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Na^+,K^+-ATPase, THE STAR OF EPITHELIAL NET TRANSPORTS: WHAT DO WE KNOW ABOUT ITS OWN POLARIZED DISTRIBUTION

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The emergence of polarization

The cells of metazoan organisms exchange nutrients and wastes with an extremely thin layer of interstitial milieu, that would be quickly spoiled, were it not for a circulatory apparatus that constantly restores nutrients and takes away waste products by carrying them to and from large areas of epithelia (intestinal, renal, lung, gills, etc.), where they are finally exchanged with the external environment. Therefore metazoans ultimately depend on the ability of their epithelia to transport substances in a net amount towards the outside of the organism or in the opposite direction.

In the second half of the Nineteen Century Emile Du Bois Raymond found that the frog skin maintains a spontaneous electrical potential across, and in 1904 G. Galeotti proposed that it was due to a higher Na^+ permeability in the inward than in the outward direction, but his suggestion was rejected lest that it would be in violation of the first and second laws of thermodynamics (*for reviews see* Cereijido and Rotunno 1971, Cereijido *et al.* 2004). To circumvent this difficulty it was proposed that energy would be afforded by cellular metabolism. Yet this was also refuted because, according to Curie's Principle, phenomena of different tensorial order cannot be coupled, i.e. since the transport of Na^+ is a vectorial phenomenon (it occurs in the outside –> inside direction) it cannot be driven by a scalar phenomenon (in those days chemical reactions were assumed to be scalar, and therefore was not expected to proceed in a given direction of space). Half a century later electric and tracer methods unambiguously demonstrated that the frog skin can actually transport a net amount of Na^+ in the inward direction, in the absence of an external electrochemical potential gradient. This demanded, of course, a closer look at the arguments that had stood in the way of accepting that metabolism can drive Na^+ transport.

Na^+,K^+-ATPase is polarized at several levels

It was realized that a protein like Na^+,K^+-ATPase is in fact vectorial at the microscopic (*first*) level, as it cannot interact with Mg^{2+}, Na^+, K^+, and ATP just anywhere on its surface, but at very distinct and specifically located sites. Na^+,K^+-ATPase is also anisotropic at a higher (*second*) level of organization, as it is inserted in the cell membrane with the binding site for a given substances facing the cytoplasm ***or*** the extracellular side, but not both **(fig. 1A)**. In turn, De Donder and van Rysselberghe proved that chemical affinity can generate a driving force, Onsager demonstrated that all fluxes and all forces present in a system can be –in principle- coupled (See Cereijido *et al.* 2004), and Kedem 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 Na^+. In keeping with the idea that gradients and fluxes of different substances can be coupled, it was found that the Na^+,K^+-ATPase is also

responsible for the vectorial flux of sugars, aminoacids, Cl^-, Ca^{2+}, H^+, etcetera, because the electrochemical potential gradient that this enzyme generates drives a host of co- and counter-transporters.

