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A Novel Type of Cell-Cell Cooperation Between Epithelial Cells

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Abstract. Ma104 cells (renal, epithelial) have a peculiar way of resisting ouabain: their Na^+,K^+-pumps bind the drug with high affinity, cellular K^+ is lost and cell division arrested, but cells do not detach as most cell types do. Then, if up to 4 days later the drug is removed, Ma104 cells recover K^+ and resume proliferation (Contreras et al., 1994). In the present work, we investigate whether Ma104 cells are able to protect ouabain-sensitive MDCK cells in co-culture. The main finding is that they do, but in this case protection is not elicited by the usual mechanism of maintaining the K^+ content of neighboring cells through cell-cell communications. Ma104 cells treated with ouabain simply remain attached to the substrate and to their MDCK neighbors, and both cells lose K^+. This attachment includes tight junctions, because the transepithelial electrical resistance of the monolayers is not abolished by ouabain. Although the β-subunit of the Na^+,K^+-ATPase is known to possess molecular characteristics of cell-cell attachment molecules, attachment between Ma104-MDCK cells does not seem to be mediated by this enzyme, as immunofluorescence analysis reveals that Na^+,K^+-ATPase is only inserted in the plasma membrane facing a neighboring cell of the same type.

Key words: Ouabain-resistance — Epithelia — Ma104 cells — MDCK cells — Tight junctions — Na^+,K^+-ATPase — K^+ content — Metabolic cooperation — Cell attachment

Introduction

The cardiac glycoside ouabain binds with high affinity and specificity to Na^+,K^+-ATPases and, as a consequence, active ion pumping stops, cells lose K^+, detach and die. Cells whose Na^+,K^+-ATPases have instead a low affinity for ouabain can live in the presence of otherwise lethal concentrations of the drug. Some ouabain-resistant cells can also protect neighboring cells in co-cultures, a phenomenon termed "metabolic cooperation" and attributed to the ability of Na^+,K^+-pumps to maintain the cytoplasmic composition of ouabain-sensitive neighbors through cell-cell communications (Ledbetter & Lubin, 1979; Ledbetter, Young & Wright, 1986; Bolivar et al., 1987).

However, in a previous article (Contreras et al., 1995) we reported that Ma104 cells have a novel way of withstanding ouabain: they bind the drug, lose most of their K^+ and stop proliferating, nevertheless remain attached; when four days later the drug is removed from the culturing medium, cells regain K^+ and resume proliferation. In the present work, we explore whether Ma104 cells are able to protect ouabain-sensitive MDCK cells in co-cultures.

Materials and Methods

Cell Culture

Ma104 (epithelial line derived from rhesus monkey kidney) were a generous gift of Dr. Enrique Rodríguez-Boulán (Cornell University Medical College, New York). 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, México, DF), and 10% foetal calf serum (GIBCO 200–6170). In the following text,

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this complete medium is referred to as CDMEM. Cells were washed three times with Ca^{2+}-free PBS, harvested with trypsin-EDTA (In Vitro, México) and plated on 24 multiwell chamber (Linbro 76-033-05, Flow Lab., VA), on glass coverslips, or on nitrocellulose filters (Millipore HA, pores 0.45 μm, Bedford, MA), as required by the experiment (*see below*). Unattached cells were washed after one hr of incubation, and monolayers were further incubated for a day before further experimental manipulation.

Cell Number

Cells grown as monolayers on multiwell dishes were detached with Ca^{2+}-free PBS and trypsin-EDTA, and counted with a Coulter Counter (Coulter Electronics, Hialeah, FL).

Immunofluorescence

Glass coverslips containing MDCK monolayers cultured under the several experimental conditions described below, were rinsed twice with PBS, fixed and permeabilized with −20°C methanol for 45 sec, washed with PBS, incubated with 3% foetal bovine serum in PBS for 30 min, and treated with rabbit monoclonal antibody against the β-subunit of Na^+,K^+-ATPase (generous gift of Dr. Michael Caplan, Yale University) for 1.0 hr. Monolayers were then rinsed 3 times for 5 min each, with PBS, incubated with a FITC-labeled goat antirabbit antibody (SIGMA Chemicals, St. Louis, MO) for 30 min, rinsed as above, mounted in a mixture of p-phenyldiamine (SIGMA)-Gelvatol (Montsanto, Indian Orchard, MA) (1:19), and examined with a confocal microscope (MRC-600, Bio-Rad). Lateral views were generated by scanning the monolayers from apical to basolateral side along a 46 μm, using steps of 0.46 μm.

