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The Polarized Distribution of Na^+, K^+-ATPase and Active Transport across Epithelia

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Epithelia Unambiguously Demonstrated Active Transport

Today's understanding of active transport of sodium through biological membranes relies on such a mind-boggling amount of experimental data that few would realize that it started as an obscure concept whose acceptance had to overcome several formidable theoretical objections: (i) In 1904 Galeotti proposed that the electrical potential of the frog skin, discovered decades earlier by Du Bois Raymond, could be explained by assuming that the epithelium has a Na^+ permeability that is higher in the inward than in the outward direction (Fig.1*A*). Yet this explanation was rejected on the assumption that it would be in violation of the first and the second laws of thermodynamics, as a preparation like the one depicted in Fig. 1*B* would constitute a *perpetuum mobile* (Cereijido, Ponce & González-Mariscal, 1989; Cereijido and Rotunno, 1970). (ii) Biologists retorted that it was not a "perpetuum" mobile, because the electrical potential would vanish in a few hours, and proposed that cell metabolism would provide the necessary energy for a vectorial transport of ions that would in turn account for the electrical potential. (iii) This explanation was also rejected, because it would violate Curie's Principle, according to which phenomena of different tensorial order cannot be coupled: Na^+ transport is a vectorial phenomenon (it occurs in the outside $\rightarrow$ inside direction) and cannot arise from a scalar phenomenon (in those days chemical reactions were assumed to be scalar, and therefore could not be expected to proceed in a given direction). (iv) In order to account for the high K^+/low Na^+ content of animal cells, a pumping mechanism placed at the cell membrane was proposed, and an enzyme, Na^+, K^+-ATPase, that would split ATP in a Na^+- and K^+-sensitive manner, was found (Skou, 1965). (v) Yet, according to Gilbert Ling (1962), the concentration of fixed electric charges and hydrogen bonding sites in the cytoplasm is sufficiently high to account for the K^+/Na^+ asymmetry in terms of a differential adsorption of these ions to nonpermeable macromolecules, and therefore he saw no reason to assume that the Na^+, K^+-ATPase was responsible for the asymmetry.

In that scenario Hans Ussing devised electric and isotopic methods to unambiguously demonstrate that the frog skin can actually transport a net amount of Na^+ in the inward direction and in the absence of an external electrochemical potential gradient (Ussing & Zerahn, 1951). Of course, this demonstration, confirmed thereafter in hundreds of laboratories, prompted a closer look at the arguments that had stood in the way of accepting that metabolism can drive Na^+ transport. Let us mention just two: (1) A protein like Na^+, K^+-ATPase cannot bind ATP, Na^+ and K^+ just anywhere on its surface, but only at very specific sites; i.e., enzymes are vectorial at the microscopic level. Yet, when studied in a solution where millions of molecules point in all directions, vectoriality is lost (Fig. 1*C*). Nevertheless, in the orderly molecular orientation of a cell membrane, Na^+, K^+-ATPase is anisotropic both, at the microscopic *and* at the macroscopic level (Fig. 1*D*). (2) Based on De Donder's demonstration (De Donder & van Ryssel-berghe, 1936) that chemical affinity can constitute a driving force, as well as on Onsager's argument (Onsager, 1931) that all fluxes and all forces present in a system can be—in principle—coupled, Kedem (1961) demonstrated that the flux of a given ion can be coupled to metabolism, so the split of ATP into ADP plus P_i can, in fact, drive the net flux of ions (Fig. 1*E*). In keeping with those ideas, it was later