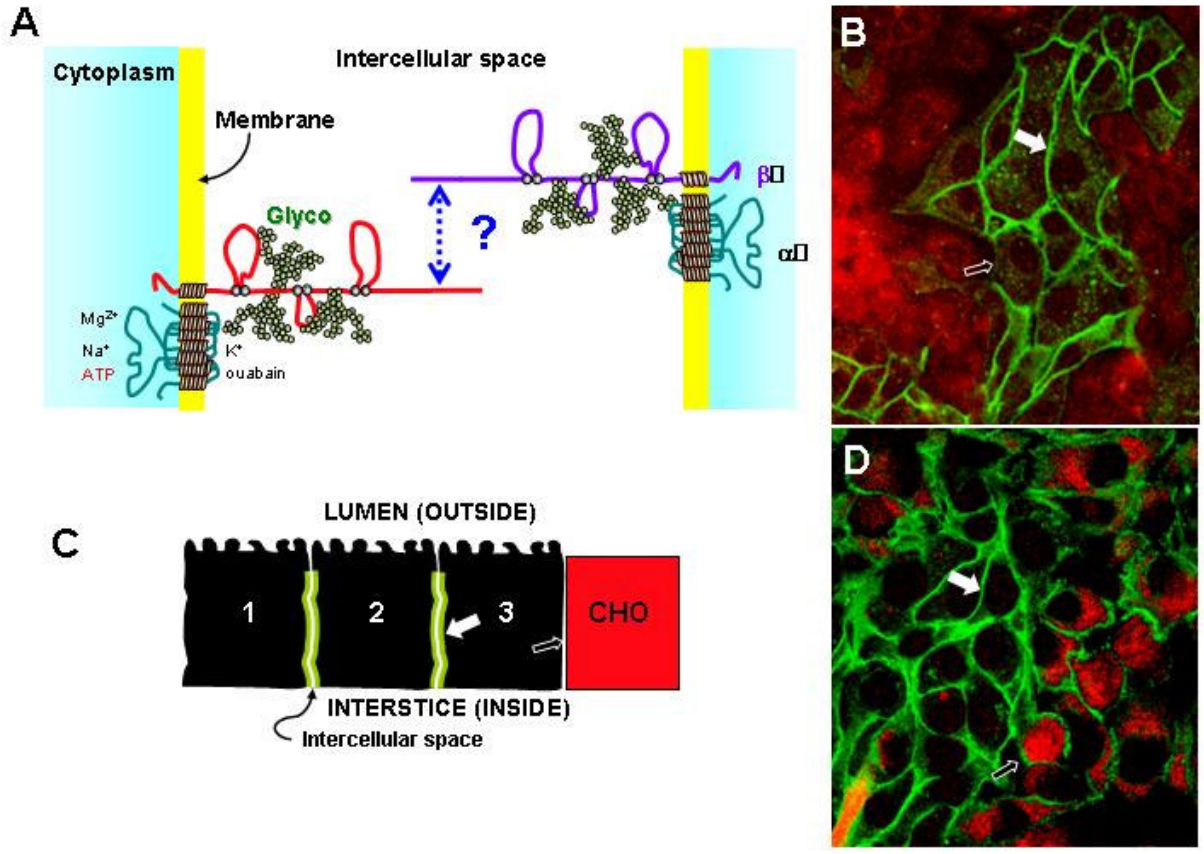

Figure 1: *Na^+,K^+-ATPase and its several polarities. (A) In this highly schematized cartoon, the β-subunit only spans the plasma membrane once; its extracellular moiety (red line) is highly glycosylated (green circles) and has three loops caused by S-S linkages. The β-subunit on the left (red) interacts with a β of the enzyme of the cell across the intercellular space (purple). At present we do not know whether this interaction is a direct one (blue ?) or mediated by another molecular species. (B) A mixed culture of chinese hamster ovary cells (CHO, red) with dog MDCK (black) shows that MDCK cells express their β-subunit at homotypic MDCK/MDCK contacts (filled arrows) but not at borders contacting a CHO (empty arrows). This is schematized in C, where MDCKs express Na^+,K^+-ATPase at homotypic contacts (1/2, 2/3). Yet MDCK cell number 3 expresses the enzyme at homotypic contact 2/3, but not at the MDCK/CHO one (empty arrow). (D) MDCK cells (black) co-cultured with hamster CHO fibroblasts (red) transfected with the β1-subunit of the dog (green), express their β-subunit at homo (filled arrow) as well as heterotypic contacts (empty arrow).*

Yet net transport of substances across the whole epithelium requires of a third type of polarization because, as proposed by Ussing and Koeffoed-Johnson, Na^+,K^+-ATPase should occupy the apical ***or*** the basolateral domain of the cell, but not both. Accordingly, in most epithelia Na^+,K^+-ATPase is found to reside on the basolateral surface. In a few epithelial cells such as those of the choroid plexus, retinal pigment epithelium, and cockroach salivary gland this enzyme is expressed on the apical side, but again, always in a polarized manner. In order to understand this *third*, apical/basolateral polarization of Na^+,K^+-ATPase, that is the one responsible for net transports across the whole epithelium,

we developed a model system of epithelial cell lines cultured as monolayers (Cereijido *et al.* 1978), and the purpose of the present article is to review the experimental keystones that led to the elucidation of this problem (Cereijido *et al.* 1981, Contreras *et al.* 1989,1995,1999,2004, Cereijido *et al.* 2001,2003,2004).

