. This solution is referred to as "K-free Ringer." Binding of [^{3}H]-ouabain (Amersham, England, TRK.429, around 16 Ci/mmole) was made in K-free Ringer, with and without a 500-fold excess of non-labeled ouabain to measure total and specific binding respectively. At the end of the incubation period, monolayers were washed three times with ice-cold 0.2 M $MgCl_2$ for 10 min, extracted with 0.5 M NaOH, and aliquots were taken for ^{3}H and protein measurements. It was found that the activity of ^{3}H in further washings does not differ from background. Although ouabain is added to the apical (free) side of the monolayer, it easily reaches the pumps, located on the basolateral domain of the plasma membrane, because monolayers cultured on nonpermeable supports do not appear to have sealed tight junctions around their whole perimeter (Cereijido et al., 1981). Further technical details and information on the reliability and accuracy of this method are given by Cereijido et al. (1980, 1981), Bolivar et al. (1987) and Contreras et al. (1989).

[^{35}S]-Methionine Uptake

This protocol is used to ensure that the use of cycloheximide (*see below*) actually inhibits protein synthesis. Monolayers plated at confluence and incubated for 24 hr were exposed to 0.5 ml of CDMEM containing 20 μCi/ml of the tracer. At different times, the medium was removed and monolayers were quickly washed 3 times with ice-cold 0.1 M $MgCl_2$ followed by three more washing periods of 5 min each. They were then extracted with 0.5 ml of 2% SDS and precipitated with 1.5 ml of 10% trichloroacetic acid, spun in a Microfuge centrifuge (Beckman Instruments, Palo Alto, CA.) and washed twice with 1.5 ml of trichloroacetic acid. The pellet was extracted for 2 hr in 1.0 ml of 0.5 M NaOH. Aliquots were taken for ^{35}S and protein measurements.

Intracellular K^+ Content

Monolayers of 2 cm^2, plated in Linbro 24-well chambers were incubated overnight with medium containing ^{86}Rb (Amersham RGS.2, England), as it was shown that this isotope behaves as a valid tracer for K^+ in this preparation (Cereijido et al., 1981). This period is long enough to equilibrate the specific activity in the cells with the bathing solutions (Bolivar et al., 1987). Monolayers were then washed five times with ice-cold 0.1 M $MgCl_2$, the last wash lasting 1.0 min, and extracted with 0.5 M NaOH for 2 hr. ^{86}Rb radioactivity was determined in 400 μl samples added to 10 ml Aquasol (New England Nuclear) and counted in a beta counter, and proteins were measured by Lowry method.

ATP Content

ATP was determined using the luciferase reaction of Wulff & Döpen (1985) as adapted by Mújica et al. (1991). Briefly 20 mg firefly lantern extract was reconstituted with 1.0 ml water by mixing for 10 min in a Vortex and centrifuged 20 min at 6,000 × *g*. The clear pale yellow supernatant fraction was decanted into a glass tube; 30 μl lantern extract was added to a vial containing 0.86 ml of the buffer solution (50 mM MOPS, 10 mM $MgCl_2$, 5 mM KH_2PO_4, pH 7.8) and mixed. The number of light flashes was recorded (background) using a Lab-Line ATP meter. Immediately, 100 μl of cell extract was added, swirled briskly, the number of light flashes was recorded, and the background value subtracted. The time between enzyme or sample addition, and the start of recording of light flashes was 5 sec.

Immunofluorescence

Glass coverslips containing cells cultured under the several experimental conditions described below, were rinsed twice with PBS, fixed and permeabilized with methanol at –20°C for 45 sec, washed with PBS, incubated with 3% foetal bovine serum in PBS for 30 min, and treated for 1.0 hr with a mouse monoclonal antibody against the β-subunit of dog Na^+,K^+-ATPase (generous gift of Dr. Michael Caplan, Yale University; *see* Gottardi & Caplan, 1993). Monolayers were then rinsed 3 times with PBS for 5 min each, incubated with a FITC-labeled goat antimouse antibody (SIGMA Chemicals, St. Louis, MO) for 30 min, rinsed as above, mounted in a 1:19 mixture of p-phenyldiamine (SIGMA)—Gelvatol (Montsanto, Indian Orchard, MA.) and examined with a confocal microscope (MRC-600, Bio-Rad).

Results are expressed as ±SEM. The number of observations (n) is given in parenthesis.

Results

Ma104 cells plated at confluence (zero time) and exposed to ouabain from the 3rd to the 27th hour (Fig. 1), remain attached at concentrations as high as 10 μM, and even 53% are still present at 1.0 mM. This behavior resembles that of ouabain-resistant MDCK cells (filled square) rather than the one of the wild type (open square).

This resistance of Ma104 cells may not be due to a lack of ouabain binding to the pumps, as a kinetic experiment performed with 1.0 μM [^{3}H]-ouabain shows (Fig. 2), that the drug attaches with a fast time course, and the number of specific binding sites per milligram of protein, as well as the half-saturating concentration (Fig. 3) are of the same order of magnitude as those found in ouabain sensitive MDCK cells (Bolivar et al., 1987).