In the case of co-cultures of two different cell types, one of them was prelabeled with 5-(and-6)-((((4-chloromethyl) benzoyl) amino) tetramethylrhodamine (Cell Tracker Orange CMTMR) (Molecular Probes, Eugene, OR, C-2927). This was achieved by incubating the cells 45 min at 36.5°C, with CMTMR in DMSO added to the medium to a final concentration of 2 μM. Cells were then washed three times with PBS, reincubated 1 hr in CDMEM, trypsinated as above, and the suspension mixed with another cell type.

^{35}S-Methionine Labeling

2.0 mCi of ^{35}S-methionine (New England Nuclear) per milliliter of culture media was 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, mixed with unlabeled cells and plated in a multiwell plate. 24 hr later, cells were thoroughly washed with PBS and prepared either for cell counting, or 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 are given by Bolivar et al. (1987).

Intracellular K^+ Content

Monolayers of 2.0 cm^2, plated in Linbro 24-well chambers were incubated overnight with medium containing ^{86}Rb (Amersham RGS.2, England). This period is long enough to equilibrate the specific activity in the cells and in the bathing solutions (Cereijido et al., 1980). 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 2.0 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.

Table 1. Rescue of MDCK cells in co-cultures with Ma104 in the presence of 10 μM ouabain

Initial cell mixture		Ouabain	^{35}S	Surviving MDCK cells
Ma104	MDCK			
(%)		(μM)	(CPM)	(%)
0	100	0	2384 ± 48	100
0	100	10	63 ± 3	3
45	55	0	1312 ± 22	100
45	55	10	670 ± 14	51

MDCK cells labeled with [^{35}S]-methionine were mixed in suspension with unlabeled Ma104, and plated at confluence. Two hr after plating, the incubation medium was replaced with a fresh one containing 10 μM ouabain. One day later, monolayers were washed, scraped and the activity of ^{35}S measured.

Transepithelial Electrical Resistance (TER)

The degree of sealing of tight junctions was assessed by measuring the transepithelial electrical resistance (TER) (Cereijido et al., 1978*a,b*) of monolayers plated on nitrocellulose filters. 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.

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

Results

To investigate whether Ma104 cells can rescue ouabain-sensitive MDCK cells in co-culture, we labeled MDCK cells with [^{35}S]-methionine, mixed them with Ma104, plated the mixture at confluence, and exposed the resulting monolayers to 10 μM ouabain. By counting the amount of ^{35}S in monolayers of pure MDCK cells, and in those with mixed cell types, we measured the percentage of MDCK cells present in the second one. Further details on this procedure are given by Bolivar et al. (1987). Table 1 (first two rows) shows that 24 hr of 10 μM ouabain treatment effectively reduces the number of MDCK cells attached to 3%. However, when monolayers contained 55% of MDCK and 45% of Ma104 cells (third row), 51% of the MDCK cells remain attached in the presence of the drug (fourth row).

The next question was whether this protection de-

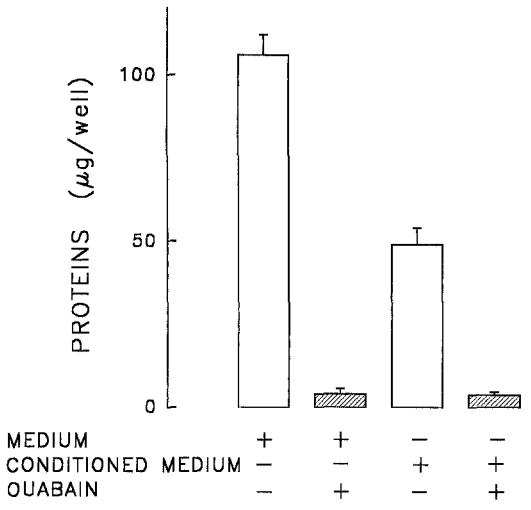

Fig. 1. Effect of conditioned media on substrate attachment of MDCK cells. One-day-old monolayers of MDCK cells cultured in 24-well multiplates were switched to CDMEM that had been 24 hr in contact with MDCK (**first** and **second** columns) or Ma104 cells (**third** and **fourth** columns), either in the absence or in the presence of 10 μM ouabain (**second** and **fourth** columns), and incubated for another 24 hr; (n = 6)

Table 2. Effect of ouabain on potassium content of monolayers of Ma104 and MDCK cells