The Na^+,K^+-ATPase

Na^+,K^+-ATPase **(fig. 1)** belongs to the P-type family, that phosphorylates/ dephosphorylates during the pumping cycle (Beauge 2001). It has a 112 kDa ***α-subunit*** that crosses the plasma membrane some 10 times, and bears the site for ATP hydrolysis, the binding sites for Mg^{2+}, Na^+, K^+ and cardiac glycosides (Garrahan *et al.* 1982). The 4 isoforms of the α-subunit presently known in mammals exhibit somewhat different properties in terms of cation binding, and are expressed in a tissue-specific and developmentally regulated manner (Cereijido *et al*. 2004).

The ***β-subunit*** spans the membrane only once, and has a long extracellular portion, with 3 conserved disulphide bonds and 3-to-9 N-glycosylation consensus sequences. The 3 β-isoforms Na^+,K^+-ATPase identified so far in mammals, exhibit a tissue specific distribution (*for a review see* Chow and Forte 1995). Without the β-subunit α does not reach the plasma membrane, nor exhibits a detectable enzymatic activity and is rapidly degraded.

Finally, the **γ*-subunit*** is a small membrane protein that spans the membrane only once, and in spite of modulating the affinity for ATP, Na^+ and/or K^+, it does not seem to be required for catalytic activity (see Cereijido *et al.* 2004).

The polarized distribution of Na^+,K^+-ATPase

Three models have been proposed to account for the polarized expression of Na^+,K^+-ATPase. The first involves intracellular sorting of newly synthesized proteins at the Golgi apparatus, followed by a vectorial delivery of the Na^+,K^+-ATPase molecules to a specific surface domain (Caplan *et al.* 1986). A second one emphasizes random delivery of newly synthesized Na^+,K^+-ATPase to the entire plasma membrane, followed by selective retention by the submembrane cytoskeleton at specific points (Hammerton *et al.* 1991). We have proposed a third possibility, that attributes a crucial role to the β-subunit (Cereijido *et al.*, 2001). This model as well as its experimental support are discussed below (Shoshani *et al.* 2005).

Te so called "epithelial transporting phenotype", constituted by tight junctions and apical/basolateral polarity, cannot be expressed in the absence of Ca^{2+} (Contreras *et al.* 1989). Upon addition of this ion ("Ca-switch", Cereijido *et al.* 1978, Contreras *et al.* 1992) it binds to the extracellular repeats of E-cadherin, triggering a signal that is transduced via two different G proteins, a protein kinase C (PKC), a phospholipase C (PLC), calmodulin and MAPK (Balda *et al.* 1991). The expression of this phenotype requires the synthesis and simultaneous assembly of TJs (Cereijido *et al.* 1981), polarized expression of ion channels (Ponce *et al.* 1991b, Moreno *et al.* 2002), Na^+,K^+-ATPase (Contreras *et al.* 1989), etc. as if depending on a common upstream process. Actually, studies by Baas *et al.* (2004) have shown that the activation of gene LKB1 triggers the distribution of TJ

proteins ZO-1 and p120, redistributes the cytoskeleton of actin, and polarizes the cell, even in the absence of cell contacts. The cascade of reactions elicited by Ca^{2+} assembles and seals the TJs so quickly, that a fraction of the Na^+,K^+-ATPase is trapped on the apical side, but is thereafter removed from this location, demonstrating that the polarized expression of Na^+,K^+-ATPase is not part of an anatomical feature, but the frozen image of a process (Contreras *et al.* 1989).

