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Epithelial Na⁺,K⁺-ATPase — A Sticky Pump

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Additional information is available at the end of the chapter

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Abstract

Na⁺,K⁺-ATPase is an ATP-powered ion pump that establishes concentration gradients for Na⁺ and K⁺ ions across the plasma membrane in all animal cells by pumping Na⁺ from the cytoplasm and K⁺ from the extracellular medium. This heterodimeric enzyme, a member of P-type ATPases, is composed of a catalytic α -subunit with ten transmembrane domains and a heavily glycosylated auxiliary β -subunit. The Na⁺,K⁺-ATPase is specifically inhibited by cardiotonic steroids like ouabain, which bind to the enzyme's α -subunit from the extracellular side and thereby block the ion pumping cycle. Na⁺,K⁺-ATPase generates ion gradients that establishes the driving force for the transepithelial transport of several solutes and nutrients. The effectiveness of this vectorial transport motivated by Na⁺,K⁺-ATPase depends on the integrity of epithelial junctions that are essential for the maintenance of the polarized localization of membrane transporters, including the lateral sodium pump. This chapter reviews the facts showing that, in addition to pumping ions, the Na⁺,K⁺-ATPase located at the cell borders functions as a cell adhesion molecule and discusses the role of the Na⁺,K⁺-ATPase β -subunit in establishing and maintaining cell-cell interactions. Furthermore, Na⁺,K⁺-ATPase is a multifunctional protein that, in addition to pumping ions asymmetrically and participating in cell-cell contacts, acts as specific receptor for the hormone ouabain and transduces extracellular signals. Thus, when bearing in mind with transporting epithelia phenotype, the importance of modulation of cell contacts by Na⁺,K⁺-ATPase can hardly be underestimated.

Keywords: Epithelial cells, Na⁺, K⁺-ATPase, Polarity, cell adhesion

1. Introduction

Epithelium is the name given to the cells that line a surface. Epithelia separate biological compartments with different composition, a fundamental role that depends on the establish-

ment of occluding junctions. Thus, epithelial cells are always contiguous with one another and are usually joined by special junctions—the tight junctions. The functions of epithelia differ markedly. Although all form a barrier, some are much more impermeable than others. The trans-epithelial movement of ions and molecules is reached by the action of a multitude of specialized transporting proteins that are asymmetrically distributed on the apical or basolateral plasma membrane domains of epithelial cells. This remarkable polarity of epithelial cells depends on the selective insertion and the recycling of newly synthesized proteins and lipids into distinct plasma membrane domains and on the maintenance and modulation of these specialized domains once they are established during epithelial development. The two basic characters of epithelial transporting phenotype are polarity and tight junctions (TJs). Polarity offers the necessary direction for substances transported across epithelia to be absorbed or secreted. TJs guarantee that the transported substances do not leak back through the intercellular space [1, 2]. The Na^+, K^+ -ATPase, known as the sodium pump, has been shown to play a central role in the transporting phenotype of epithelia. Today, we already know its crystal structure, the chemical composition as well as the spatial arrangement of its three subunits (α , β , and γ) (Fig. 1A), the relationship between ATP hydrolysis and ion movement, and several diseases related to its malfunction. Surprisingly, despite such detailed information, we keep finding new remarkable properties and new physiological functions of the Na^+, K^+ -ATPase. The present chapter is focused on three recently found properties: its expression at the lateral membrane of epithelial cells due to the self-adhesive property of the β -subunit, its role as hormone receptor, and its ability to modulate several types of cell contacts.

2. Na^+, K^+ -ATPase in epithelia

The history of Na^+, K^+ -ATPase can be compared to a double-sided step ladder, one climbed by biologists that investigated its intrinsic mechanisms and subunits the other by accomplished physicists who first criticized but finally solved immense theoretical obstacles in the road toward active transport. Thus, for a long while, the peculiar composition of the cytoplasm was attributed to presumed membrane impermeability to Na^+ . This alternative was invalidated right after the Second World War, when radioisotopes became available for biological research, and it was discovered that tracer Na^+ added to the bathing solution readily penetrates and distributes into the cell. This revived the question why Na^+ in the cytoplasm remains at a concentration much lower (~ 15 mM) than in the extracellular water (~ 140 mM). The simplest assumption was that there should be an enzyme in the plasma membrane that dissipates metabolic free energy to pick up Na^+ from the cell water and pump it out. This response was not readily accepted because it appeared in violation of the Curie principle: “Processes of different tensorial order cannot be coupled.” In simple worlds, metabolism and ATP hydrolysis are chemical reactions, which at that time were taken to be *scalar* processes; hence, they could not drive a *vectorial* process like the extrusion of Na^+ from the cytoplasm. Later on, it was argued that, in fact, chemical reactions are vectorial at microscopic level. However, this vectoriality is masked at macroscopic scale, in particular, when working with homogenized tissues where pumps point in all directions. However, if they were ordered in a membrane,

the asymmetry would be recovered, and a macroscopic flux would take place. A model put forward by Koefoed-Johnson and Ussing [3] proposed a pump located asymmetrically on the basal side of the epithelial cell that together with the specific Na-permeability of the outer cell membrane and the specific K-permeability of the inner facing membrane is responsible for the net movement of Na⁺. This made theoreticians happy, yet where was “the pump,” i.e., the membrane molecule, that would align and be responsible for the sided, asymmetrical movement of Na⁺? On 1957, Jens Christian Skou prepared an extract of crab tissue that contained an enzyme that splits molecules of ATP (hence deserving the name “ATPase”) into ADP + Pi, provided the medium contains K⁺ and Na⁺ ions at concentrations that compare with those in the cell and in the surrounding extracellular space. Therefore, the enzyme was aptly named Na⁺,K⁺-ATPase. Interestingly, Skou [6] was able to inhibit the ATP splitting activity of his extract by adding *ouabain*, a substance of vegetal origin that was found a few years earlier to inhibit active potassium and sodium transport in erythrocyte membrane [5]. By performing the Na⁺/K⁺ translocations cyclically, Na⁺,K⁺-ATPase transfers those ions in a net amount toward the extracellular medium and toward the cytoplasm, respectively, so it was justified to call it “pump” [6]. Another experimental/theoretical conflict occurred with sugars and amino acids transported in a net amount across epithelia. Since this transport occurs in a net amount and can be inhibited with ouabain, for a while, it was taken as a proof that there exists a glucose pump as well as other pumps for diverse amino acid species. Yet eventually, it was demonstrated that carriers for sugars and for amino acids are not pumps as they are not *directly* coupled to metabolism. Therefore, the Na⁺,K⁺-ATPase is the *primum movens*, responsible for the exchange of substances between the metazoan and the environment, across transporting epithelia, as well as for net exchange between the internal milieu and the cytoplasm.

2.1. Structure–function relationship of Na⁺,K⁺-ATPase

Na⁺,K⁺-ATPase is expressed in all animal cells and is one of the most important members of the P-type ATPases. The Na⁺,K⁺-ATPase creates the Na⁺ and K⁺ concentration gradients across the plasma membranes of most higher eukaryotic cells. Per cycle, it pumps three Na⁺ ions out and two K⁺ ions into the cell, coupling the energy derived from the hydrolysis of one ATP molecule. The Na⁺ and K⁺ gradients originated and maintained by Na⁺,K⁺-ATPase are the energy source for secondary active transport, which are used for the maintenance of cell osmolarity and volume, for the generation of action potentials along nerve cells, and for many other cellular purposes. The functional Na⁺,K⁺-ATPase is a heterodimer of α - and β -subunits. In addition to the $\alpha\beta$ heterodimer, there are tissue-specific regulatory γ -subunits, also known as FXYD proteins [7]. In this section, we will review the general structural and functional characteristic of each subunit and how the pumping of Na⁺ and K⁺ is achieved. Important findings about the function are briefly discussed in the light of several X-ray crystallography studies of Na⁺,K⁺-ATPase published in recent years [8–10] (Fig. 1A).