Cell mixture		Ouabain	K content
Ma104	MDCK		
(%)		(μM)	(μmole · mg^{-1})
100	0	0	1.68 ± 0.04
0	100	0	1.63 ± 0.17
50	50	0	1.91 ± 0.06
100	0	1	0.30 ± 0.02
0	100	1	0.14 ± 0.01
50	50	1	0.31 ± 0.03

Cell suspensions were mixed, plated at confluence and the incubating medium was changed 1 hr later for a fresh one, containing 2.0 μCi · ml^{-1} of ^{86}Rb. One day later ouabain was added to this medium. Cells were incubated for 6 more hr, washed with ice-cold 0.1 mM $MgCl_2$, digested with 0.5 mM NaOH and counted. Protein was measured with Lowry method. Results are expressed as μmole per milligram of protein.

pends on direct cell-cell attachment, or is elicited instead through a putative hydrolase released by Ma104 cells to the medium that would destroy ouabain. The first two columns of Fig. 1, corresponding to control and ouabain-treated monolayers of MDCK cells, confirm that MDCK cells are highly sensitive to ouabain. The third and fourth column correspond to MDCK cells incubated with medium that was conditioned through 24 hr of exposure to confluent monolayers of Ma104 cells. The clear difference between first and third columns ($P < 0.001$) indicates that MDCK cells are affected by this medium. Nevertheless, the ouabain contained in the conditioned medium is fully active, as detachment of MDCK cells is almost complete (column 3 *vs.* 4; $P < 0.001$).

Generally, a cell rescues a neighbor by maintaining its ion content through intercellular junctions (Ledbetter et al., 1986). Therefore in a second step we investigated the fate of K^+ in the mixture of cells treated with ouabain. Cells were mixed and equilibrated for one day with ^{86}Rb, used as a marker for potassium, then treated for another day with 10 μM ouabain. ^{86}Rb was present at the same specific activity in all solutions until the experiment was terminated. Table 2 shows that control monolayers of mixed or pure cell types contain 1.63 to 1.91 μmole of K^+ per milligram of protein. Ouabain produces a drastic decrease of K^+ in monolayers of pure Ma104 (1.86 *vs.* 0.30 μmole/mg), in the few cells that remained attached in cultures of pure MDCK (1.63 *vs.* 0.14 μmole/mg) and in the mixed monolayer (1.91 *vs.* 0.31 μmole/mg).

To the best of our knowledge no information is available on why the blockage of Na^+,K^+-pumps with ouabain results in detachment of ouabain-sensitive cells. The possibility exists that this detachment is mediated by the loss of K^+. To test this possibility, MDCK cells were incubated in CDMEM under control condition (Fig. 2, left, open squares) and in the presence of 1 μM ouabain (filled squares), and in a medium in which Na^+ was replaced with K^+, both in the absence (open triangles) and in the presence of 1 μM ouabain (filled triangles). High K^+ in the medium does not help to keep MDCK cells attached, but it rather promotes detachment even in the absence of ouabain. A similar experiment performed with Ma104 cells (Fig. 2, right) confirms that attachment is not disturbed by the drug, and indicates that it does not depend on K^+.

Taken together, results in Tables 1 and 2, and Fig. 2 indicate that Ma104 cells, in spite of losing their K^+, remain attached, and rescue MDCK cells through a mechanism that does not require the presence of high concentrations of this ion in the cytoplasm. Another conclusion that may be reached, is that not only the types of attachments that enable Ma104 cells to anchor themselves to the substrate (plastic) are impervious to ouabain, but those involved in cell-cell adhesion as well.

One of the main forms of cell-cell contacts between cells of transporting epithelia is the tight junction (TJ), that seals the interspace at the level of the apical/basolateral limit and confers a transepithelial electrical resistance (TER) (Cereijido, 1991; Contreras et al., 1992). To assess the integrity of TJs under ouabain treatment we measured TER across the monolayer (Cereijido et al., 1978*a,b*). Figure 3 shows the value of TER across monolayers of MDCK (left) and Ma104 cells (right), represented as fractions of control values: 184 ± 5 (22) and 34 ± 2 (10) Ω · cm^2, respectively. Ouabain (1.0 μM) produces a drastic and fast reduction of TER in mono-