Figure 4 shows the effect of 1.0 μM ouabain on the growth of Ma104 cells plated at low density (10,000 cells/cm^2). Open circles and filled squares illustrate

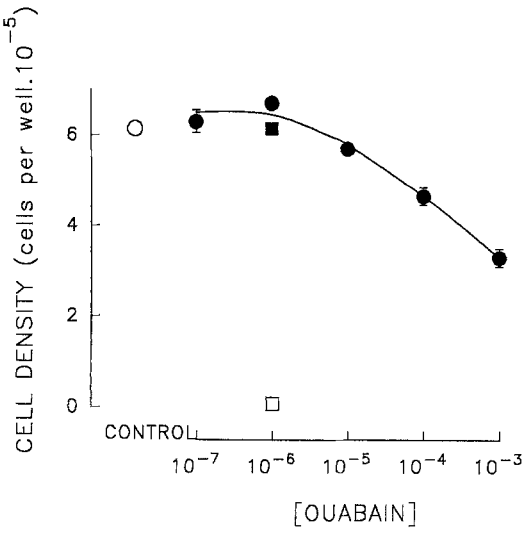

Fig. 1. Filled circles: Number of Ma104 cells that survived a continuous exposure to ouabain for 24 hr at the concentrations indicated in the abscissa. Cells were plated at confluence in multiwell plates (2.0 cm^2 per well). Exposure to ouabain was started at the third hour after plating, and was terminated by detaching the cells with trypsin-EDTA. Cells were then suspended in Ca-free PBS and counted. Open circle: control monolayers that were not exposed to ouabain. Filled and open squares: monolayers of ouabain-resistant and wild type MDCK cells respectively; (n = 4). In this and subsequent figures, when standard errors do not show, it is because they are smaller than symbols.

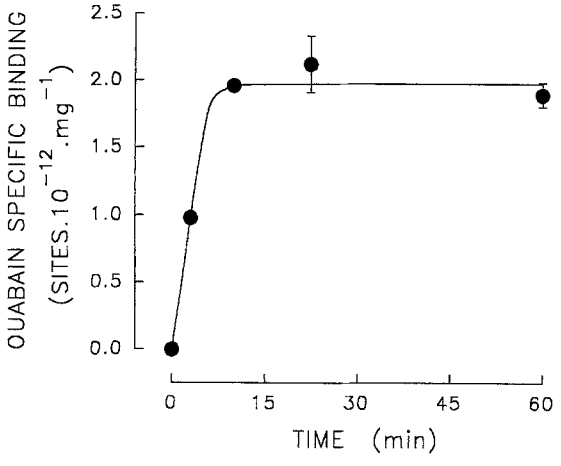

Fig. 2. Time course of specific binding of ouabain to Ma104 cells in confluent monlayers. Two sets of monolayers were exposed to 1.0 μM ouabain containing [^{3}H]-ouabain, one in the absence and the other in the presence of a 500-fold excess of cold ouabain. The difference in labeling between the two sets was used to calculate the number of specific pumping sites; (n = 3).

growth in control and ouabain containing (1.0 μM) media, respectively. Although the drug drastically slows down proliferation, cells remain attached. Upon ouabain removal after 4 days of exposure (open squares) proliferation is resumed. It may be speculated that although

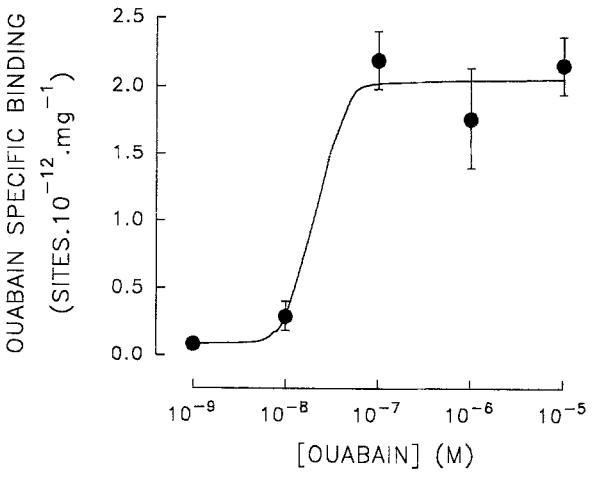

Fig. 3. Specific binding of ouabain to pumping sites of Ma104 cells as a function of its concentration. The exposure time was 6 hr. Each point represents the difference between groups of 10–16 monolayers exposed as indicated in Fig. 2. The curve was fitted by eye.