2.1.1. The α -subunit

The catalytic α -subunit is composed of approximately 1000 amino acid residues with a molecular mass of about 110 kDa. Since the first sequencing and cloning of the α -subunit of

the Na⁺,K⁺-ATPase [11], many biochemical studies pointed out to a model of ten transmembrane domains (M1–M10) and three cytoplasmic domains: A (actuator), N (nucleotide binding), and P (phosphorylation domain) (Fig. 1A). Another myriad of studies identified motifs crucial for cation binding and conformational transitions [12]. Four distinct isoforms of the α -subunit have been identified (α_1 – α_4) in which sequence differences are minor. Each isoform has different kinetic properties which may be essential in adapting cell Na⁺,K⁺-ATPase activity to specific physiological requirements [3]. The major form α_1 is found in most tissues and is the main or only form in kidney and most other epithelia.

The Na⁺,K⁺-ATPase *A domain* consists of the N-terminal segment plus the loop between M2 and M3 (Lys212–Glu319), which form a distorted jelly roll structure plus two short helices in the 40 residues of the N-terminal segment. The movements of this domain, especially of the loop M2–M3, are determinants for the TM conformational rearrangements, needed for the occlusion and release of cations (see Fig. 1B) [14]. *N domain* contains the ATP-binding site and extends from the phosphorylation site Asp376, with the 377-KTGTL sequence, to the C-terminal 593-DPPR hinge motif. Finally the *P domain* can be described as a six-stranded parallel β -sheet and contains three important motifs: 376-DKTGTL, which contains the aspartic acid residue that phosphorylates during catalysis; 617-TGD on strand 3, which associates with the phosphorylation motif during the conformational transition E1 to E2; and 715-TGDGVND, which terminates the sixth β -strand of the P-domain where Asp717 is required for binding of Mg²⁺ and phosphorylation (Fig. 1B).

The first crystal structure of Na⁺,K⁺-ATPase α -subunit published was that of Rb⁺-bound pig renal Na⁺,K⁺-ATPase. Each of the three cytoplasmic domains and transmembrane helices of α -subunit are superimposable with Ca²⁺-ATPase (SERCA). The two sites for K⁺ binding are found between helices M4, M5, and M6, and many of the residues involved had already been identified in various studies as cation-binding residues [8]. The first complete high-resolution crystal structure of Na⁺,K⁺-ATPase was obtained in a potassium-bound state and provided further detail into the molecular basis for K⁺ specificity [9] (Fig. 1A). The carboxy terminal part of the L7/8 loop is the primary interaction site with the β -subunit, in which a consensus sequence 901 SFGQ, proposed as a key interaction site is located [15].

2.1.2. The β -subunit

The β -subunit of the Na⁺,K⁺-ATPase was initially identified as a glycoprotein associated with the α -subunit in purified functional enzyme preparations [16]. The association between α - and β -subunits is relatively strong and remains stable in most non-ionic detergents. All of the known β -subunit species and isoforms share a common domain structure: a short N-terminal cytoplasmic tail, a single transmembrane segment, and a large extracellular C-terminal domain containing six extracellular cysteine residues forming three disulfide bridges, whose locations are completely conserved among the isoforms. Na⁺,K⁺-ATPase β -subunit is composed of approximately 310 residues with an apparent molecular mass of 55 kDa due to N-glycosylation. The β -subunit of the Na⁺,K⁺-ATPase has three isoforms designated β_1 , β_2 , and β_3 . Among these isoforms, there are various degrees of difference, and although all β -subunits are glycosylated, the number of N-glycosylation sites varies with the isoform. The β_1 isoform of

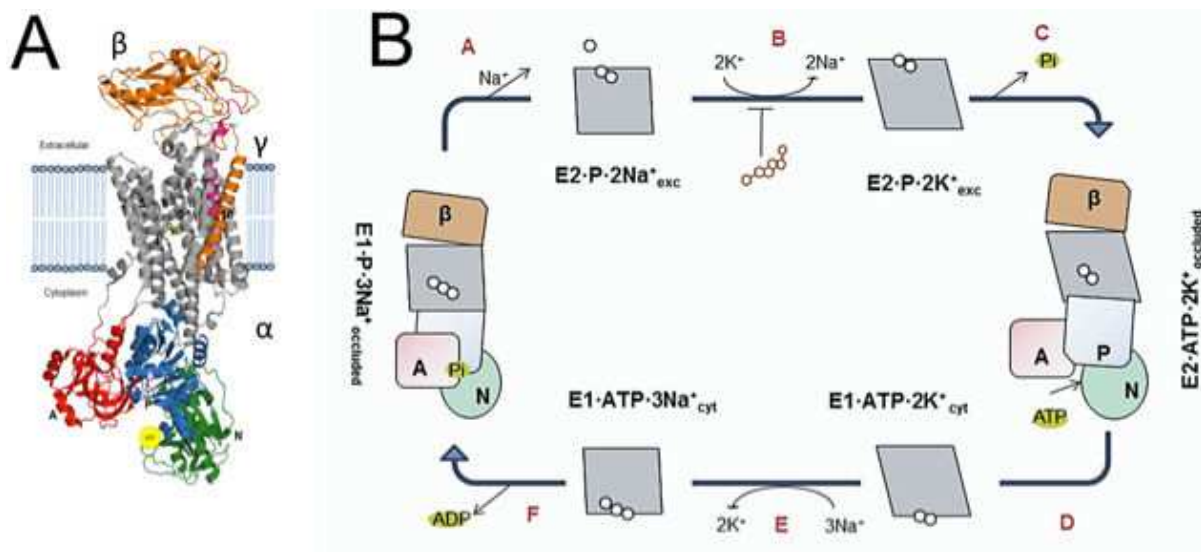


Figure 1. Structure of the Na⁺,K⁺-ATPase. (A) Ribbon model of the crystal structure of shark Na⁺,K⁺-ATPase [9; PDB code: 2ZXE], indicating α -subunit transmembrane domains in gray and cytoplasmic domains A, N, and P in red, green, and blue, respectively. The β -subunit is colored in orange and lacks most of its N-terminal cytoplasmic domain as in the crystal. The γ -subunit is colored in magenta. Alpha subunit transmembrane helices are numbered with the exception of TM 1 and 9, which are not visible from this projection. The two potassium ions occluded in the crystal structure are depicted in yellow. In the N domain, the ATP binding site is also indicated in yellow. (B) Schematic depiction of the catalytic cycle of Na⁺,K⁺-ATPase. A structural rearrangement, especially in domain A (pink-colored), is suggested by the cartoons at both conformational states E1 and E2. For simplicity, the rest of the cycle stages are represented only with the TM region in gray. (A) The outward transport of three Na⁺ ions is coupled to the E1 to E2 transition. (B) Two K⁺ ions bind at binding sites positioned to the extra cellular space. (C) Extracellularly bound K⁺ ions activate dephosphorylation that in turn results in ion occlusion. Ouabain (cardiotonic steroid silhouette in brown) binds at this conformational state halting the cycle. (D) ATP with a low affinity triggers the acceleration of inward transport of K⁺ ions through the pore as the pump enters the E1 conformational transition with low affinity for K⁺. (E) Three Na⁺ ions bind the intracellularly oriented sites. (F) Phosphorylation from ATP occurs, and Na⁺ ions are occluded. Several transitional substates exist during the cycle; nevertheless, they are not depicted for simplification.

Na⁺,K⁺-ATPase, which is consistently predicted to have three N-linked glycosylation sites, has been most extensively studied. All three consensus sites of β_1 are glycosylated [17, 18], and the oligosaccharides are terminally sialylated. Treuheit et al. [19] showed by mass spectrometry that the oligosaccharides of the β_1 -subunit from dog and lamb kidney are of tetra-antennary structure with extensions of 2–4 *N*-acetylglucosamine units, and the units of extension seemed to differ between the dog and the lamb β_1 -subunits. Nevertheless, detailed information is not available for the oligosaccharide composition of the two other isoforms, but it seems clear that there is a high degree of species variability for the β_2 isoform too. This isoform contains 7–9 N-glycosylation sites. It holds the high mannose-type carbohydrate epitopes L3 and L4 [20] also present on the neural recognition molecules L1, MAG, and P0 that mediate adhesion among neural cells [21]. Indeed, the β_2 subunit was originally identified as an adhesion molecule, AMOG, in glial cells [22].