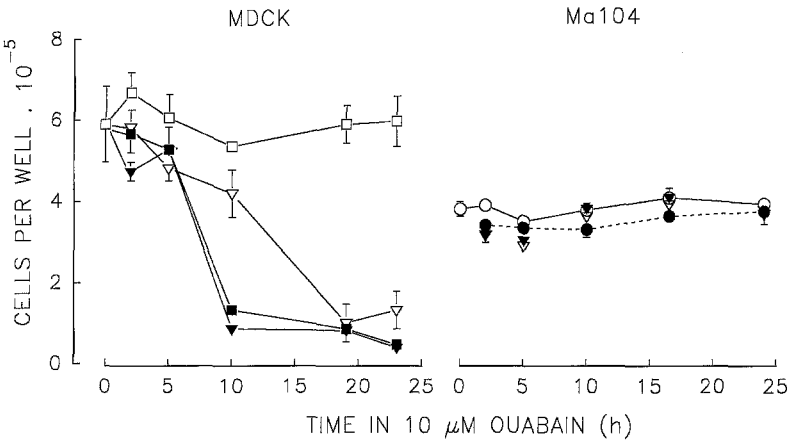

Fig. 2. Effect of exposure to ouabain and/or high K^+ content in the incubation media. Cells were grown as confluent monolayers in flat bottom multiwell dishes, and exposed to the experimental conditions since time = zero: Left, MDCK cells incubated in media with normal K^+ (squares) and Na^+ replaced by K^+ (triangles). Filled symbols refer to the same conditions but in the presence of 10 μM ouabain. Right: a similar experiment performed with Ma104 cells. Notice that although the two cell types were plated at approximately the same density (*ca* 10^6/ml), the amount of Ma104 ones per well is smaller. This can also be observed in Fig. 8 of the accompanying article.

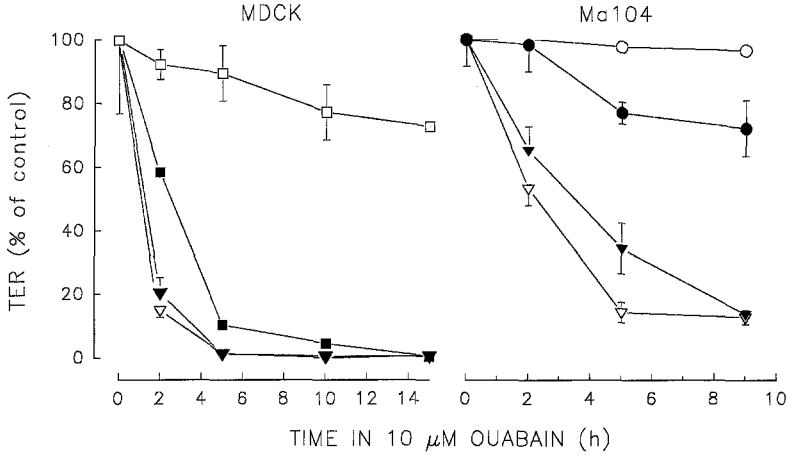

Fig. 3. Effect of exposure to ouabain and/or high K^+ content in the incubation media on tight junctions. Cells were grown as confluent monolayers in 24 multiwell dishes with millipore filters as substrate, and exposed to the experimental conditions since time = zero. Symbols as in Fig. 2.

layers of MDCK cells, but only a minor and slow decrease in those of Ma104 ones (filled symbols). Keeping extracellular K^+ at high levels (triangles), that presumably reduces the loss of cytoplasmal K^+, does not prevent the decrease of TER.

Mixed monolayers of 50:50 MDCK and Ma104 cells should have a TER_{mix} of 57 $\Omega \cdot cm^2$, according to the equation:

$$1/TER_{mix} = 0.5/184 + 0.5/34 \quad (1)$$

The value of 44 ± 2 (12) $\Omega \cdot cm^2$ found experimentally (Fig. 4) is not far from the theoretically expected one. This is in keeping with the observation of González-Mariscal et al. (1989) that TJs may be established between cells from different animal species and different sealing capacities. Ouabain reduces this value to 15 ± 1 (10) $\Omega \cdot cm^2$ ($P < 0.001$), indicating that while cell-cell attachments between the two cell types are sufficiently resistant as to permit the retention of MDCK cells in the monolayer, TJs are more sensitive to the effect of the drug.