The role of β-subunit in the polarized distribution of Na^+,K^+-ATPase

The experimental evidence indicating that the β-subunit is the key factor in the polarized distribution of Na^+,K^+-ATPase (Shoshani *et al.* 2005) can be summarized as follows: (1) MDCK cells do not express Na^+,K^+-ATPase over their entire basolateral side, but just on the ***lateral*** domain, as if the contact with its neighbors were crucial for the expression of this enzyme **(fig. 1)**. (2) MDCK cells co-cultured with other epithelial types express the enzyme in all (100%) homotypic MDCK/MDCK borders, but rarely in heterotypic ones with cells derived from human, cat, dog, pig, monkey, rabbit, mouse, hamster or rat. (3) Accordingly, MDCK cells never express Na^+,K^+-ATPase at contacts with Chinese hamster ovary cells (CHO). Yet when these are transfected with β_1-subunit from the dog (CHO-β), MDCK cells do express the enzyme at heterotypic CHO-β/MDCK contacts. (4) The β-subunit has the typical structure of an attachment protein: a short cytoplasmic tail, a single transmembrane segment, and a long and highly glycosilated extracellular portion **(fig. 1)**. (5) In keeping with this property, we have demonstrated that CHO-β cells are more adhesive than their CHO counterparts. (6) The expression of β_1-subunit in CHO-β cells forces the co-expression of endogenous α-subunit (Shoshani *et al.* 2005). Taken together, our results indicate that α and β subunits, that bind to each other right after their synthesis, and remain attached thereafter, including the journey to the plasma membrane, are expressed on the lateral side of epithelial cells through a highly specific β-β adhesiveness **(fig. 1)**.

The Hormone Ouabain

Knowledge about ouabain started in the 18^{th} century as an arcane component of foxglove (*Digitalis purpurea*) used to treat hearth congestive failure. Once purified, ouabain became a useful experimental tool to specifically inhibit the membrane enzyme Na^+,K^+-ATPase. This specificity together with its low Km (10^{-8} M) led to suspect that there must exist endogenous compounds resembling ouabain. These compounds have been found, and there is now compelling evidence that ouabain is in fact a hormone (Hamlyn *et al.* 1991, Schoner *et al.* 2003). This prompted a vigorous effort to detect and characterize its physiological roles. We will briefly mention just three of the most fascinating ones already found.