The essential function of the β -subunit is acting as the molecular chaperone of the α -subunit. Na⁺,K⁺-ATPase α -subunit is inactive without its β -subunit. It has been demonstrated that the association of the β -subunit facilitates the correct packing and membrane integration of the newly synthesized α -subunit. Also, as the intimate partner of α -subunit, it modulates cation-

binding affinity [23, 24]. The complete crystal structure of Na⁺,K⁺-ATPase explains, at least partially, previous implications of the β -subunit in modulation of cation transport. The transmembrane helix of the β -subunit runs slightly separated from those of the α -subunit and is rather inclined than perpendicular to the membrane. It forms several interactions with M7 and M10 helices of the α -subunit. The extracellular domain of the β -subunit contains the well-conserved YYPYY motif that mediates several salt bridges with α -subunit L7/8 loop and also contains at least three additional clusters of residues interacting with α -subunit [9]. In epithelia, in addition to the classical chaperone function of the β -subunit, a cell-to-cell adhesion function has been ascribed to the β_1 subunit. This novel role of the β_1 -subunit will be described in a separate part of this chapter.

2.1.3. The γ -subunit

In addition to the $\alpha\beta$ heterodimer, there are tissue-specific regulatory γ -subunits, which are small membrane proteins characterized by an FXYD sequence, and therefore known also as FXYD proteins, of approximately 80–160 residues [24]. FXYD proteins mainly consist of a single transmembrane helix and an N-terminal extracellular domain where the FXYD motif is located. This domain anchors to the β -subunit extracellular and transmembrane domains. These proteins modulate the function of Na⁺,K⁺-ATPase adapting kinetic properties of cation active transport to the specific needs of different tissues [7]. The most studied FXYD proteins are FXYD1 or phospholemman mainly expressed in heart and skeletal muscle and is involved in heart contractility. In epithelia, kidney-specific FXYD2 decreases affinity of Na⁺,K⁺-ATPase for sodium and FXYD4 or CHIF, expressed in colon and kidney epithelia acts as a modulating several ion transport mechanisms that have Na⁺,K⁺-ATPase as a common denominator [26, 27].

2.2. The pumping catalytic cycle

Ion movements through the Na⁺,K⁺-ATPase have been studied by biophysical experiments for many years [28–30]. Those studies were incorporated into the conceptual framework called post-Albers cycle that depicts the sequence of reaction steps that couple ion transport and ATP hydrolysis (Fig. 1B). Na⁺ and K⁺ transport follow a “ping-pong” mechanism, wherein the two ion species are transported sequentially. Pumping of ions is achieved by alternation between two major conformational states, E1 and E2 [31, 32]. In E1, the cation-binding sites have high affinity for Na⁺ and face the cytoplasm; in E2, the cation-binding sites have low affinity for Na⁺ but high affinity for K⁺ and face the extracellular. As it is the case for all P-type ATPases, Na⁺,K⁺-ATPase autophosphorylates and dephosphorylates during each reaction cycle. In E1, after three Na⁺ ions are bound at the cytoplasmic face, the phosphoryl group is transferred to a conserved aspartic residue in the P-domain. At this point, the pump enters the E1P state with occluded Na⁺ ions, and when ADP leaves, another conformational change occurs, and the Na⁺ ions are released at the extracellular face. At this stage, the enzyme is no longer sensitive to ADP addition but to aqueous hydrolysis (E2P state) so that Pi is released at the catalytic site and so are two K⁺ ions occluded at the extracellular face. To complete the cycle, ATP binds the phosphorylation site leading to the departure of the two K⁺ ions in the cytoplasm. At this point, the pump returns to the E1 state with high affinity for Na⁺ ready to launch a new catalytic cycle (Fig. 1B).

The first crystal structures of Na⁺,K⁺-ATPase have been obtained in the E2 state, which is more stable [8, 9]. Nevertheless, they lack information about the Na⁺-bound state and in particular the location of the third Na⁺ site. Two recently published crystals both of Na⁺,K⁺-ATPase from pig kidney are of E1 states. These crystals are stable analogues of the transition state (E1P-ADP⁺•3Na⁺) preceding E1P•3Na⁺. The molecular comparison of the two states, E1 and E2, show that the α -subunit suffers important conformational changes in its cytoplasmic domains, especially in the A domain, which is rotated around an axis nearly perpendicular to the membrane. The transmembrane helices involved in cation binding also undergo important conformation changes, most of all TM 4, 5, and 6 (Fig. 1B). Based on these structural evidences, the third Na⁺-binding site has been clearly localized, and a cooperative process for the sequential binding of Na⁺ has now been formulated in detail [33, 34].

2.3. Mechanism of Na⁺,K⁺-ATPase polarity in epithelia

The apical and basolateral plasma membrane proteins of epithelial cells are synthesized in the endoplasmic reticulum (ER) and then sorted in the trans-Golgi network (TGN) to be sent into different carrier vesicles to apical or basolateral domain [35, 36]. The polarity of those routes depends significantly on specific signals encoded inside the membrane proteins. The basolateral proteins have short peptides sequences in the cytoplasmic domain. Some signals resemble endocytic signals (dileucine, YXX ϕ , and NPXY), while others are unrelated to endocytic signals (the tyrosine motifs in LDL receptor [37] and the G-protein of the VSV [38]). Early studies demonstrated that the Na⁺,K⁺-ATPase, comprised of α - and β -subunits, is sorted in the TGN and delivered directly to the basolateral membrane without significant appearance at the apical surface in certain strains of the Madin–Darby canine kidney cells (MDCK) [39, 40]. Therefore, a basolateral signal was assumed to exist in the α -subunit of the Na⁺,K⁺-ATPase. The Na⁺,K⁺-ATPase and the H⁺,K⁺-ATPase are highly homologous ion pumps, yet in LLC-PK1 cells they are polarized to the basolateral and the apical domains, respectively. The polarized expression of chimeric constructs of the α -subunit of the H⁺,K⁺-ATPase and the Na⁺,K⁺-ATPase in LLC-PK1 cells has been studied [41–43]. Uncommonly, an apical sorting information in the α -subunit of the H⁺,K⁺-ATPase was recognized within the fourth transmembrane domain. Swapping this domain into the Na⁺,K⁺-ATPase resulted in the redirection of that basolateral pump to the apical surface of LLC-PK1 cells [44]. Nevertheless, these studies do not clarify whether the α -subunit of the Na⁺,K⁺-ATPase contains a basolateral sorting signal in its fourth transmembrane domain. Therefore, it seems that a non-canonical polarity signal is involved in the basolateral targeting of Na⁺,K⁺-ATPase.

Clathrin plays a fundamental role in basolateral sorting. It interacts with endocytic or basolateral proteins through a variety of clathrin adaptors [45]. It has been shown that the adaptor involved in basolateral protein sorting is the epithelial cell-specific AP-1B (adaptor protein 1B). Nevertheless, the basolateral localization of the Na⁺,K⁺-ATPase is independent of AP-1B expression because its localization was not significantly affected by knocking down clathrin expression and it remained localized to the basolateral surface in both the μ 1B-deficient cell line LLC-PK1 [46] and in MDCK cells in which μ 1B expression had been suppressed via RNAi [47]. By taking advantage of the SNAP tag system to reveal the trafficking itinerary of the newly synthesized Na⁺,K⁺-ATPase, it was shown that the basolateral delivery of the Na⁺,K⁺-ATPase

is very fast (at 5 minutes after Golgi release, 50% of newly synthesized Na pump is colocalizing with the PM) and does not involve passage through recycling endosomes en route to the plasma membrane. Moreover, Na⁺,K⁺-ATPase trafficking is not regulated by the same small GTPases as other basolateral proteins [48]. Some membrane proteins may achieve polarity by selective retention at the apical or basolateral surface. Although less well understood, this polarity may reflect interactions with extracellular ligands or with intracellular scaffolds, such as cytoskeletal elements or arrays of PDZ domain-containing proteins [35, 49–51]. As described and discussed below, this is also the case of the epithelial Na⁺,K⁺-ATPase, which is retained at the lateral membrane domain due to *trans* adhesion of its β_1 subunits on neighboring cells.