To study the relationship of MDCK and Ma104 cells

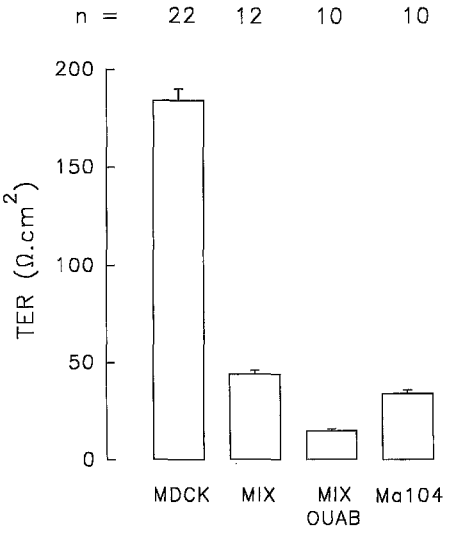

Fig. 4. Transepithelial electrical resistance (TER) in two-day-old monolayers of pure MDCK or Ma104 cells, that have been plated at confluence on millipore supports. *Mix:* monolayers prepared with mixed suspensions of both cell types in a 50:50 proportion. Under control conditions, or in the presence of 10 μM ouabain during the second day.

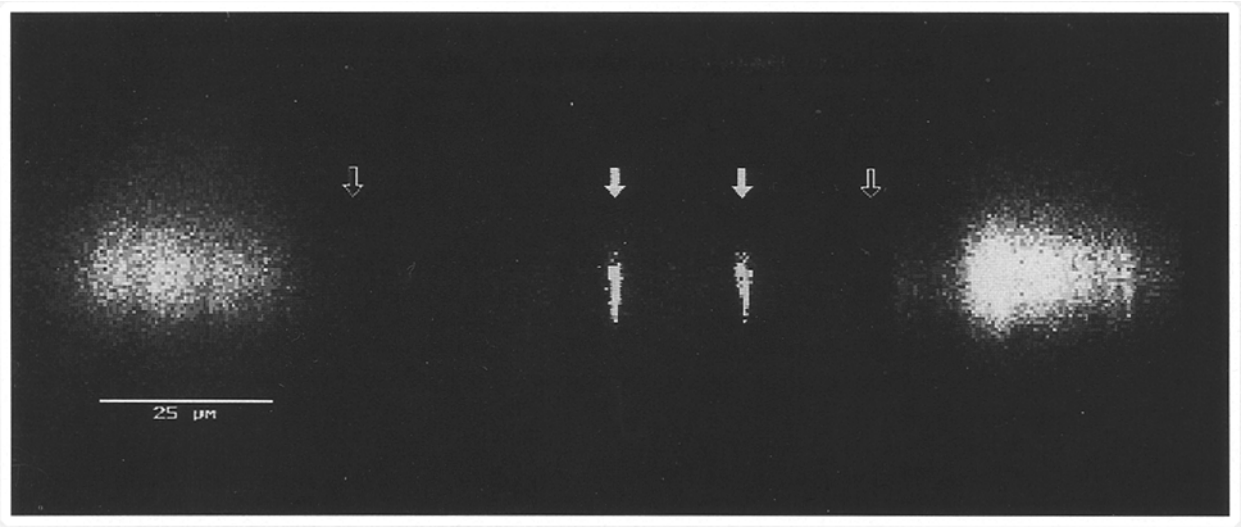

Fig. 5. Lateral view of the distribution of Na^+,K^+-ATPase in a mixed monolayer of MDCK and Ma104 cells. Ma104 cells were stained beforehand with an orange fluorescent tetramethylrhodamine derivative (CMTMR), so they can be easily identified by their red cytoplasm. However, this picture was prepared for publication in black and white. In this particular picture, the sequence (left to right) is Ma104/MDCK/MDCK/MDCK/Ma104. Limits between Ma104/MDCK are indicated by open arrows, and those between MDCK cells by filled arrows. Mixed monolayers were then incubated for 24 hr, fixed with methanol at –20°C, and stained first with a mouse antibody against the β-subunit of dog Na^+,K^+-ATPase (a generous gift of Dr. Michael Caplan, Yale), and then with a FITC-labeled goat antimouse antibody (SIGMA). The Na^+,K^+-pumps of Ma104 cells stain very weakly with this antibody, as it was developed against dog Na^+,K^+-ATPase, and are not seen. Notice that MDCK cells only express the enzyme at their borders with another MDCK cell.

as well as the distribution of Na^+,K^+-ATPases in mixed monolayers, we prestained the cytoplasm of Ma104 cells with the Cell Tracker Orange CMTMR. Figure 5 shows a lateral view of one of such monolayers having the following sequence (left to right): Ma104-MDCK-MDCK-MDCK-Ma104. Staining of the Na^+,K^+-ATPases as above only shows the distribution of the ones belonging to MDCK cells, as the ones of Ma104 cells stain too weakly to show at this intensity. Interestingly, Na^+,K^+-ATPases are only found at homologous contacts between the two MDCKs cells (filled arrows), but not at the MDCK-Ma104 borders (open arrows). Therefore it appears that a cell only expresses its pumps at the cell membrane, provided a neighboring cell of the same type also does.

