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**Na⁺,K⁺-ATPase is the putative membrane receptor of hormone ouabain**

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At 10 nM, ouabain elicits changes in cell contacts, which are independent and usually in opposite direction to effects occurring at μM levels, suggesting that these depend on entirely different mechanisms. However, this does not discard the possibility that in both instances ouabain would act on the same receptor. We demonstrate that such is the case by comparing the response of wild and ouabain-resistant MDCK cells on a very special type of cell contact, the tight junction (TJ).

The affinity and specificity of Na⁺,K⁺-ATPase for inhibitor ouabain are so high, that led to suspect that there might exist endogenous analogues. In keeping with this possibility Hamlyn et al. demonstrated the presence in plasma of a substance that, so far, cannot be distinguished from ouabain by mass spectrometry. This endogenous ouabain (EO) is first and foremost an adrenocortical hormone that may be also synthesized and secreted by the hypothalamus. EO was found to increase during exercise, salty meals and pathological conditions such as arterial hypertension and myocardial infarctions, raising the possibility that ouabain would constitute a new hormone (“new” in the sense that its status was only recently recognized), and prompting efforts to discover its physiological role.

In 1999 we observed that toxic levels of ouabain (≥1.0 μM) elicits retrieval from the plasma membrane of adhesion molecules to cell-cell and cell-substrate contacts between MDCK cells, and cause cell detachment (the so called "P→A mechanism", from pump/adhesion). On these basis we speculated that perhaps non-toxic levels of ouabain (≤50 nM) would act on molecules involved in cell adhesion, and enable hormone ouabain to modulate cell contacts. In keeping with this possibility we have recently found that 10 nM ouabain modifies the TJ through signaling pathways involving c-Src and ERK1/2, and distinctly modulating the expression as well as the pattern of distribution of specific claudins. This selective modulation is also reflected by the fact that participation of these signal molecules on the regulation of flux of ions (as gauged through TER) is different from the modulation of the flux of neutral Dextran. Interestingly, these hormonal effects elicited by 10 nM ouabain, are just the opposite of toxic ones caused by levels 50 times higher of ouabain. Therefore toxic and hormonal effects differ so much that leads to wonder whether they depend on different receptors.

Our basic strategy is to compare TER, a parameter that gauges the degree of tightness of the TJ, in the presence of inhibitors of signaling pathways, in wild (W-MDCK) and ouabain-resistant epithelial cells (R-MDCK). R cells constitute a stable line produced by Soderberg et al. by selection for growth in MDCK cells exposed to 2 μM ouabain, and have up to 30,000-fold higher K₅₀ for this substance. Other characteristics of Na⁺,K⁺-ATPase such as the rate of synthesis and amount of enzyme per unit of cell protein was unaltered in the mutants. In the batch of MDCK cells that we use in our laboratory, W cells are totally detached from the monolayer by ouabain concentrations ≤1 μM in less than 15 hours; a similar effect on R cells would take concentrations two or three orders of magnitude higher.
c-Src (but Not IP3K) Participates in the Effect of Ouabain

Receptors are usually associated to molecules that convey information to diverse cell structures and mechanisms. In this respect the Na⁺,K⁺-ATPase associates to a variety signaling pathways, notably through c-Src, IP3K, PLC and ERK1/2. Therefore we explored the possibility that these molecules would also participate in the transmission of no toxic effects. This was tested by studying the sensitivity to specific inhibitors of c-Src, ERK ½; IP3K and EGFR in W- and R-MDCK cells. Figure 2A shows the TER across monolayers in control condition and stimulated by 10 nM ouabain (white and grey columns), a systematic control that accompanied every test in the present work. Third and fourth columns show the value of TER in the presence of 10 μM PP2, an inhibitor of c-Src (light blue) and 10 nM ouabain in the presence of the inhibitor (dark blue). Blocking c-Src prevents the response to ouabain. Fifth and sixth columns are represented in different shades of red, to illustrate that by itself, the inhibition of phosphoinositide 3-kinases (PI3K) with 25 μM LY294002 does not affect the value of TER (pink), and the stimulation of this parameter with 10 nM ouabain is not prevented when added simultaneously with the inhibitor (red). In summary: while the route of c-Src participates in the modulation of the TJ permeability to ions, IP3K does not.