3. Na⁺,K⁺-ATPase β subunit as an adhesion molecule

3.1. The β_1 -isoform is a self-adhesion molecule in epithelia

The β -subunit is a glycoprotein of 40–60 kDa that was shown to be involved in the structural and functional maturation of the holoenzyme [52, 53] and subsequent transport of the α -subunit to the plasma membrane [54–56]. Ion transport requires the participation of both α - and β -subunits [54, 57]. The β -subunit has a short cytoplasmic tail, a single transmembrane segment, and a long extracellular domain heavily glycosylated, a typical structure of a cell-attachment protein [25]. Fig. 1A depicts the position and arrangement of the three subunits of Na⁺,K⁺-ATPase: α -subunit, β -subunit, and γ -subunit obtained by crystallography. Note that the β -subunit is mostly exposed toward the intercellular space, while most of the α -subunit is contained in the cytoplasm [9]. Observations made in MDCK cells suggested that the β -subunit is a cell–cell attachment protein: (1) As most transporting epithelia, the monolayer of MDCK expresses Na⁺,K⁺-ATPase polarized toward the basolateral side [58]. Nevertheless, confocal immunofluorescence analysis of Na⁺,K⁺-ATPase localization shows that the pump is not located on the *basal* domain of the plasma membrane, but only in the *lateral*, at cell–cell contacts (Fig. 2A). (2) Upon previous treatment with EGTA, the confocal images show the apparent single green line splits into two indicating that in order to express Na⁺,K⁺-ATPase at a cell–cell contact both neighboring cells have to contribute part of the enzyme. (3) The expression of the Na⁺,K⁺-ATPase at a given lateral borders is observed when both contributing neighboring cells are homotypic and from the same species, for instance, MDCK/MDCK (dog/dog, Fig. 2A) but not MDCK/NRK (dog/rat) [59] (Fig. 2B). (4) When CHO cells (fibroblasts from Chinese Hamster Ovary) were transfected with a gene coding for the β_1 -subunit of the dog (CHO-dog β_1), these cells become more adhesive, as estimated by aggregation assays [60]. (5) On the other hand, mixed monolayers of MDCK and NRK-dog β_1 show that MDCK cells expose the Na⁺,K⁺-ATPase at the heterotypic border (Fig. 2C).

All together, these observations indicated that the lateral localization of the Na⁺,K⁺-ATPase in MDCK cells depends on the recognition and adhesion between the β_1 -subunits of neighboring cells [60] (Fig. 2D). Of course, the first question that arises is whether two corresponding β -subunits from different cells would get close enough to be able to span the intercellular space and interact directly as proposed. To answer this question, several protein–protein interaction

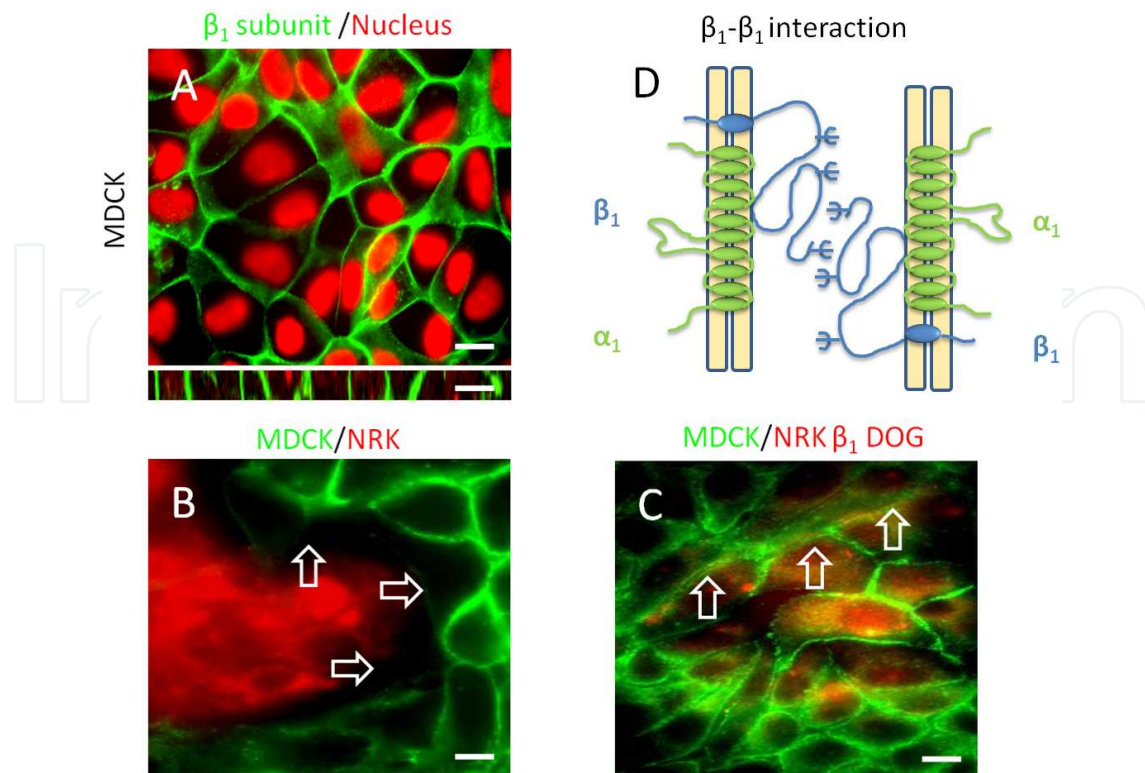


Figure 2. Hints to propose a model for the polarized distribution of Na⁺,K⁺-ATPase in transporting epithelia. (A) Monolayer of MDCK cells in a horizontal and a transversal section. Na⁺,K⁺-ATPase is stained in green, and nuclei in red, showing that the pump is expressed on the lateral membrane of the cells. (B) A confocal image of a monolayer prepared with a mixture of MDCK cells and NRK (normal rat kidney) cells; notice that the MDCK cells surrounding the NRK cell (previously stained in red with CMTMR) in the center only express their Na⁺,K⁺-ATPase on the membrane contacting MDCK cells, but not on the side contacting the NRK epithelial cell (indicated by arrows). (C) A confocal image showing a mixture of MDCK and NRK cells transfected with dog β₁-subunit. Arrows indicate the presence of Na⁺,K⁺-ATPase at heterotypic borders. (D) Proposed model for the polarized distribution of Na⁺,K⁺-ATPase in transporting epithelia. Scheme showing Na⁺,K⁺-ATPase α- and β-subunits expressed at the lateral border, where they are anchored by the β-subunits interaction at the intercellular space. Scale bar: 10 μm.

assays have been performed: (1) By pull-down assay, it was shown that dog β₁-subunit immobilized on Ni-beads could specifically bind to the soluble extracellular domain of β₁-subunits of the same animal species (dog). (2) Co-IP experiments have shown that rat β₁-subunits on NRK cells co-precipitate with rat YFP-β₁ subunit transfected in MDCK cells. (3) FRET (fluorescence resonance energy transfer) analysis of monolayers with a mixed population of MDCK cells transfected with a β₁-subunit fused to a cyan fluorescent protein (CFP), or with a β₁-subunit fused to yellow fluorescent protein (YFP), has shown that energy can be transferred from the first to the second cell type; in other words, two β₁-subunits can interact directly at <10 nm, thereby anchoring the whole enzyme at the cell membrane facing the intercellular space. Taken together, these evidences [61] supported by works from other groups [62-65] indicated that the β₁ subunit is indeed an adhesion molecule in epithelia.

In Moloney sarcoma virus-transformed MDCK cells (MSV-MDCK) that have an invasive phenotype, the level of Na⁺,K⁺-ATPase β₁-subunit is reduced as well as the expression level of E-cadherin. As expected, these transformed cells are also deficient in tight and desmosome

junctions. Interestingly, transfection of both E-cadherin and Na⁺,K⁺-ATPase β_1 -subunit induces the formation of junction complexes, reestablishes epithelial polarity, and suppresses invasiveness and motility, suggesting that β -subunit and E-cadherin are required to maintain the polarized epithelial phenotype [66]. Furthermore, stable adherens junctions are a requisite for proper tight junction function. In this regard, improving the Na⁺,K⁺-ATPase β_1 - β_1 interaction by reducing the complexity of the N-glycans of the β -subunit increases the resistance to detergent extraction of junction proteins and decreases the paracellular permeability. In other words, the fewer the branches are in β -subunit's N-glycans, the tighter are the intercellular junctions. Conversely, the impairment of the β_1 - β_1 binding by removing the N-glycans or altering the amino acid sequence of one of the interacting proteins decreases detergent resistance and increases the paracellular permeability, indicating that stability of adherens, and in turn tight junctions, does depend on β_1 - β_1 interaction [65, 67].