Discussion

In a companion article (Contreras et al., 1995), we report that the resistance to ouabain of Ma104 cells is not due to a lack of affinity for the drug, but is attributable instead to their ability to remain attached to the substrate. We now see that Ma104 cells also remain attached to neighboring MDCK cells, thus presenting a novel mechanism of metabolic cooperation. This cooperation may not be attributed to the usual mechanism of keeping a high-K^+ concentration in the rescued cell, via gap junctions, because both cell types (Ma104 and MDCK) lose most of this ion in the presence of ouabain. These observations on the Ma104 pose the question of why other cell types detach when their Na^+,K^+-pumps are arrested for a sufficiently long time. There is at present almost no information to answer this question, but Contreras et al. (1995) suggested that it is conceivable that a blocked pump would send signals to different kinds of attaching molecules, ordering them to release their grip from whatever they are attached. Therefore this order should be sent firstly to cell/substrate attaching molecules, because in monolayer of pure MDCK cells treated with the drug these cells detach from the substrate. A second target of such signals would be cell/cell attaching molecules, because in the present work we observe that in monolayers of pure MDCK cells ouabain detaches them from each other. A third target must be constituted by the tight junction (TJ), because the value of transepithelial electrical resistance (TER) in such condition is abolished.

One may wonder whether one of such cell/cell attaching molecules may be the Na^+,K^+-ATPase itself, as it was pointed out that the β-subunit of this enzyme has many of the characteristics of such molecules, namely a single hydrophobic transmembrane domain, and a long and heavily glycosilated extracellular tail (Gloor et al., 1990). Furthermore, when the β-subunit is purified and incorporated into liposomes, it specifically binds to certain types of neurons (Antonicek et al., 1988), a property that led Schmalzing and Gloor, (1994) to suggest that this subunit mediates *trans*-stimulation of adjacent cells. Therefore one may speculate that when the Na^+,K^+-pump is blocked, its β-subunit receives an order to release neighboring cells, and that this may conceivably be

one of the mechanisms that fails in Ma104 cells, that can thus remain attached in spite of having their pumps arrested by ouabain.

In a previous work (Cereijido et al., 1981), we have shown that MDCK cells have their Na^+,K^+-ATPases in a polarized distribution on the basolateral side. However, if the monolayer is incubated in media with a low Ca^{2+} concentration (1–5 μM), TJs are absent and Na^+,K^+-ATPases are distributed at random over the whole plasma membrane. Upon transfer to normal Ca^{2+} (1.8 mM), the enzyme is removed from the apical domain, and a new enzyme is added to the basolateral side (Contreras et al., 1989, 1992; González-Mariscal et al., 1990). Nelson et al. (1990) have shown that this is due to the fact that Na^+,K^+-ATPases is only retained in the membrane when it forms a complex with fodrin and ankyrin, which in turn is only kept in close association to the lateral membrane, because of its attachment to the cytoskeleton of actin. MDCK cells maintained in suspension form aggregates and express the enzyme exclusively in the lateral membrane (Rodríguez-Boulan and Nelson, 1989). In the present work, it is observed that this positioning is very specific, as no enzyme is detected at MDCK/Ma104 boundaries.

In summary, in the previous article (Contreras et al., 1995) we report that the resistance to ouabain of Ma104 cells, is not due to lack of affinity of Na^+,K^+-ATPases for the drug, and reveals instead a peculiar relationship between this enzyme and the mechanisms of attachment. We ventured that a relationship between pumping and attachment, albeit not with the characteristics it has in Ma104 cells, would exist also in other cell types and would play an important role in differentiation and morphogenesis. In the present work, we observe that this relationship between pumping and attachment involves cell-substrate and cell-cell attachments, as well as TJs, and enables Ma104 to rescue MDCK cells in co-cultures treated with ouabain. Circumstantially, we also observe that cells only express Na^+,K^+-ATPases in a given lateral membrane, provided the neighboring cell is of the same type, a feature that is in keeping with the fact that the β-subunit has structural and functional properties resembling cell-cell attachment molecules.

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