With a similar protocol, in Figure 2B we blocked ERK1/2 with 10 μM PD98059 and observed that by itself it lowers the control value of TER in monolayers of W-MDCK cells (light green), but does not completely block the effect of ouabain on TER (dark green). The studies described in the previous paragraph show that ouabain stimulation of Na⁺,K⁺-ATPase activates c-Src, a molecule that can also transactivate the epidermal growth factor receptor (EGFR). The EGFR autophosphorylation can be prevented with 10 μM PD153035 a specific inhibitor. Yet the last two columns in Figure 2B (lila shades) show that the inhibitor by itself (lila) does not perturb TER, and when

Figure 1. 10 nM Ouabain increases TER in monolayers of W-MDCK but not R-MDCK. (A) TER as measured in monolayers incubated in the absence or presence of ouabain in MDCK W and MDCK-R for 3 days. (B) Effect on TER of several concentrations of ouabain on R-MDCK (squares) and 1.0 μM on W-MDCK cells (circles).

ouabain Increases TER in W-MDCK Cells But Not in R Ones

In Figure 1A circles correspond to W and squares to R-MDCK cells. Open and filled symbols indicate control and ouabain treated cells respectively. Untreated W and R cells have a similar degree of tightness of the TJ (TER at time zero). Yet 10 nM ouabain gradually increases TER in W cells (gray circles) but not in R ones (gray squares). The effect of ouabain on W cells is a slow one, reflecting the fact that hormonal effects depend on protein synthesis. In the three day period studied the effect does not reach saturation. To study the possibility that R cells would be able to respond to ouabain albeit at higher concentrations, in Figure 1B we tested up to 1 μM, and corroborated that R cells do not respond to hormone ouabain. Notice that at this (toxic) concentration W cells do not respond by increasing the tightness of the TJ, but open this attaching structure so it becomes completely relaxed (black circles). Therefore, R cells, whose Na⁺,K⁺-ATPases cannot bind ouabain, are not stimulated by 10–100 nM of the hormone either, as if the pump were also the hormonal receptor.

Figure 2. c-Src and ERK1/2 participate in the effect of ouabain on TER; EGFR autophosphorylation and IP3K do not. To ease comparisons, a given pair of related columns are represented in light and dark shades of the same color. (A) TER across monolayers of W-MDCK cells with or without ouabain, in the absence (white and grey columns) or presence of PP2 (light and dark blue columns) or LY294002 (pink and red columns). (B) TER across monolayers of W-MDCK cells with or without ouabain in the absence (white and grey columns) or presence of PD98059 (light and dark green columns) or PD153035 (lila and purple columns). Samples were taken at the 2nd day of adding ouabain.
receptor for the modulation that ouabain exerts on cell contacts. Ogawa et al. have recently described a crystal structure of Na+,K+-ATPase from the shark rectal gland with ouabain bound by soaking the extract with 20 mM ouabain for 5 h. They found a single site for ouabain binding, with this molecule deeply inserted into the transmembrane domain with the lactone ring very close to the bound K+. Structural changes caused by ouabain binding are rather small and limited to transmembrane helices M1-M4, and are essentially the same for the high and the low-affinity ouabain-bound state.

![Figure 3](image)

**Figure 3.** Relationship between Na⁺,K⁺-ATPase (pink silhouette), the plasma membrane and several signaling routes and physiological effects (bottom). The silhouette corresponds to the contour of the crystal structure published by Ogawa et al. (2009). It shows also the position of ouabain (red), as well as the site where a cysteine residue in W cells was mutated in R ones, causing a marked decrease of their affinity for ouabain that allow them to survive in the presence of high concentrations of ouabain (black circle). In the second Na⁺,K⁺-ATPase (left to right) c-Src (red oval) is occluded and cannot phosphorylate EGF receptor nor ERK1/2 for example. Upon addition of ouabain, the c-Src is unmasked and is able to phosphorylate (red stars) both of them. c-Src and ERK1/2 cause a modification in the distribution of claudins 1 and 2 that changes the permeability of the TJ to ions and neutral Dextran. The third Na⁺,K⁺-ATPase illustrates the ability of ouabain to bind to this receptor and cause the release of Ca²⁺ from endoplasmic reticulum, that originates two basic responses; the first one is the transfer of NFκB to the nucleus, where it modifies gene expression, and the second is the oscillation of calcium levels in the cytoplasm. These changes affect both apoptosis and proliferation. The Na⁺,K⁺-ATPase in the extreme right of the plasma membrane represents the well known toxic effect of treatment with μM levels of ouabain, which blocks this molecule as pump as well as ATP hydrolyzing enzyme. The consequence is of course an inhibition of Na⁺ and K⁺ pumping.