Studies in *Drosophila* have also shown that the β -subunits (in *drosophila* are named Nrv1, Nrv2, and Nrv3) are determinant of the Na⁺,K⁺-ATPase subcellular localization as well as function. Of the three *Drosophila* isoforms, Nrv1 and Nrv2 are localized in epithelia, while Nrv3 is expressed in the nervous system. Remarkably, while Nrv1 is expressed in the basolateral membrane of almost all epithelial cells, Nrv2 is localized at the septate junctions (tight junctions in insect) and co-localizes with coracle [68]. Furthermore, it has been shown that the extracellular domain of Nrv2 regulates the function of septate junctions and the size of the tracheal tube in a free manner independent of the pumping task [69].

3.2. β_2 /AMOG is a heterophilic adhesion molecule in nervous system

The Na⁺,K⁺-ATPase β_2 -subunit was first described in the nervous system. Schachner's group identified a cell surface glycoprotein and named it as Ca²⁺-independent adhesion molecule on glia (AMOG). AMOG was shown to mediate the neuron-to-astrocyte adhesion in the process of granule cell migration [70–72]. Further analysis revealed that AMOG is an isoform of the Na⁺,K⁺-ATPase β -subunit, named as the β_2 -subunit [22]. A remarkable characteristic of the β_2 -subunit is the multiple N-glycosylation sites in the extracellular domain [13]. Treatment with endoglycosidase H produces the shift of the apparent molecular weight from 50 to 35 kDa [70,20]. In a mass spectrometry analysis of the endoglycosidase H, released oligosaccharides from the β_2 -subunit three molecular ions were found corresponding to oligosaccharides composed of one N-acetylglucosamine and 5, 6, or 7 mannoses [20]. The β_2 -subunit promotes the neurite outgrowth by AMOG-to-neuron binding [73]. Schachner's group has assayed different partners for AMOG association in *trans*. They found that AMOG-containing liposomes only bind to small cerebellar neurons. When L1 and N-CAM antibodies were added to a monolayer of cerebellar neurons, none of these antibodies inhibited binding of AMOG-containing liposomes to neurons. Also, cells preincubation with an AMOG-antibody prior to addition of AMOG-containing liposomes did not reduce AMOG-containing liposomes-to-neurons adhesion [71]. These experiments suggest that neither L1 nor N-CAM is the β_2 -subunit ligand; thus, AMOG/ β_2 -subunit is a heterophilic CAM. As mentioned above, β_1 - β_1 adhesion in epithelial cells is homophilic. Accordingly, when assaying β_1 to β_2 adhesion, we found a null binding between these two isoforms, as well as between two β_2 -subunits (our unpublished

results). These findings are in agreement with previous studies [71]. As heterophilic CAM, the β_2 -subunit was shown to *cis*-interact with an oligomannose binding lectin, basigin. Basigin or CD147 is an ancillary protein of the monocarboxylate transporters 1, 3, and 4-isoforms [74–77] and, as a receptor molecule for high mannose carbohydrates, basigin binds specifically with oligomannoside carrying glycoproteins and neoglycolipids [78]. Kleene and coworkers showed that PrP, the AMPA receptor subunit GluR2, the astroglial α_2/β_2 ATPase, basigin, and the MCT1 form a functional complex at the plasma membrane of astrocytes. In this regard, the β_2 -subunit and basigin interact by means of the carbohydrate structure of the β_2 -subunit. The functional interplay of PrP, GluR2, the α_2/β_2 ATPase and basigin regulates the lactate transport via MCT1. Moreover, they observed that disturbing the oligomannose-mediated interaction of the β_2 -subunit and basigin leads to a deregulated and thus elevated glutamate-independent lactate transport [79].

3.3. β_2 isoform and apical polarity

The α_1 -subunit holds a basolateral sorting signal that commands the traffic of the epithelial sodium pump to this membrane domain [43]. However, the role of other α -subunit isoforms in the sorting of the Na⁺,K⁺-ATPase has not been studied yet. On the other hand, little is known about sorting signals in any of the β -subunit isoforms, yet the β_1 and β_3 isoforms have exclusive basolateral localization in epithelial cells [80], and instead, the apical distribution of the sodium pump correlates with the expression of the β_2 isoform [81–84]. In this regard, studies from our laboratory provide evidence showing that the apical polarity of the Na⁺,K⁺-ATPase in the retinal pigment epithelium (RPE) is related to the expression of the α_2 - and β_2 -subunits (Fig. 3A and B). Moreover, the time-dependent β_2 -subunit expression in the RPE model cells ARPE-19 correlates with the epithelialization of these cells (our unpublished results).

As we mentioned before, the β_2 -subunit possess up to 9 N-glycosylation sites (upon the species). Numerous studies have indicated the role of N-glycans in the polarity mechanism of apical proteins. For instance, the mutagenic removal of N-glycosylation sites in the gastric H⁺,K⁺-ATPase β -subunit [85], bile salt export pump [86], and glycine transporter 2 [87, 88], significantly decreased their apical content and increased their intracellular accumulation. Also, it has been shown that addition of N-glycans to various proteins changed their cellular localization toward the apical membrane domain. For example, a truncated occludin and a chimeric ERGIC-53 residing inside the Golgi in their nonglycosylated forms were apical redistributed after addition of N-glycans [89]. Indeed, engineering the β_1 -subunit by adding the N-glycosylation sites of the β_2 isoform leads to apical localization of the pump in HGT-1 cells [90]. All these evidences are consistent with the important role of N-glycosylation in apical polarization of Na⁺,K⁺-ATPase.

3.4. Structural insights into the self adhesion mechanism of Na⁺,K⁺-ATPase β_1 subunits

The shark Na⁺/K⁺-ATPase crystal structure in the E2 state published by Shinoda and coworkers was the first resolving the atomic structure of the extracellular domain of the β -subunit (PDB: 2ZXE) [9]. The extracellular C-terminal domain of the protein folds into an Ig-like β -sheet sandwich as predicted in silico [91]; actually, deletion of this C-terminal domain abolishes the

ARPE-19, 4 weeks

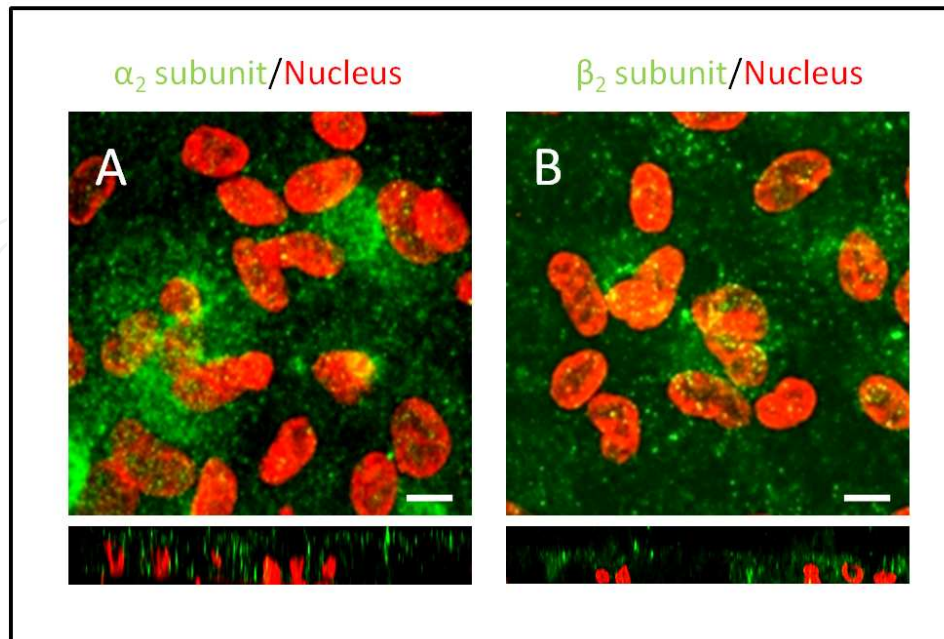


Figure 3. Na⁺,K⁺-ATPase expression at the apical domain of ARPE-19 cells (human retinal pigment epithelium). ARPE-19 cells were cultured on laminin-coated inserts for 4 weeks and treated for IF analysis. Confocal image of a monolayer stained with specific antibody against the α₂ subunit (A) and against β₂ subunit (B). Notice the preferential distribution on apical domain of both subunits. Scale bar: 10 μm

β₁ adhesion capacity (unpublished observations). However, a large number of adhesion and nonadhesion proteins contain domains with an immunoglobulin-like topology (CATH database). Structural alignments of the β₁ subunit extracellular domain against other well-studied cell adhesion molecules reveal no structural homologue of β-subunits of any kind. Detailed inspection of the ectodomain structure uncovers several features distinctive to β-subunit family members. Namely, its Ig-like fold has a unique topology given that its β-sheet sandwich is interrupted by a long α-helix secondary structure and has an atypical β-sheet disposition in relation to classical Ig folds. Also, the β-subunit fold contains extensive loops and therefore its length is twice as that of a typical Ig domain. Furthermore, the β₁ subunit is structurally compromised with the catalytic α-subunit in such a way that the C-terminal fold must be more rigid than the typical flexibility of whole adhesion domains such as in cadherins. Altogether, these observations suggest that the β-subunit of the Na⁺,K⁺-ATPase must possess an adhesion mechanism that is particular to this family, as shown in Figure 4.