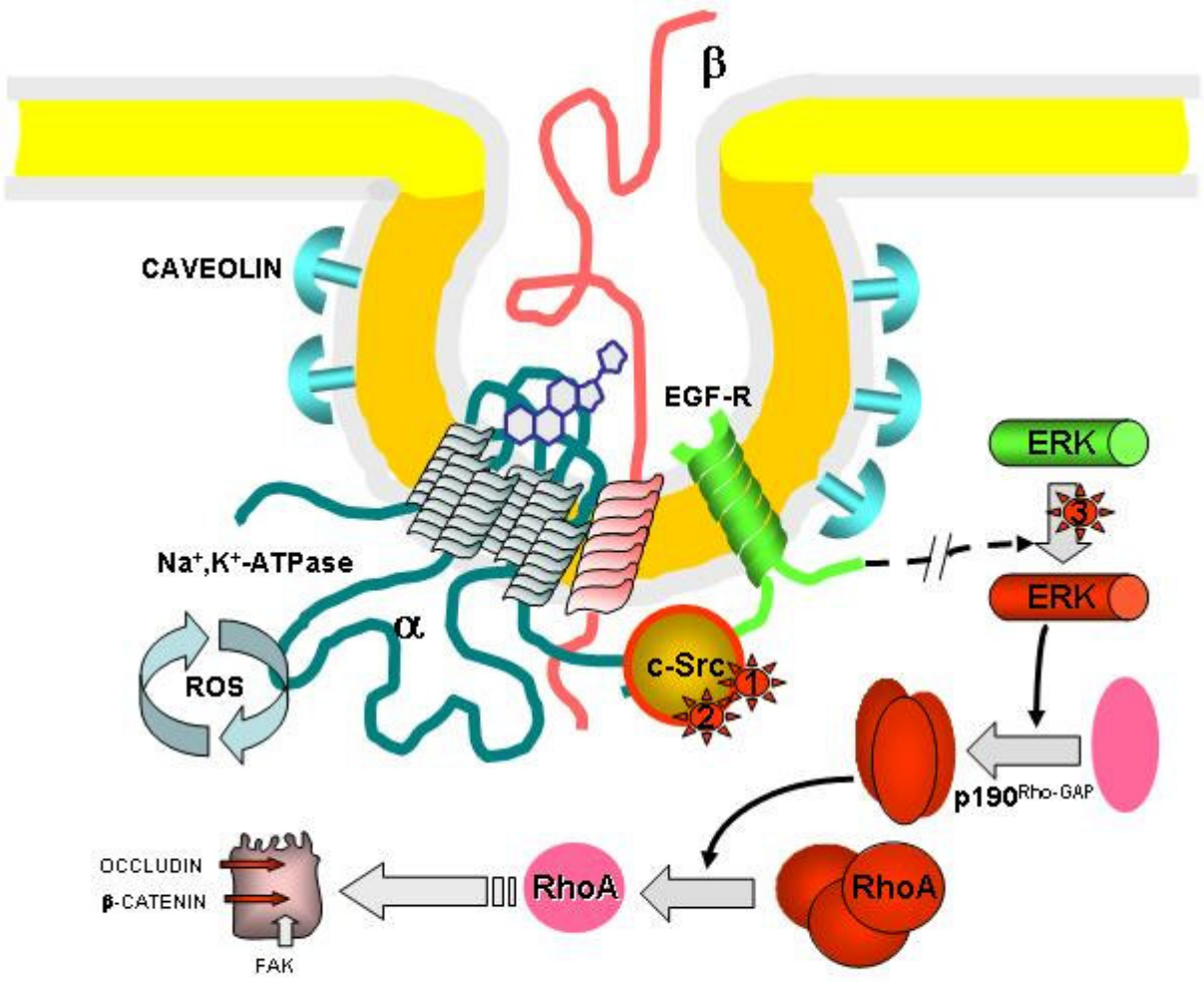

Figure 2: P–>A mechanism. *Ouabain (silhouette) induces the formation of a caveola (dark yellow) coated with caveolin, and including the α and β-subunits of Na^+,K^+-ATPase, as well as the EGF receptor. Red suns indicate the inhibition of tyrosine kinases, c-Src and extracellularly regulated kinases (ERK1/2), caused by genistein (1), PP2 (2) and PD98059 (3) respectivelly. Blue circling arrows represent reactive oxygen species pathway (ROS) which are involved in cell response to ouabain challenge. Ouabain results in activation of c-Src which in turn transactivates EGF-R which activates the ERK1/2 cassette, an effect that is followed by an increase in RhoA GTPase-activating protein p190 (p190Rho-GAP), with a consequent inhibition of RhoA. This signaling pathway prompts retrieval of adhesion molecules occludin, β-catenin and FAK, that results in detachment of the cell.*

The P–>A mechanism. We have found that ouabain binding to the pump (P), causes a release of the cell attachment (A) from its neighbors and from the substrate, through the so called "P–>A mechanism" **(fig. 2)** (Contreras *et al.* 1999, 2004). The binding of ouabain provokes accumulation of at least part of the Na^+,K^+-ATPase in caveolae, where it interacts with the epithermal growth factor receptor (EGF-R), and with caveolin (Wang *et al.* 2004, Xie 2003). As discussed above, expression of Na^+,K^+-ATPase of one cell causes the positioning of another Na^+,K^+-ATPase of a neighboring cell, right across the interspace (Cereijido *et al.* 2001, Shoshani *et al.* 2005). Yet we do not know whether the pull of Na^+,K^+-ATPase that occurs in the initial steps of P–>A snaps β-subunits free from their interaction with those in neighboring cells. The possibility exists that it would occur the other way around: the breakage of the β-β interaction may be the last straw that triggers

the endocytose of a caveolae, and this would be followed by a cascade of phosphorylations, along which ERK1/2 are activated, the cellular content of $p190^{Rho\text{-}GAP}$ increased, and these changes would in turn reduce the amount of RhoA bound to GTP. The final stage of the P–>A mechanism is the modification of the degree of phosphorylation and location of occludin, β-catenin and FAK (the chosen representatives of TJs, adherens junctions, and focal adhesion to the substrate respectively), which are thereby retrieved and cause detachment of the cell. Detached cells do not show signs of undergoing apoptosis (Contreras *et al.* 1999), as they can be collected, washed in ouabain-free media, and re-seeded, with a considerable plating efficiency.