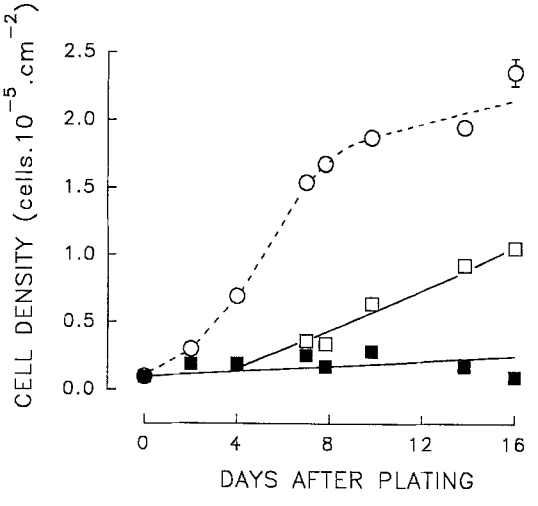

Fig. 4. Effect of 1.0 μM ouabain on cell proliferation. Ma104 cells were plated at a low density (10^4 cells · cm^{-2}) in Petri dishes and periodically harvested for counting. Open circles and filled squares correspond to control cells, and cells that had been exposed to ouabain since the third hr of plating respectively. Open squares: correspond to cells that were exposed to ouabain until the fourth day, and then switched to control medium.

the concentration of ouabain used in the experiment of Fig. 4 achieves a maximal labeling of a population of pumps (Fig. 3), cells may have a second population of pumps of lower affinity for the drug, that suffices to maintain normal K^+ concentration. However, Fig. 5 shows that monlayers exposed to 1.0 μM ouabain for 24 hr lose a large fraction of K^+, and that upon switching to control CDMEM recover a normal content of this ion. Fig. 6 illustrates the time course of K^+ recovery in cells that had been exposed to 1.0 μM ouabain for 4 days.

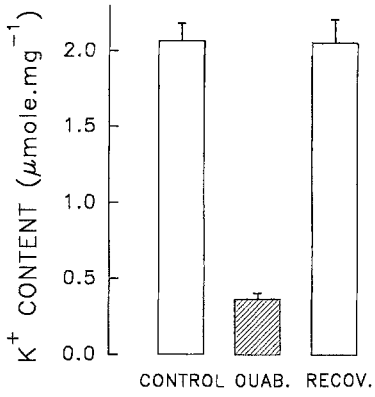

Fig. 5. Effect of 1.0 μM ouabain on potassium content. Cells were plated at confluence. 24 hr later the resulting monolayers were divided into three sets: one was left as control and the other two were switched to a ouabain-containing medium. At the 48th hr, one of the ouabain-exposed sets was switched back to control medium (*recov.*). The hatched column corresponds to the set of monolayers exposed to ouabain from the 24th to the end of the experiment. All media contained ^{86}Rb at the same specific activity. At the 76th hour the experiment was terminated and K^+ and protein content of each monolayer were measured through the activity of ^{86}Rb and the method of Lowry respectively.

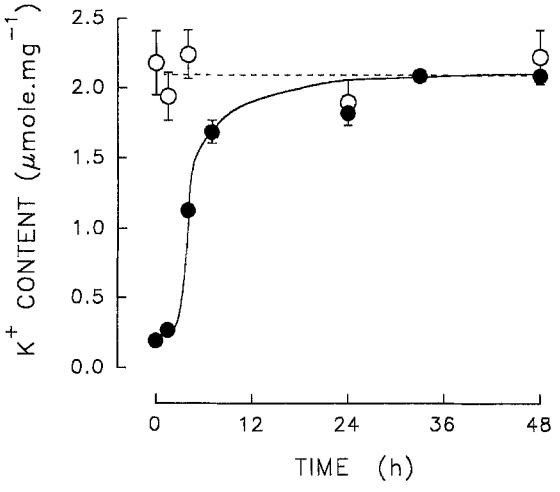

Fig. 6. Recovery of intracellular potassium following a 4-day exposure to 1.0 μM ouabain added 3 hr after plating at confluence (filled circles). At the fourth day of exposure to ouabain, cells were switched to a medium also containing ^{86}Rb. Zero time in the abscissa corresponds to the switch of all monolayers to control medium without ouabain. Open circles: monolayers prepared and cultured in control medium without ouabain, (n = 4).

A priori, the most probable cause of recovery of cellular K^+ would be the detachment of ouabain from the Na^+,K^+-ATPases, that would thus regain the ability to pump. In this respect, Fig. 7 shows that the use of strophanthidin, a drug that has a much lower affinity for the pumps (Kyte, 1972; Sweadener, 1979) and that is consequently released with a much shorter time course upon