The first attempt to clarify this adhesion mechanism on a molecular base is related to the regions of the ectodomain involved in β₁-β₁ recognition. Given that the interaction between two β₁ subunits of the same species (dog-dog or rat-rat) is more effective than the interaction between rat and dog β₁ subunits [61], Tokhtaeva and colleagues [92] looked for surface-exposed species-specific amino acids in the sequence of β₁ subunit and identified four residues, which are different between both species and are contained in the 198–207 segment. Rat-like amino acid substitutions introduced in the dog β₁ subunit weakens its interaction with the

endogenous dog β_1 subunit, whereas the insertion of the rat-specific Thr202 into the exogenous dog β_1 subunit impairs its interaction with the endogenous dog β_1 subunit to the level observed between dog and rat native subunits. The opposite effect is observed in the rat β_1 subunit upon the introduction of dog-like residues and the deletion of Thr202. These results suggest that the amino acid residues important for β_1 - β_1 binding are located upstream and downstream of the Thr insertion position. The insertion or removal of the Thr residues in one of the two interacting subunits probably misaligns these binding residues, and thus causes the characteristic difference in affinity between the two species [92].

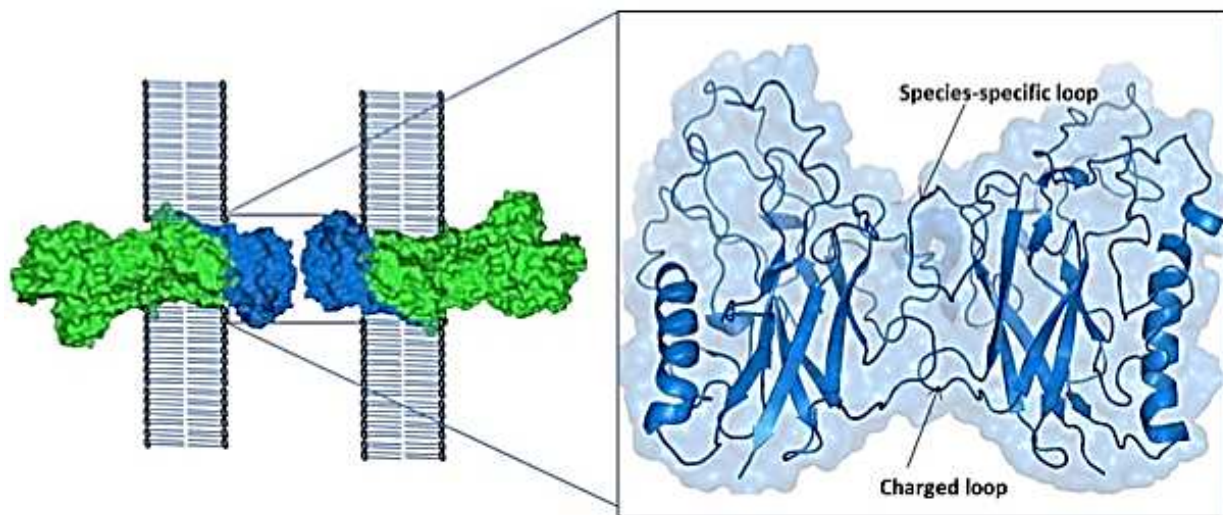


Figure 4. The intercellular adhesion between Na⁺,K⁺-ATPase β_1 subunit. A surface model based on the crystal structure illustrating the association of Na⁺,K⁺-ATPase dimer (α in green and β in blue) at the intercellular space. The magnified square shows one of the representative models resulting from the docking algorithm performed for the coupling of two Na⁺,K⁺-ATPase β_1 subunits structures obtained from the crystals. In this specific model, two loops form the core of the interaction, namely, the one containing the species-specific residues identified by [92] and the other comprised of an unusual sequence of eight consecutive charged residues (214KRDEDKDR221). This charged loop and other regions adjacent to the species-specific loop are suggested as the potential interface for β_1 - β_1 interaction.

How specie-specific residues adjacent to Thr202 coordinate with residues residing at surrounding regions on the same β_1 -subunit and with its interacting partner have yet to be elucidated. The segment 198–207 constitutes one of the characteristic protruding loops in the connecting β -strands B and C of the β_1 -subunit extracellular fold. The majority of the ectodomain surface-exposed residues located most distal from the membrane reside within loops interconnecting β -strands, some of which must be involved in the dimer interface in conjunction with segment 198–207. Since the crystal structure of the Na⁺,K⁺-ATPase β_1 subunits now available [33;93], we modeled and predicted interacting surfaces on β -subunit and thus identified putative amino acids that participate in β_1 - β_1 interaction. This approach will soon lead us to uncover a detailed adhesion mechanism, which is of great importance for epithelial physiology.

4. The Na^+, K^+ -ATPase is the receptor of hormone ouabain

4.1. Cardiotonic steroids (CTSs)

The CSTs have been used for at least 200 years to treat heart failure and tachycardia due to their inotropic effect on the heart [94]. They are specific steroids and are extracted from plants of genus *Digitalis* and *Strophanthus* and from vertebrates such as several species of toads [95]. The CSTs have a steroid nucleus and can sort as cardenolides (with a five-membered lactone ring) or bufadienolides (six-membered lactone ring) and contain various combinations of hydroxyl, sulfate, or carbohydrates groups (Fig. 5) [96]. All types of CST bind with its receptor, the α -subunit of Na^+, K^+ -ATPase, in a pocket formed by transmembrane segments M1–M6. The best affinity for the CST is of the E2P conformation [97]. The sensitivity of the sodium pump to CSTs is controlled by multiple elements mainly by the tissue specific distribution of α and β isoforms and by the glycosylation of CSTs. Thus, in the case of digoxin and digitoxin (Fig. 5), the affinity toward the Na^+, K^+ -ATPase improves with up to fourfold preference for α_2/α_3 over α_1 isoforms [98].

Many studies have demonstrated the endogenous productions of CSTs in mammals. Thus, ouabain was detected in plasma [99], digoxin in urine [100], and marinobufagenin in plasma [101]. An interesting feature of Na^+, K^+ -ATPase is the highly conserved nature of the CST-binding site, suggesting that this site plays a significant physiological role [94]. The normal ranges for circulating ouabain vary between 2500 ± 500 pmol/l and $176\,000 \pm 68\,000$ pmol/l, depending on the measuring condition and the test used [102]. Interestingly, the binding of cardenolides and bufadienolides to the α -subunit of the Na^+, K^+ -ATPase results not just in the inhibition of Na^+, K^+ -ATPase ion transport activity but also in the activation of signaling cascades [103]. Moreover, endogenous ouabain is synthesized and secreted by the hypothalamus [104, 105] and the adrenocortical gland [106–107]. A status of hormone was recommended for the endogenous CSTs as it was demonstrated that it increases during exercise [108], salty meals [109–111], and pathological conditions such as arterial hypertension and myocardial infarction [112]. To confirm the hormone-like function, Arnaud-Batista and colleagues [113] showed that ouabain and bufalin induce diuresis, natriuresis, and kaliuresis, mediated by signal transduction in the isolated intact rat kidney. Furthermore, at the systemic level, cardenolides and bufadienolides have been implicated in many physiological and pathophysiological mechanisms, including cell growth and cancer, body or organ weight gain, mood disorders, vascular tone homeostasis, blood pressure, hypertension, and natriuresis [114].