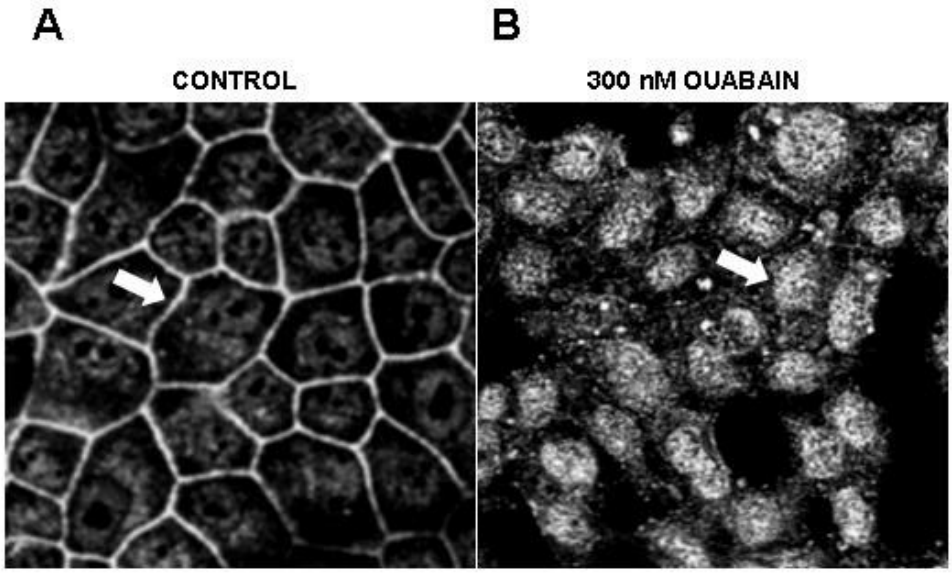

***Figure 3: Effect of ouabain on Nucleus and Adhesion Complexes (NACos). (A):** Monolayer of MDCK cells under control condition, showing the distribution of β-catenin mainly (arrow) on the lateral borders of the plasma membrane and, to a lesser extent, on the nucleus. (B): upon treatment with ouabain, β-catenin shuttles to the nucleus (arrow). In this location β-catenin co-localizes with other nuclear markers (not shown).*

Ouabain and the NACo family (***n***uclear and ***a***dhesion ***co***mplexes). NACos comprise a family of proteins with two different cellular locations. One of them is the submembrane scaffold of adhesion complexes such as TJs, adherens junctions and focal adhesions. The other location is the nucleus. Upon relaxation of the adherent grip with neighboring cell, a NACo releases from the submembrane scaffold and switches to the nucleus (Cereijido *et al.* 2004, Contreras *et al.* 2004) where it promotes the activation/silencing of specific genes **(fig. 3)**. A common experimental way to release this grip is to lower the concentration of Ca^{2+} in the bathing solution. We have found that a similar relaxation can be elicited by ouabain through the P–>A mechanism (Contreras *et al.* 2004). Toxic concentrations of ouabain, typically used to produce a total blockade of the Na^+,K^+-pump (1-10 ∝M), provoke a ***complete*** cell detachment; yet at nanomolar concentrations, i.e. closer to its hormonal levels, ouabain triggers the transfer of β-catenin to the nucleus. One of the NACos is β-catenin, that is primarily found at the lateral membrane of epithelial cells, as a member of the scaffold of adherens junctions. This protein participates also in the Wnt/Wingless signaling pathway involved in proliferation, differentiation, migration, embryonic development and cancer (Behrens and Lustig 2004). Therefore the importance of hormone ouabain stems from the possibility that, through its ability to switch NACos to

the nucleus, it would control these processes.