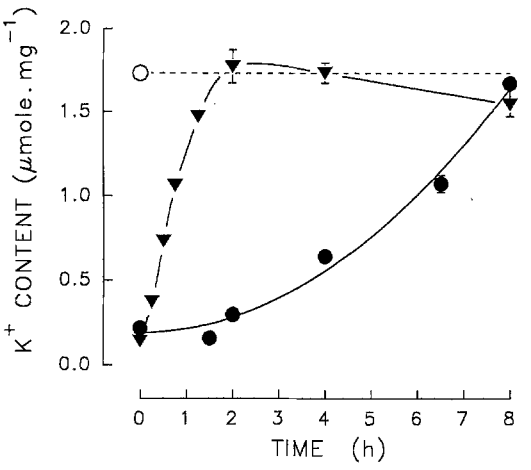

Fig. 7. Recovery of intracellular K^+ upon removal of the pump blocking drug. Monolayers exposed to 1.0 μM ouabain (filled circles) or 100 μM strophanthidin (triangles) for 2 days were loaded with ^{86}Rb and left with the drug and the tracer for another day. At this time, they were thoroughly washed and incubated with ^{86}Rb-containing CDMEM at the same specific activity (zero time). Open circles correspond to control monolayers, (n = 3–12).

switching to ouabain-free media, permits a considerably faster recovery of intracellular K^+ (triangles).

The interpretation that recovery is due to ouabain detachment from the pumps in response to its removal from the bathing medium, and that K^+ is recovered with the same set of pumps that had been previously blocked, implies that ouabain-blocked pumps remain in the plasma membrane and are continuously exposed to the bathing medium. This is unlikely, because in most cell types blocked pumps are endocyted. Thus, Fig. 8 (top), shows that Na^+,K^+-ATPases occupies the lateral membrane of MDCK cells in a control monolayer (left), but is retrieved towards the cytoplasm in ouabain-treated ones (right). Na^+,K^+-ATPases of Ma104 cells exhibit instead an entirely different behavior (Fig. 8, bottom), as they remain at the cell-cell border even in the presence of ouabain. Of course, this does not necessarily mean that the Na^+,K^+-ATPases visualized with antibodies at a given moment, are the same ones that have been binding ouabain from the beginning of the experiments, i.e., hours or days earlier; the possibility exists that Na^+,K^+-ATPases that have bound ouabain were efficiently endocyted and destroyed, and those seen at the cell-cell borders in Fig. 8 have been inserted and not yet endocyted.

To test this possibility, we studied the effect of an inhibitor of protein synthesis on the recovery of Ma104 cells. As a preliminary control, we assayed the effect of 10 μM cycloheximide on the uptake of $[^{35}S]$-methionine. Figure 9 shows that cycloheximide reduces—but does not completely suppress—the uptake of the amino acid. Fig. 10 describes the effect of 10 μg/ml cycloheximide