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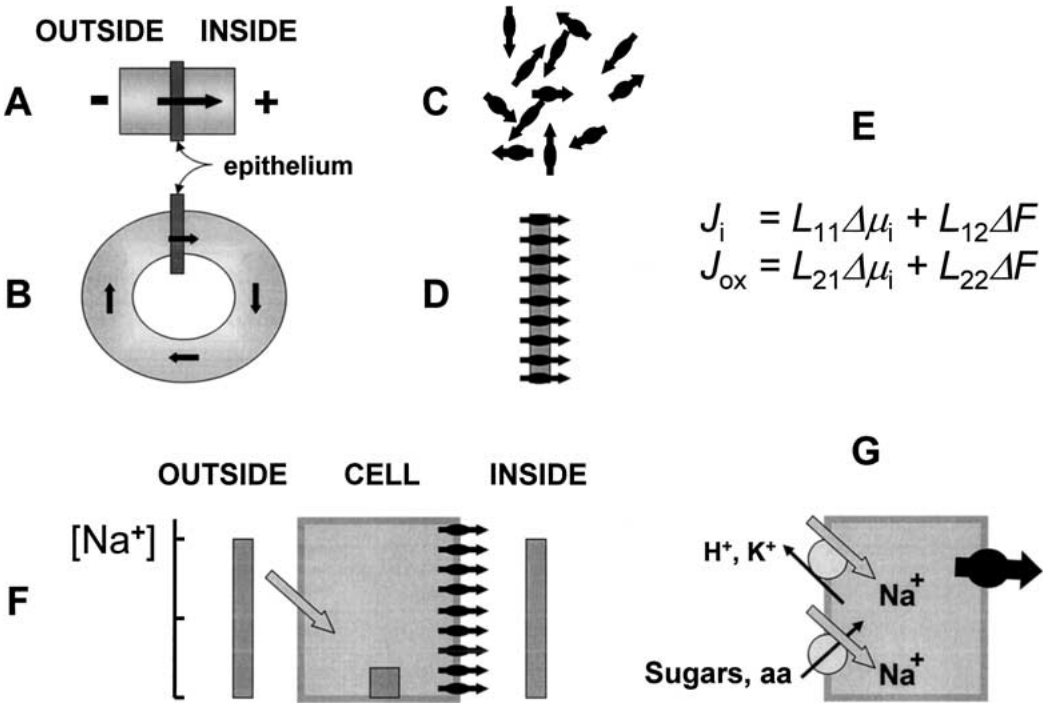

Fig. 1. Hans Ussing, polarity and active transport. (*A*) Galeotti proposes that epithelia may have asymmetric properties. (*B*) The asymmetric permeability to Na^+ would originate a *perpetuum mobile*. (*C*) Chemical reactions, which are vectorial at the microscopic level, loose vectoriality at the macroscopic one. (*D*) Vectoriality may be recovered by ordering enzymes in a cell membrane. (*E*) The flux of an ion (J_i) may be driven not only by its own electrochemical potential gradient ($\Delta\mu_i$) but by the driving force (ΔF) of a chemical reaction that can be gauged through the consumption of oxygen (J_{ox}). L_{12} and L_{21} are coupling coefficients. (*F*) In the model of Koefoed-Johnson and Ussing, Na^+ penetrates into an epithelial cell thanks to its concentration gradient, and is pumped on the opposite side against a concentration and an electrical gradient. (*G*) The penetration of Na^+ into the cytoplasm drives counter- and co-transports of K^+, H^+, sugars, amino acids, etc. Furthermore, the pump also helps to maintain a membrane potential (*not represented*) that affects the movement and distribution of all ions, including H^+, and thereby affects all chemical reactions in the cell through the pH.

found that the Na^+ gradient originated by the Na^+, K^+-ATPase can also drive the unidirectional fluxes of other substances via co- and counter-transporters (Fig. 1*G*).

The First Model of Active Transport across a Cell

Koefoed-Johnson and Ussing (1958) put forward a model that can account for the net transport of Na^+ across an epithelium (Fig. 1*F*) whose crucial characteristic is the polarized expression of the pump constituted by the Na^+, K^+-ATPase. The side-specific expression of this enzyme was later on confirmed by a host of experimental studies on the specific binding of ^{3}H-ouabain and of markers that can be observed under optical and electron microscopes, as well as by studies on membrane fractions of the apical and the basolateral side.

Therefore epithelial Na^+, K^+-ATPase has a double anisotropy: it is mounted in the plasma membrane with its Na^+- and ATP-binding sites toward the cytoplasm, and its K^+-binding site facing the extracellular milieu, and the enzyme is only expressed at the basolateral side of the cell.

Na^+, K^+-ATPase Expression is Polarized even in Cultured Epithelial Cells

The asymmetric orientation of Na^+, K^+-ATPase in the plasma membrane of all eukaryotic cells results from the way the peptides that constitute its subunits are inserted during translation in the endoplasmic reticulum. So the next question was, why does Na^+, K^+-ATPase occupy a restricted position on the basolateral side of epithelial cells and is not homogeneously distributed all over its surface? The answer to this question is part of an overall problem: how do epithelial cells distribute their membrane components (pumps, channels, receptors, etc.) in a polarized way into an apical and a basolateral domain? This polarization is in turn one of the two main features of the so-called "epithelial transporting phenotype", the other one being the making of tight junctions (TJs) that restrict the backflow of substances through the intercellular space (Cereijido, 1991), a feature that enables epithelia to separate biological compartments of widely different chemical composition. To study the process of polarization and junction formation we developed a preparation using epithelial cell lines, which express these two