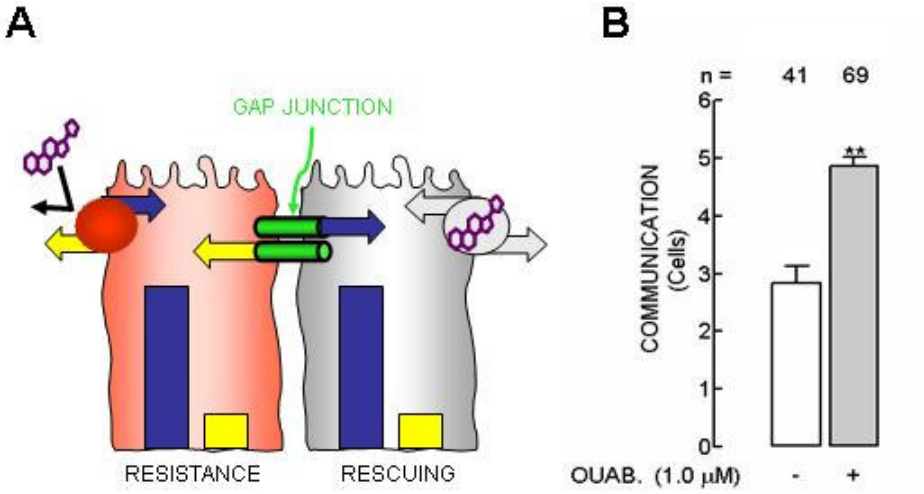

Figure 4*: Metabolic cooperation. (A) The Na^+,K^+-ATPase of a ouabain-resistant epithelial cell (left) has no affinity for this drug (silhouette), and keeps the control levels of K^+ (blue) and Na^+ (yellow). A cell whose enzyme is blocked by ouabain (right), manages to keep its ion levels thanks to gap junctions. (B) The scale indicates the number of cells connected to a one injected with neurobiotin, a fluorescen probe for intercellular conexion. Ouabain treatment increases the number of cells connected to the injected one.*

Metabolic cooperation. Cell-cell cooperation has a paramount physiological relevance, as it enables a cell to profit from substances that it does not synthesize, but can be provided by neighboring cells, or takes advantage of regulatory mechanisms that only the neighbor operates. It is involved in immune T-cells response, and is lost in cancer cells (Neijssen *et al.* 2005). Such is the case of cells that differentiate and engage in transport of substances across an epithelium thanks to the energy provided by a mitochondria-rich neighbor **(fig. 4)**. Metabolic cooperation can be easily studied experimentaly in cocultures where ouabain resistant cells (R) rescue ouabain sensitive ones (W) that would otherwise be detached and killed. We have shown that ouabain has a profound effect on the expression of cell-cell communication and thereby on metabolic cooperation (Bolivar *et al.* 1987, Cereijido *et al.* 1985, Contreras *et al.* 1995). This poses the fascinating question of how does a cell "know" that a given neighbor (or itself) is in danger and it would be helpful to express gap junctions?

Interest in the interaction of ouabain with Na^+,K^+-ATPase was further aroused by the observation that it triggers several signaling pathways, such as those involving reacting oxygen species (ROS) (Valente *et al.* 2003).

Concluding remarks

The cell membrane separates the mindboggling order of a cell from its comparatively chaotic surrounding. Na^+,K^+-ATPase is a central piece of the mechanism that, either directly throug the pumping of Na^+ and K^+, or indirectly through the electrochemical potential difference that drives co- and counter-transporters, keeps this order within the cytoplasm. Its importance is compounded in multicellular organisms, because pumping

should be performed in such a way that it will propel a multitude of net fluxes across the whole epithelium. The way Na^+,K^+-ATPase performs this role in epithelia depends crucially on its peculiar polarized expression on the lateral borders of the cells. In the present article we describe briefly that this is primarily due to the specific binding of α to the β subunit, and the homotypic binding of the β in one cell to the β of an Na^+,K^+-ATPase in the cell across the intercellular space. This enzyme also plays a role of receptor to hormone ouabain, that in the interim has also switched its status from a toxic of plant origin, to a hormone that can in principle affect cell proliferation, differentiation and cancer.

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