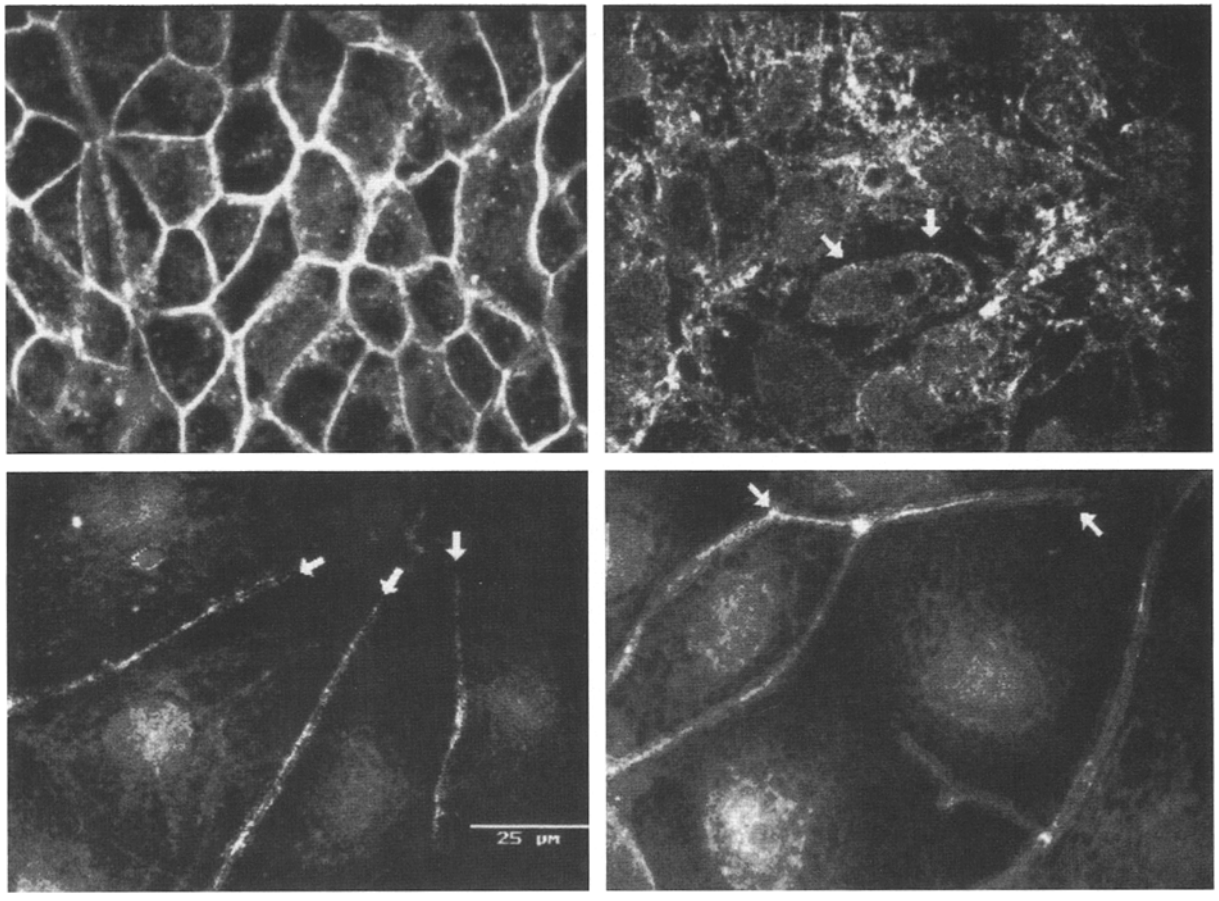

Fig. 8. Distribution of Na^+,K^+-ATPase in confluent monolayers of MDCK (above) and Ma104 cells (below), under control conditions (left), and treated with 10 μM ouabain for 8 h (right). In spite of the fact that Ma104 cells have a high pump content (Fig. 4), staining is much weaker than in MDCK cells, because the first antibody was prepared against dog pumps. Ma104 cells are larger than MDCK ones, or spread to a larger area. In both cell types Na^+,K^+-ATPase shows a characteristic position at the cell-cell border. Ouabain distorts this pattern in MDCK cells, as a large fraction of the enzyme is distributed throughout the cytoplasm, arrows point at opened intercellular spaces. In Ma104 monlayers, exposed to ouabain pumps remain at the cell-cell borders (arrows). The image obtained when using only one of the antibodies by itself, suggests that the signal on the nucleous is due to autofluorescence (*not shown*), and that the signal recorded instead in the cytoplasm may be in fact due to the presence of Na^+,K^+-ATPase.

on the specific binding of 1.0 μM ouabain. Open circles and filled symbols correspond to the specific binding in control monolayers, and to monolayers that had been exposed to cold ouabain, respectively. Filled squares represent ouabain binding to cells continuously exposed to cold ouabain, that were switched to ouabain-free medium to perform the usual measurement of $[H^3]$-ouabain binding. Obviously, in this group the time elapsed since the removal of cold ouabain is too short to allow recovery of pumping sites. When monolayers are allowed to recover in the absence of ouabain and of cycloheximide (filled circles), the number of specific binding sites progressively increases with time, reaching control levels by the 24th hour. Cycloheximide markedly suppress, albeit not completely, this recovery (filled triangles).

Figure 11 shows a complementary study: the effect of cycloheximide on the recovery of K^+ content. When monolayers that had been previously incubated with ouabain are switched to control CDMEM (filled circles) they progressively recover K^+. Yet if cycloheximide is present (filled triangles), restoration of this ion is drastically reduced (full circles *vs.* triangles at the 8th hr; $P < 0.001$).

The possibility exists that Ma104 cells incubated with ouabain would keep inserting Na^+,K^+-ATPases, either newly synthesized, or that are coming from an intracellular reservoir. When cells are transferred to ouabain-free media, newly inserted pumps would help to start a recovery, and as more pumps become inserted, ionic conditions in the cytoplasm would be further improved, until control levels of K^+ are restored. To test this possibility, monolayers were incubated with 1.0 μM

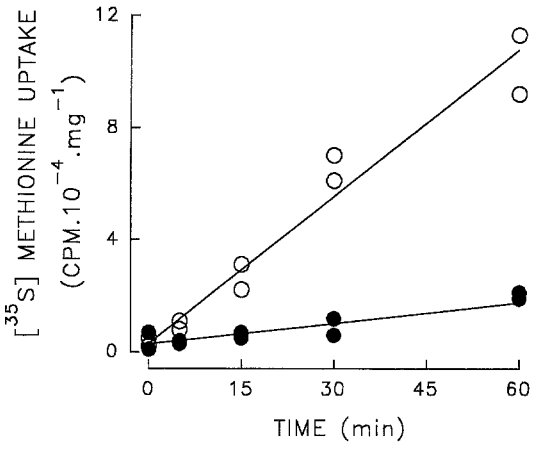

Fig. 9. Effect of cycloheximide on the uptake of [^{35}S]-methionine. Open and filled circles correspond to control cells and to cells exposed to 10 μg/ml of cycloheximide, respectively. Exposure to cycloheximide started 5 min before the addition of [^{35}S]-methionine. Each point represents an individual monolayer.