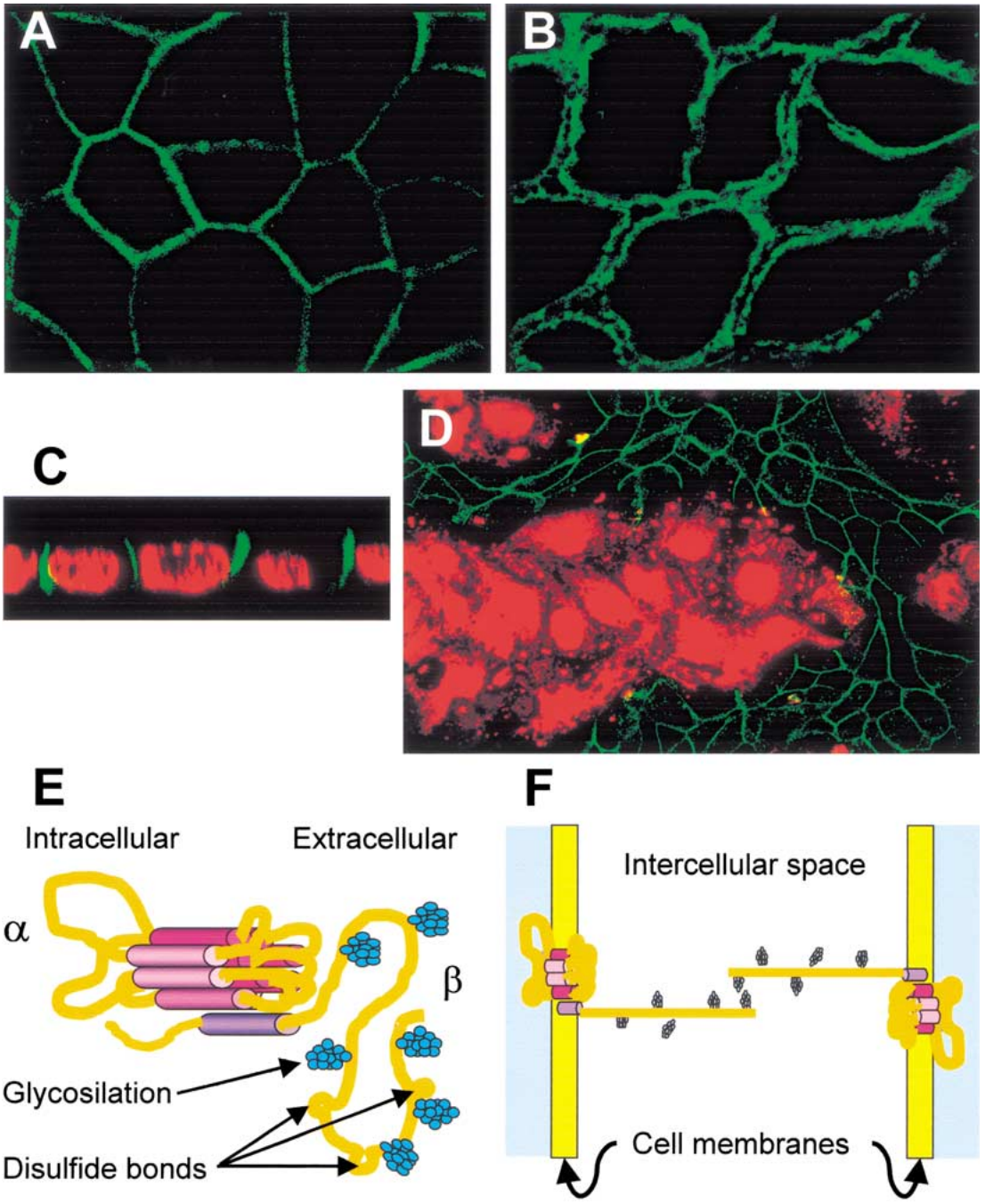

Fig. 2. Na^+, K^+-ATPase polarity. (*A*) In MDCK cells cultured in monolayers, Na^+, K^+-ATPase is expressed at the lateral borders. (*B*) Cell detachment provoked by 2.0 mM EGTA shows that each cell carries Na^+, K^+-ATPase in its borders, implying that the image in Fig. 2*A* is given by the enzyme present in the two neighboring cells. (*C*) The enzyme is expressed at the lateral but not at the basal border. (*D*) A monolayer prepared with a mixed population of MDCK cells and Mal04 cells, which were previously stained with the red dye CMTMR. The use of a mouse antibody specific for the β-subunit of MDCK, followed by a goat anti-mouse fluoresceinated antibody, indicates that this subunit only occupies the cellular border of an MDCK cell, provided the neighboring cell is another MDCK one, but not the border of an Ma104 cell. (*E*) The α-subunit of Na^+, K^+-ATPase is predicted to span the membrane 10 times, and is inserted with the Na^+-, Mg^{2+}- and ATP-binding sites toward the cytoplasm, and the K^+- and ouabain-binding sites facing the extracellular medium. The β-subunit has the characteristic structure of cell-attachment molecules: only one transmembrane domain, a long and glycosilated extracellular segment. (*F*) Na^+, K^+-ATPases in neighboring cells are assumed to interact through their β-subunits with a high level of specificity.

characteristics, lose them upon harvesting with trypsin and EDTA, but regain them in a few hours of reseeding (Cereijido et al. 1978). In this preparation Na^+, K^+-ATPase is not just expressed on the basolateral side (Cereijido et al., 1980, 1981), but restricted in addition to the plasma membrane of the

intercellular spaces (see Fig. 2*C*) (Contreras et al., 1989, 1999).

The Mechanism of Na^+, K^+-ATPase Polarization

Once cells are attached to the support, the removal of Ca^{2+} from the medium inhibits their ability to establish TJs and to polarize. In such condition, Na^+, K^+-ATPase is mainly retained inside the cell, and the few molecules remaining in the plasma membrane show no polarized distribution. Yet 20 hrs later, the addition of Ca^{2+}, acting on the extracellular fragment of E-cadherin, promotes cell attachment, and sends a signal via two different G