4.2. The physiological role of hormone ouabain in epithelia

Fifteen years ago, the evidence that ouabain is a hormone was convincing enough as to start wondering what may its physiological role be. Our search was oriented by the observation that (MDCK) epithelial cells exposed to high concentrations of ouabain (≥ 1 μM) do not show sign of damage, but retrieve from the plasma membrane molecules involved in cell–cell and cell–substrate attachment, and detach from each other and from the substrate. These observations suggested that there is a mechanism that relates the occupancy of the pump (P) by ouabain to adhesion mechanisms (A). Accordingly, this mechanism was called $P \rightarrow A$. We

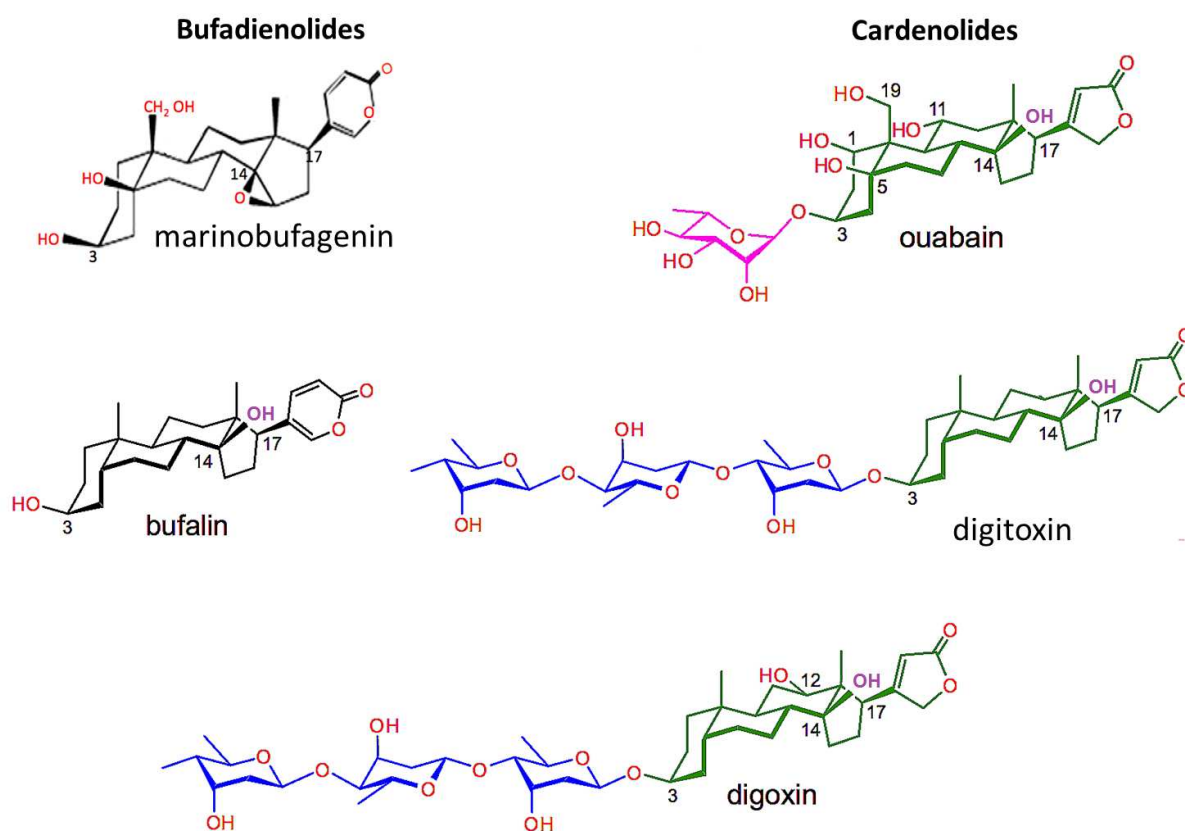


Figure 5. Structural features common for cardiotonic steroids (CTSs). All CTSs include a *cis-trans-cis* ring fused steroid core, which adopts a U-shaped conformation with a convex β -surface, a hydroxyl group at C14 (OH14 β ; purple). CTSs are classified as Cardenolides and Bufadienolides based on a five- or six-membered lactone ring in a β -conformation at position C17. Some CTSs have a carbohydrate moiety of one to four residues attached to C3. Ouabain, the most hydrophilic CTSs, is constituted of a steroid core with four hydroxyl groups at the β -surface (in blue), a hydroxyl group at the α -surface (purple), an unsaturated lactone ring of five members (green), and a rhamnose sugar moiety (pink). The structures of two members of the bufadienolides (marinobufagenin and bufalin) and three members of the cardenolides (ouabain, digitoxin, and digoxin) are illustrated.

discovered that P → A mechanism is associated with several signaling proteins such as cSrc and ERK1/2 (Fig. 6), and it consists of a loss of cytosolic K⁺, an increase of cytosolic levels of Na⁺ and Ca²⁺ and the activation of protein tyrosine kinases and ERK1/2. Ouabain binding also increases p190Rho-GAP, which enhances the GTPase activity of RhoA [115]. Detachment may not be ascribed to the ensuing decrease of K⁺ content because lowering the K⁺-content by incubating the cells in media with only 0.1 mM K⁺ (instead of the regular 4.0 mM) does not cause cell detachment [116]. Therefore, we put forward the working hypothesis that ouabain at nanomolar concentrations, i.e., within the hormonal range in mammalian plasma, may act on the same junctional structures without provoking irreversible damages. To explore the plausibility of this idea, we experimentally tested the effect of ouabain on different cell-cell adhesion complexes starting with TJs. While toxic concentration of ouabain open the TJ, physiological concentrations of ouabain increase its hermeticity. Interestingly, the first effect depends on the pumping activity of Na⁺,K⁺-ATPase, whose inhibition perturbs the ionic balance of the cell. On the contrary, physiological concentrations of ouabain (i.e., in the

nanomolar range) neither inhibit K^+ pumping nor disturb the K^+ balance of the cell [117]. At these concentrations, the effects of ouabain depends mainly on the activation of the receptor complex of Na^+,K^+ -ATPase. While toxic levels of ouabain regulate the opening of TJs through endocytic and degradation processes, physiological concentrations of ouabain modulate TJs through changes in the molecular composition of the TJ through processes that provoke changes in transcription rate and expression of its proteins [118] (Fig. 6A). Another prominent cell–cell contact is the adherens junction (AJ) and one of the scaffolding proteins of this junction is β -catenin, a key member of the Wnt signaling pathway (Fig. 6B). During the activation of this pathway, β -catenin is translocated to the nucleus, where it modifies gene expression [119]. Interestingly, 10 nM and 1 μ M ouabain provoke the translocation of β -catenin to the nucleus of MDCK cells [116]. Liu and co-workers [120] have recently found evidence that Na^+,K^+ -ATPase, and E-cadherin are closely associated, indicating that E-Cadherin could be part of the signalosome of the Na^+,K^+ -ATPase. To further explore the hypothesis that nanomolar concentrations of ouabain modulate cell–cell contacts, the effect of 10 nM ouabain have been studied on another type of cell–cell contact, the gap junction. In MDCK cells treated with this concentration of ouabain cell–cell communication have been increases by up to 510% in one hour. Moreover, inhibitors of transcription and of translation do not affect the induction of Gap junction communication (GJC) by ouabain, indicating that cells express a sufficient level of connexins to account for the rapid enhancement of GJC [121].