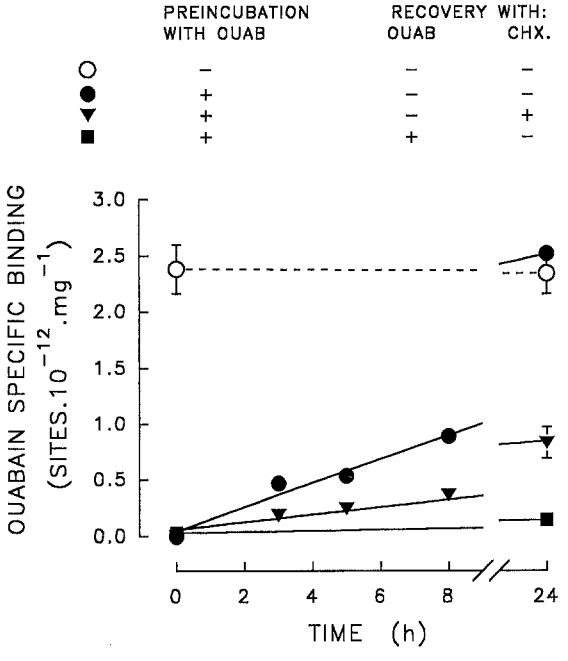

Fig. 10. Effect of cycloheximide on the specific binding of [^{3}H]-ouabain measured as in Figs. 2 and 3. Monolayers were incubated for 24 hr in the absence (open circle) or in the presence of 1.0 μM cold ouabain (filled symbols). They were then washed and allowed to recover membrane pumping sites for an additional period of 24 hr in the absence of ouabain, with (filled triangles) or without cycloheximide (filled circles). Filled squares correspond to monolayers exposed to cold ouabain throughout. The concentration of [^{3}H]-ouabain used to label the number of sites at a given period was also 1.0 μM, and was competed or not with 5×10^{-4} M nonlabeled ouabain; (n = 5–6).

[^{3}H]-ouabain both, in the absence and in the presence of 5×10^{-4} M cold ouabain. [^{3}H]-ouabain binding was measured at 1 and 24 hr of exposure. As described in Fig.

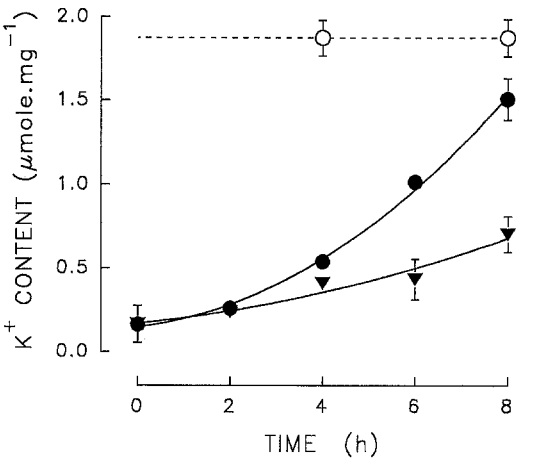

Fig. 11. Potassium content of monolayers that were exposed for one day to 1.0 μM ouabain (filled symbols). At zero time ouabain was removed and incubation pursued with control medium in the absence (filled circles) or in the presence (filled triangles) of 10 μg/ml cycloheximide. Open circles: K^+ measurements in monolayers left as control; (n = 4).

12, total binding is slightly but significantly higher in monolayers exposed for 24 hr, yet this is accounted for by the difference in nonspecific binding, as specific binding in 1 and 24 hr of exposure do not show an appreciable difference. Experiments depicted in Fig. 13, show that when cells are incubated in ouabain-containing media (1.0 μM during 3 days) with 10 μM chloroquine for 5 hr, a drug known to block exocytosis in MDCK cells (González-Mariscal et al., 1990, Contreras et al., 1989, Ponce et al., 1991), the number of pumps that can be labeled with [^{3}H]-ouabain is severely reduced.

One would expect that the blockade of the pumps would drastically reduce ATP consumption and thereby increase its concentration in the cytoplasm. Yet the Table shows that cells treated for 3 days with 1.0 μM ouabain have a lower content of ATP, suggesting that cells respond to the decreased demand of triphosphates by down regulating its synthesis. The fact that Ma104 cells remain attached in spite of this decrease of cytoplasmal ATP, may constitute another peculiarity of their Na^+,K^+-ATPase, as ATP depletion in epithelial cells results in a rapid release of cell-cell junctions and a subsequent decrease in TER (Canfield, Geerdes & Molitoris, 1991; Mandel, Bacallao & Sampighi 1993).