proteins, a protein kinase C, a phospholipase C, and calmodulin that triggers the development of TJs and polarizes the cell (Balda et al., 1991, 1993; González-Mariscal et al., 1990). The assembly and sealing of TJs occur so quickly, that a fraction of the Na^+, K^+-ATPase is trapped on the apical side, but is thereafter removed from this location (Contreras et al., 1989). The polarized insertion of Na^+, K^+-ATPase is blocked by inhibitors of protein synthesis, yet the proteins whose synthesis is required are neither the α- nor the β-subunits of Na^+, K^+-ATPase (Contreras et al., 1989).

Newly synthesized Na^+, K^+-ATPase is directly addressed to the basolateral membrane domain in MDCK cells (Caplan et al., 1986; Gottardi and Caplan, 1993). This targeting seems to be determined by the impossibility of this enzyme to board the glycosphingolipid (GSL)-rich rafts that assemble in the Golgi complex and form vesicles that carry proteins towards the apical domain. This exclusion can be overcome by endowing the Na^+, K^+-ATPase with a sequence signal from the 4th transmembrane segment of the α-subunit of H^+, K^+-ATPase (TM4), which suffices to redirect the Na^+, K^+-ATPase towards the apical domain. Yet, there are nongastric H^+, K^+-ATPases that, in spite of lacking the TM4 signal, are nevertheless addressed towards the apical domain. On the basis of their work with chimeras, Dunbar and Caplan (2000) convincingly suggest that these differences in the polarized expression of ATPases can be explained as follows: (i) gastric H^+, K^+-ATPase has the TM4 apical addressing signal already formed; (ii) nongastric H^+, K^+-ATPases lack the TM4 signal, but can form it through a particular twist of the molecule that would join two separated segments and thereby complete the signal sequence; (iii) also Na^+, K^+-ATPase lacks a TM4 but, at variance with nongastric H^+, K^+-ATPases, it is unable to form one by reconfiguring the α-subunit in space; only the insertion of a TM4 by molecular engineering achieves its apical expression (Dunbar et al., 2000; Dunbar and Caplan, 2000). Once Na^+, K^+-ATPase arrives at the basolateral membrane it interacts with ankyrin, and becomes anchored to the cytoskeleton, which stabilizes the enzyme in this position (Hammerton et al., 1991).