Ouabain effects through signaling were observed also in cardiac myocytes when nontoxic concentrations of ouabain, that partially inhibit the Na^+,K^+ -ATPase, activate signaling pathways that regulate growth [122, 123]. Ouabain can activate signal cascades that vary between cell types, depending on the dose and the α -subunit isoform expressed in the cell [124, 103]. The existence of two pools of Na^+,K^+ -ATPase within the plasma membrane with two distinct functions have been proposed: the classical ion pump whose partial inhibition by ouabain provokes an increase in $[Ca^{2+}]_i$, and the second, the signal transducing pool which through protein–protein interactions regulates cell growth, proliferation, differentiation, and apoptosis. Part of the nontransporting Na^+,K^+ -ATPase is located in the caveolae. cSrc is usually bound to the Na^+,K^+ -ATPase in caveolae. Ouabain binding to the pump located in caveolae, stimulates cSrc activation, which consequently activates other downstream signaling pathways [125]. Signaling through Src is supported by the discovery that in a cell-free system, the addition of ouabain modifies the Na^+,K^+ -ATPase cSrc complex and activates cSrc [128]. Alongside, the epidermal growth factor receptor (EGFR) is transactivated upon ouabain binding to Na^+,K^+ -ATPase and additional signaling occurs that activate downstream targets including She, Grb, Ras, Raf, MEK, and ERK [125, 127] (Fig. 6). These signaling pathways regulate early response genes associated with cell growth and also regulate cell motility and a number of metabolic pathways [123,126]. Another signaling role was found by Aizman and coworkers [126]. In epithelial cells, the Na^+,K^+ -ATPase interacts with the inositol 1,4,5-triphosphate receptor (IP3R) within the signaling microdomain. They show that interaction of ouabain with the signaling Na^+,K^+ -ATPase provokes synchronized Ca^{2+} oscillations rising from the modification of such interaction. Those slow oscillations activate NF- κ B.

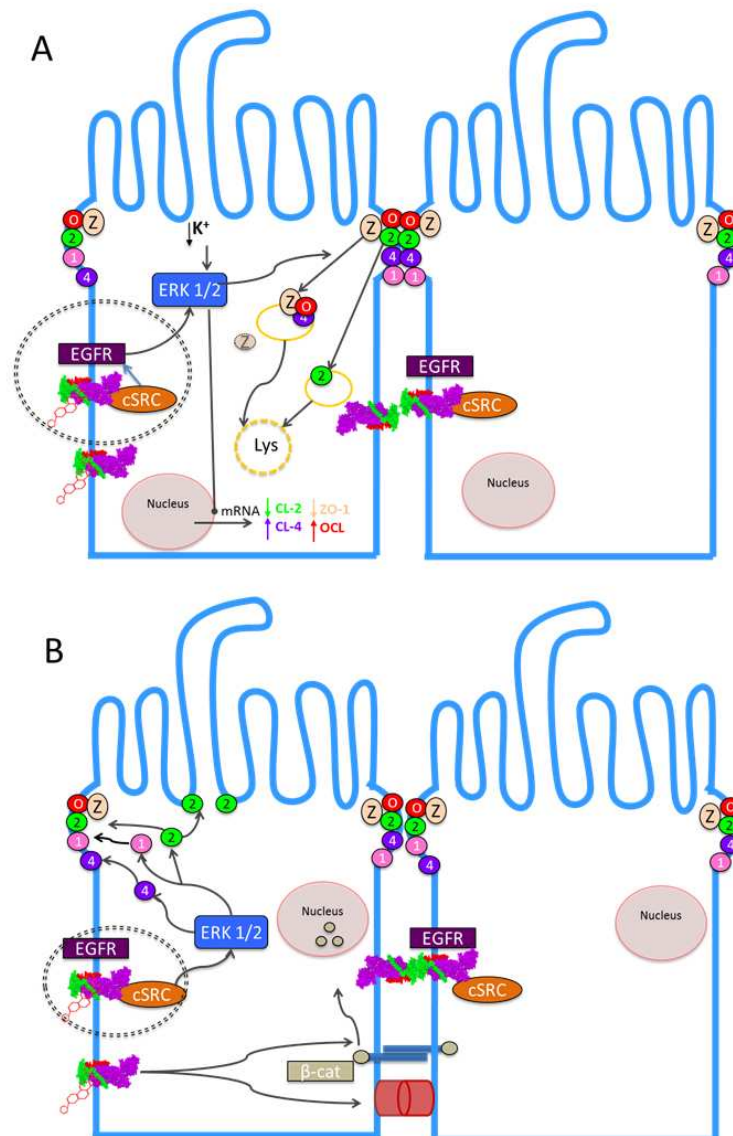


Figure 6. Signaling in the ouabain-induced modulation of cell contacts. Ouabain (red silhouette) induces the formation of a signalosome, a caveolar complex (discontinued grey line) including the Na⁺,K⁺-ATPase, its associated cSrc (cSRC) and the EGF receptor (EGFR). (A) Ouabain (300 nM) activates cSrc, which in turn transactivates the EGFR pathway, causing a phosphorylation of ERK1/2. The inhibition of the pump alters the ionic gradient that also contributes to the activation of ERK1/2. The activation of ERK1/2 is crucial for the clathrin- and dynamin-dependent endocytosis of TJ components. Two possible types of endocytic vesicles are formed: one containing a core complex with essential TJ proteins, such as ZO-1 (encircled Z); occludin (encircled O) and Claudin-4 (encircled 4) and a second one entailing components such as Claudin-2 (encircled 2) that makes TJs permeable to water and Na⁺. ERK1/2 activation is required to reduce the levels of Occludin, Claudin-4 and ZO-1 proteins, but not that of Claudin-2. ERK1/2 is also necessary to reduce Claudin-2 and ZO-1 mRNA levels. Notably, the cellular content of Claudin-4 and occludin mRNAs increases, during the opening of the TJs induced by Ouabain. (B) Epithelial cells treated with 10 nM of Ouabain (hormonal concentration) show increased tight junction sealing [117]. Activation of ERK 1/2 modulates the expression of Claudins (1,2 and 4) at the tight junction and promotes the expression of Claudin-2 in the cilium. Moreover, under this condition, cell-communication by gap junctions (red cylinder) is also increased by a mechanism still not well understood and β-catenin (khaki circles), a component of the Adherens junctions travels to the nucleus and modulates the expression of genes involved in cell-junction regulation.

Signal cascades vary between different cells types. For example, in cardiac myocytes and renal cell lines derived from the porcine kidneys (LLC-PK1) and the opossum kidneys (OK), ouabain-mediated activation of a signaling cascade has been demonstrated to be dependent upon the activation of Src, MAPK, and PI-3K pathways [129,122], whereas in human breast (BT20), prostate (DU145) cancer cells, and PY-17 cells, ouabain activate Src and MAPK pathway, but not PI-3K pathway [130]. Downstream in the ouabain-activated signaling cascade, the level of complexity increases due to the activation of several cell-specific secondary messengers and the cross talk between distinct pathways [131]. For each cell type, different pathways and branches are activated and only part of their complexity is known. In the MAPK pathway, several secondary messengers, downstream targets of ERK1/2, have been identified. Upon activation, ERK1/2 is able to migrate to the nucleus and activate several transcription factors (STAT1/3, c-fos, CREB, Elk-1) or in the cytoplasm modulate ion channels, receptors, or cytoskeleton proteins by direct phosphorylation.

5. Concluding remarks

Although without its β -subunit the Na^+K^+ -ATPase could not be expressed in the plasma membrane, nor have an enzymatic activity, no convincing role was detected for this subunit beyond of helping the α one to cage K^+ . We have shown that, due to its adhesiveness, the β_1 -subunit may establish a linkage with an identical subunit located in a neighboring cell across the intercellular space, and be thereby responsible for the polarized expression of Na^+K^+ -ATPase in epithelial cells. Furthermore, it has been demonstrated that β - β interaction stabilizes and maintains cell-junctions integrity in transporting epithelia. The molecular mechanism by which this interaction occurs is still far from being elucidated. Nevertheless, it is clear that both N-glycans and specific sequences exposed on the polypeptide surface are implicated. The observation that $\text{P} \rightarrow \text{A}$ mechanism is involved in the shuttling of β -catenin to the nucleus and thus in the Wnt/Wingless cascade, in the growth factor signaling pathways, as well as the ability of ouabain to enhance intercellular communication through gap junctions speaks of the important physiological role played by the hormone ouabain. The importance of this mechanism is compounded by the fact that in the meanwhile ouabain was shown to be a hormone that varies in response of several physiological and pathological conditions. Therefore, we may postulate that ouabain may determine the retrieval of the β -subunit from the plasma membrane and down regulates the expression of Na^+K^+ -ATPase in the cell membrane and thus, indirectly regulates the absorption and secretion of ions and nutrients. Therefore, ouabain should be added to the list of hormones that affect transepithelial transport, along with aldosterone, antidiuretic hormone, and the like.

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