Discussion

Cells of different species vary in their sensitivity to ouabain (Repke et al., 1965). Since this drug binds to the α-subunit of the Na^+,K^+-ATPase (Ruoho & Kyte, 1974), species variations in ouabain sensitivity may be attributed to differences in the structure of the Na^+,K^+-ATPase

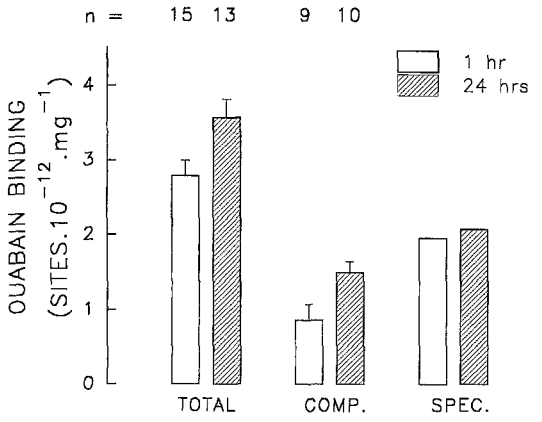

Fig. 12. [^{3}H]-ouabain binding in 1 μM ouabain CDMEM with (*comp.*) or without (total) 5×10^{-4} M cold ouabain, as determined after 1 hr and 24 hr (hatched) of exposure to the tracer. The last two bars on the right represent the difference between total and competed binding at each time.

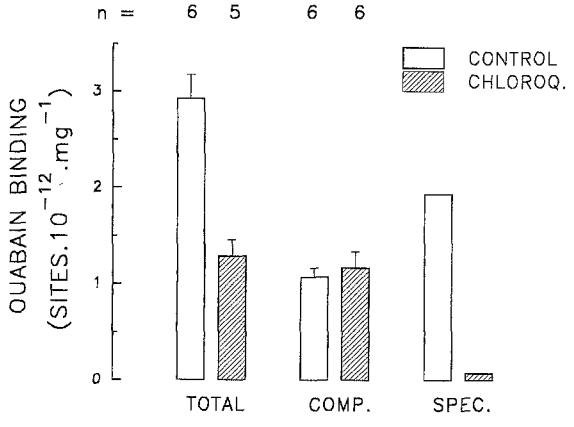

Fig. 13. [^{3}H]-ouabain binding in the presence **(comp.)** or absence **(total)** 5×10^{-4} cold ouabain, as determined in cells that had been in 1 μM ouabain CDMEM for 3 days. Cells were treated with (hatched) or whitout (white) 10 μM chloroquine 10 min before starting a 5 hr labeling period; the drug remained present throughout (n = 6).

Table 1. ATP content in Ma104 cells exposed to 1.0 μM ouabain

	ATP content	n
	(nmole · mg^{-1})	
Control	20.17 ± 0.97	(15)
1.0 μM ouabain	10.49 ± 4.42	(15)*

* $P < 0.002$ ATP was determined using the luciferase reaction, in a Lab-Line ATP meter.

α-subunit, and hence in the nucleotide sequence of the gene encoding this protein (Levenson et al., 1984, Pressley & Edelman, 1986). However, ouabain-resistance of Ma104 cells may not be ascribed to lack of affinity for the drug (*see* Figs. 2 and 3).

Another type of ouabain-resistance is exhibited by cells that keep their K^+ content with the aid of translocating mechanisms other than the regular Na^+,K^+-ATPase. Thus OR-6 cells cultured in the presence of low concentrations of ouabain express a ouabain-resistant, amiloride-sensitive K^+-transport system (English et al., 1986; Schultz & Cantley, 1988; Epstein & Lechene, 1988). Yet this mechanism does not seem to be the one conferring ouabain-resistance to Ma104 cells, as these lose most of their K^+ when challenged with the drug (Fig. 5). A similar reasoning may discard also a relatively recently discovered H^+,K^+-ATPase, present in cells that face a lumen with a high concentration of K^+, and that can be inhibited by ouabain (Wills, 1985). This mechanims may be ruled out on the basis that this H^+,K^+-ATPase is also sensitive to ouabain and therefore would not be able to maintain intracellular K^+ in the presence of the drug (Jassier, Horisberger & Rossier, 1993; Jassier et al., 1993).

Ouabain decreases cell survival in the 1 μM to 1 mM range (Fig. 1), whereas specific ouabain binding is "saturated" at 0.1 μM (Fig. 3), suggesting that cells may contain additional Na^+,K^+-pumps that have a very low affinity for ouabain, and a fast dissociation rate constant, so that they would bind little radioactive ouabain and this small amount would dissociate during the wash in the binding measurements. These "invisible" pumps might in principle be responsible for the remaining activity that would confer their ouabain resistance to these cells, and might be the ones imaged by the antibodies.

Arrest of proliferation can be easily explained by the fact that Ma104 cells lose K^+, and that a suitable concentration of this ion in the cytoplasm is required for cell division (for a review *see* Nilius & Droogmans, 1994). However, Ma104 cells not only *withstand* ouabain for at least four days, but also *recover* when this drug is removed, and resume proliferation. Therefore, the present results pose two main questions: why do Ma104 cells remain attached in spite of the blockade of their ionic pumps, and the loss of their K^+? and how do they recover from ouabain after three to four days of dormancy with their pumps blocked?