While these observations shed light on crucial steps of Na^+, K^+-ATPase polarization, we still ignore why Na^+, K^+-ATPase expressed at the lateral borders binds to the cytoskeleton, but the same enzyme does not bind to this structure when expressed at other cell borders.

We took as hints that (i) when a monolayer is treated with 2.0 mM EGTA, the image of Na^+, K^+-ATPase (Fig. 2*A*) splits into two moieties and each neighboring cell retrieves its own (Fig. 2*B*), as if the expression entailed a Na^+, K^+-ATPase/Na^+, K^+-ATPase interaction. (ii) MDCK cells express Na^+, K^+-ATPase at the lateral but not at the basal borders (Fig. 2*C*), as if in this location the enzyme could not find an attaching partner on an opposite cell. (iii) When cocultured with Mal04 cells, (epithelial cells from monkey kidney) an MDCK cell only expresses Na^+, K^+-ATPase on a given border, provided its neighbor is another MDCK cell (Fig. 2*D*), suggesting that the putative Na^+, K^+-ATPase/Na^+, K^+-ATPase interaction is a specific one. (iv) Gloor et al. (1990) have shown that the β_2-subunit of glial cells acts as a cell adhesion molecule and has the corresponding structure: a long and glycosilated extracellular fragment and a single transmembrane domain (Fig. 2*E*). MDCK cells have instead the β_1 isoform, but its structure is almost identical to that of β_2. This suggests that the β-subunit of Na^+, K^+-ATPase may establish a cell-cell contact as suggested in Fig. 2*F*. (v) As shown below, Na^+, K^+-ATPase does participate in cell attachment.

Na^+, K^+-ATPase is Involved in Cell-Cell and Cell-Substrate Attachment

We have observed that Na^+, K^+-ATPase (the pump, *P*) is involved in cell attachment in yet another way. When it is blocked by ouabain, it sends a signal to cell-cell and cell-substrate attachment molecules (*A*); these become phosphorylated, are retrieved from the plasma membrane, and the cell detaches and dies, suggesting the presence of a $P \rightarrow A$ mechanism (Contreras et al., 1999). This is shown in Fig. 3*A* where MDCK cells are blotted with an antibody that binds to proteins phosphorylated on PY aminoacids. Ouabain (10 μM, 8 hours) enhances the phosphorylation level of 195, 175, and 100 kDa peptides, and decreases that of a 79 kDa one. This causes the retrieval of cell-cell and cell-substrate attachment molecules, illustrated in Fig. 3*B* through the immunofluorescence image of E-cadherin. In turn, retrieval of attachment molecules detaches MDCK-W cells from neighbors and the support (Fig. 3*C*).