In the present article we provide evidence that Na⁺,K⁺-ATPase is also a receptor for the modulation that ouabain exerts on cell contacts. Ogawa et al. have recently described a crystal structure of Na⁺,K⁺-ATPase from the shark rectal gland with ouabain bound by soaking the extract with 20 mM ouabain for 5 h. They found a single site for ouabain binding, with this molecule deeply inserted into the transmembrane domain with the lactone ring very close to the bound K⁺. Structural changes caused by ouabain binding are rather small and limited to transmembrane helices M1-M4, and are essentially the same for the high and the low-affinity ouabain-bound state. **Figure 3** shows the contour of the crystallized Na⁺,K⁺-ATPase as well as the contour of ouabain (red) that we traced to ease discussion. Canessa et al. have found that substituting a cysteine present in the wild type first transmembrane segment, by a tyrosine or a phenylalanine increases the inhibition of ERK1/2 (but not the autophosphorylation of EGFR) participates in the effect of hormone ouabain. Therefore, while ERK1/2 is one of the effectors of the pump, and participates in TJ modulation.

It should be taken into account that the purpose of the present studies is not to systematically explore all signaling routes involved in the effect of hormone ouabain, but further explore whether Na⁺,K⁺-ATPase may be the receptor of hormone ouabain, as in this case it should activate specific signaling routes and muscle fibers. The fact that this effect cannot be elicited in R-MDCK whose pumps have an extremely low affinity for ouabain due to a specific mutation (Fig. 1A and B) and ouabain does not engage signaling routes known to be associated to the pump (Fig. 2A and B), is in keeping with the possibility that the pump is the receptor of ouabain.

The physiological role of Na⁺,K⁺-ATPase is important in several respects: (1) It develops a Na+/K+ across the plasma membrane that gives rise to an electrical potential that is fundamental for the membrane electrical potential in all cells, in particular excitable ones like neurons and muscles fibers. (2) Na⁺,K⁺-ATPase is responsible for the low concentration of Na⁺ in the cytoplasm, that provides the driving force for most co- and counter-transporters that operate the flux of nutrients (e.g., Na⁺/glucose, Na⁺/aminoacids, Na⁺/protons, Na⁺/Ca²⁺, Na⁺/K⁺, etc.) (3) It is also responsible for the exchange of substances across the whole transporting epithelium that makes metazoan life possible.

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constant of ouabain-resistant MDCK cells by more of three orders of magnitude. We marked the position of this mutation with a black circle (Fig. 3) which is very close to the position occupied by ouabain. These findings rule out the possibility of two different sites, one for blocking the enzyme, and a second one for triggering hormonal effects. In R-MDCK the affinity of this site for ouabain is too low, and cannot act as hormonal receptor.

A couple of decades ago, cell contacts were regarded as mere mechanical structures that strengthen the tissues, lest these would disintegrate upon deformation. Today that there is considerable information on the large number and wide variety of protein species in tight, gap and adherent junctions, as well as synapses, the role of cell contacts can be hardly overestimated, as they are involved in differentiation, proliferation, communication, wound healing, cancer, metastases, etc. Sufﬁce to say that the most complex object in the universe, the brain, assembles itself and its circuits on the basis of what cell touches what other, when and how.

In the last decade a growing body of information indicated that ouabain and a series of very closely related analogs are usually present in diverse tissues, as well as vary in physiological and pathological conditions, most probably constituting a hormone.29 Following our demonstration that toxic levels of ouabain, besides touching what other, when and how.

Transepithelial electrical resistance (TER). The degree of sealing of TJ to ions was assessed by measuring the TER of the cells grown on Transwell permeable supports using an EVOM (World Precision Instruments). TER was measured after 1–3 days of ouabain treatment. Final values were obtained by subtracting resistance of the bathing solution and an empty support, and the results are expressed as ohms per square centimeter (Ω/cm²).12,15

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