It is difficult to answer the first question, because even when ouabain cytotoxicity is attributable to a decrease of intracellular K^+ (Baker, 1976), the mechanism detaching ouabain-sensitive cells as a consequence of the ionic unbalance elicited by the drug is unknown. In fact, Ma104 cells withstand low K^+ content for days without losing the ability to remain attached and to resume proliferation. Our results open instead the possibility that detaching may be associated to the β-subunit of Na^+-K^+-ATPase. This subunit, whose exact role is not fully understood, may be needed for the correct targeting of the α-subunit (Fambrough, 1988) as well as for its correct insertion and folding (Noguchi et al., 1987). Moreover, cloning and sequencing analysis of β-subunits revealed

that this peptide has the characteristics of a Ca^{2+}-independent cell-cell attaching molecule (Gloor et al., 1990); thus purified β-subunits incorporated into liposomes specifically bind to certain types of neurons, suggesting that it recognizes a putative neuronal receptor (Antonicek & Schachner, 1988). This led Schmalzing and Gloor (1994) to speculate that β-subunits may mediate *trans*-stimulation of adjacent neurons.

Regardless of whether adhesion is exerted through its own β-subunit, or through a yet unknown molecule that would affect other attaching molecules, a relationship between Na^+-K^+-ATPase and attachment would offer a distinct advantage. Thus, a sick epithelial cell poses a serious risk to the whole organism, as it would constitute a breach in the barrier between the interstice and the lumen (Cereijido, 1991; Gumbiner, 1991; Hammerton & Nelson, 1991). Therefore it would not be surprising that if such a sick epithelial cell lacks the necessary ATP for the pump, or pumping is impaired for other reasons (e.g., binding of ouabain or ouabainlike molecules), the α-subunit may somehow send a signal to the β-subunit and to other cell attaching molecules, that would result in releasing the attachment to neighbors and substrate, and the cell would be thus ready for detachment and replacing. In this context, the results described in the present article may be explained simply by a failure of this putative mechanism. Na^+,K^+-ATPase in Ma104 cells treated with otherwise toxic concentrations of ouabain, would stay in the plasma membrane exposed to the bathing medium, so that when hours or days later the drug is withdrawn, it would be easily washed away, and the cell would thus recover its cytoplasmal K^+ and pursue proliferation.

Results in Fig. 12 might not support the idea that Ma104 cells in the presence of 1.0 μM ouabain keep inserting new pumps in the cell membrane. However, the fact that protein synthesis and membrane recycling are required for the recovery of the cell, may indicate that the cell is continuously synthesizing and inserting new Na^+,K^+-ATPases, then retrieving and addressing them to lyzosomes, but that this cycling would be blocked if the enzyme, once in the plasma membrane, combines with ouabain. Yet, as this does not involve a covalent bond, ouabain would eventually detach, and the pump would thus be endocyted and destroyed. Since this retrieved pump would not be carrying [^{3}H]-ouabain, it would not modify the estimated number of pumps, as observed in Fig. 12.

In a previous study (Contreras et al., 1989), we have shown that when MDCK cells that have been incubated in Ca-free medium are switched to a Ca^{2+}-containing one, they incorporate new pumps into the plasma membrane from preexisting intracellular pools. Although, it was observed that this process depends on the synthesis of proteins and exocytic fusion, but protein synthesized does not seem to be the Na^+,K^+-ATPase itself. Therefore, the possibility exists that the new peptide(s) required for recovery from ouabain, observed in the present work, might not necessarily be the Na^+,K^+-ATPase.

Taken together, our observations lead us to suspect that most cell types have a mechanism to trigger detachment when their pumps are blocked, and that Ma104 may have lost this ability. Alternatively, the type of ouabain-resistance we find in Ma104 cells might not reveal an actual *failure,* but perhaps a useful property. Thus the main role of Na^+,K^+-transport systems is the maintenance of an ionic balance, but they also participate in the control of proliferation and differentiation (Rozengurt & Hepel, 1975; Smith et al., 1982; English et al., 1983). Furthermore, morphogenesis during development depends not only on proliferation and differentiation of some cells, but also on the removal of other cell types, or of cells in a given position within a tissue. Therefore, the ability to remain attached in the presence of inhibitors of the Na^+,K^+-pump and to proceed with proliferation when these inhibitors are removed, may allow for the removal from a given tissue of other cells lacking this mechanism. It is therefore conceivable that the property we describe here for Ma104 cells would operate during tissue growth, specialization and repair.

Of course, being a foreign substance, ouabain cannot participate in such processes. However, endogenous digitaloids that circulate in the blood stream (Haupert et al., 1984; Graves & Williams, 1987) may in principle play such role. Hamlyn et al. (1991) have purified and identified by mass spectroscopy an endogenous substance from human plasma that is indistinguishable from ouabain. Therefore, the wide range in the sensitivity to ouabain afforded by the combinations of isoforms of α and β-subunits of the Na^+,K^+-ATPase, together with the differential attachment demonstrated in the present work, are likely to play a role in differentiation and organogenesis.

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