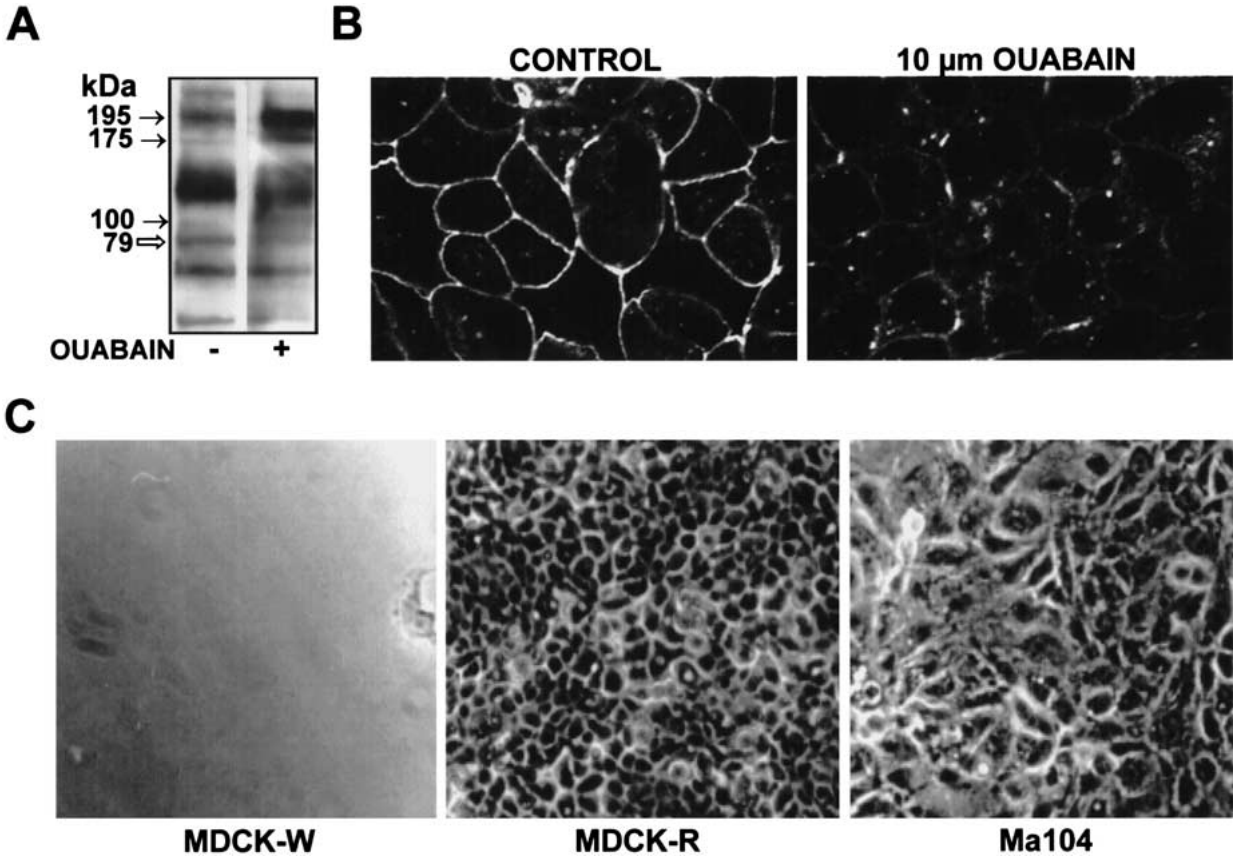

Fig. 3. Na^+, K^+-ATPase and Cell Attachment. (*A*) Binding of the hormone ouabain to the ion pump triggers a cascade of phosphorylations of peptides of 195, 175, and 100 kDa (*arrows*) and dephosphorylation of peptide of 79 kDa (*empty arrow*). It also causes retrieval of E-cadherin from the cell membrane (*B*). As a consequence, wild-type MDCK cells detach (*C*). The ion pumps of MDCK-R cells have an extremely low affinity for ouabain, and do not detach. Ma104 cells have a high affinity for the drug; yet they have a faulty $P \rightarrow A$ mechanism and do not change the phosphorylation levels. Accordingly, molecules involved in cell attachment are not retrieved from the membrane and the cells remain attached to each other and to the substrate.

To further investigate the $P \rightarrow A$ mechanism, we have an MDCK cell (MDCK-R) that resists ouabain in a "classical" way, i.e., due to lack of affinity of the Na^+, K^+-ATPase for ouabain (Bolivar et al., 1987). These cells do not undergo the changes in ion composition or the wave of phosphorylation and molecular retrieval. Fig. 3*C* (center) shows that MDCK-R cells treated with ouabain remain in the monolayer. We found another type of cells (Ma104, epithelial from monkey kidney) that is also resistant to ouabain, but in a new way, which is independent of the high affinity of the pump for the drug: its Na^+, K^+-ATPase binds ouabain, ion pumping stops, K^+ is lost and Na^+ and Ca^{2+} are gained, but attachment molecules fail to increase their level of phosphorylation and the cells remain adhered to each other and to the substrate (Fig. 3*C*, right) (Contreras et al., 1995a,b, 1999). We have also observed that this defect in the $P \rightarrow A$ mechanism enables Ma104 cells to rescue ouabain-sensitive MDCK cells in cocultures (Contreras et al., 1995a): Ma104 cells simply hold on to MDCK ones. As a complementary observation, we have shown that MDCK cells treated with a cell-detaching dose of ouabain do not undergo a process of apoptosis (Contreras et al., 1999).

Given that the cell can assemble its pumps by resorting to at least three isoforms of the α-subunits, that these isoforms vary in their affinity to ouabain, and that ouabain is a hormone readily available in the extracellular environment, it is conceivable that the $P \rightarrow A$ mechanism could play a role in tissue architecturing and in the readiness of some tumor cells to detach and produce metastasis.

In summary, the Na^+, K^+-ATPase of epithelial cells is involved in cell attachment. This attachment can in turn participate in the peculiar expression of this enzyme at the lateral border of the cell. Its β-subunit has the structural characteristics of a cell-attachment molecule. Whether this β-subunit is responsible for the lateral expression of Na^+, K^+-ATPase remains to be seen.

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