Chapter 8
The Polarized Distribution of the Na^+,K^+-ATPase

M. Cereijido, R.G. Contreras, M.I. Larre, and L. Shoshani

Abstract The life of a cell depends on the perennial inflow of metabolites and outflow of catabolites, ultimately driven by membrane pumps or by the electrochemical potential gradients that these pumps generate. Metazoans have cells in which pumps have an additional function: they accumulate in a certain domain of the membrane to induce *polarity*. Surprisingly, the polarized distribution of Na^+, K^+-ATPase does not arise only from canonical signals or classical mechanisms but also from the peculiar affinities between its own subunits. For example, subunits α and β have an affinity for each other that binds them together right after synthesis, and they then migrate through the endoplasmic reticulum and the Golgi apparatus and are delivered to the plasma membrane. In keeping with this role of subunit affinities, we have shown that the polarized distribution of the whole enzyme at the plasma membrane facing the intercellular space arises from the very specific affinity of one β subunit for another. In addition to being distributed in a polarized manner, Na^+,K^+-ATPase participates in cell polarization by acting as a receptor for the ouabain hormone, thereby promoting ciliogenesis; obviously, the enzyme can act as a receptor because this is polarized toward the blood side where hormones come from. In this chapter, we review the polarized distribution of Na^+,K^+-ATPase and suggest that the very existence of higher metazoans depends on this polarized expression of pumps.

Keywords Claudin • c-Src • Hormone • Na,K-ATPase • Ouabain

8.1 Introduction

The information gathered through more than a century of research on the permeability of the plasma membrane required several parallel efforts, including those needed to elucidate the movement of ions and molecules, because the information could not be understood on only the basis of the electrochemical potential gradient

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between the two sides of the plasma membrane. This challenged the imagination of physiologists, who conceived theoretical mechanisms such as pores, carriers, and pumps and assigned to them properties that frequently appeared to be in violation of the fundamental laws of physics. A case in point was the use of chemical reactions (in those times thought to be scalar phenomena) to produce the vectorial movement of ions, which was clearly demonstrated once J. Ch. Skou discovered the presence of Na^+,K^+-ATPase in crab nerves (Skou 1957; Onsager 1967). Another more recent example became evident after we introduced an experimental model system to study the development of the two fundamental features of the transporting epithelial phenotype: tight junctions (TJs) and polarity (de Donder and Van Rysselberghe 1936; Cereijido et al. 1978; Boulan and Sabatini 1978). The knowledge we gained on the signals and mechanisms that are accounted for by the polarized distribution of most membrane proteins was insufficient to explain the polarized distribution of Na^+,K^+-ATPase. In a way, this was ironic because this enzyme is responsible not only for its own polarity but, together with the polarized expression of other transporter proteins, for the polarized (net) flux of glucose, amino acids, and ions across plasma membranes and epithelial membranes (Csaky and Thale 1960; Crane et al. 1961, 1965; Kedem and Essig 1965; Schultz and Curran 1970). Fortunately, the polarized distribution of this enzyme was recently elucidated, and the data demonstrated that the distribution is due to mechanisms that seem to be specific to this pump (Cereijido and Rotunno 1970; Shoshani et al. 2005; Padilla-Benavides et al. 2010).

8.2 Incongruences, Stumbling Blocks, and Apparent Violations of Thermodynamics (Cereijido et al. 1971)

In the second half of the nineteenth century, du Bois Reymond (Bois-Reymond 1848; Berridge et al. 2003) demonstrated that frog skin maintains an electrical potential difference ($\Delta\Psi$) between the inner and outer sides of the epithelium (Fig. 8.1a), a feature no inert membrane can show when mounted between identical saline solutions. Half a century later, Galeotti (Galeotti 1904; Griffiths 2007) attributed this asymmetry to a higher Na^+ permeability in the inward versus outward direction (Fig. 8.1b). This attribution was disregarded on the basis that it would violate the first principle of thermodynamics and create a *perpetuum mobile*: Na^+ would forever rotate counterclockwise in the *gedankenexperiment* illustrated in Fig. 8.1b. Yet, after the Second World War, radioisotopes became available to measure unidirectional fluxes, and it was easy to demonstrate that the inward flux of sodium across frog skin is up to 20 times higher than the outward flux, a feature that many took as solid evidence that life would never be explained on the basis of pure physical laws. Later on, biologists demonstrated that $\Delta\Psi$ is by no means perpetual and lasts as long as the epithelium is alive and proposed that the net flux of Na^+ (influx *minus* outflux) across an epithelium depends on the energy generated by

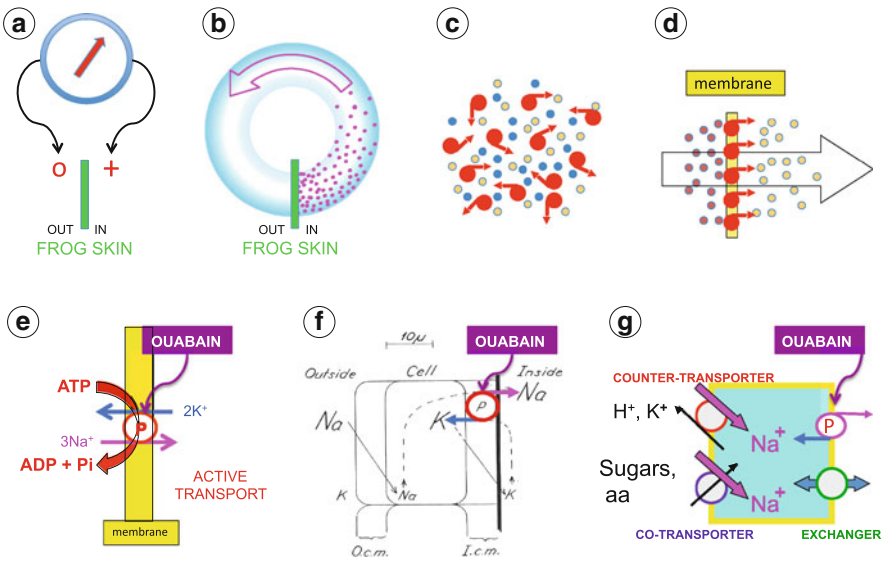

Fig. 8.1 Historical cornerstones toward the polarity of the Na^+,K^+-ATPase. (**a**) Emile du Bois Reymond discovered that frog skin maintains an electrical potential difference between its outer and inner surfaces. (**b**) Galeotti proposed that such electrical potential could be explained by assuming that frog skin has a higher permeability to Na^+ in the inward than in the outward direction. His proposal was discarded on the basis that this would increase the concentration of this ion (*magenta dots*) on the inner side and the ensuing diffusion would originate a counter-clockwise *perpetuum mobile*. (**c**) Avoiding a skirmish with Curie's principle, pumping is vectorial at the microscopic level (i.e., each pump works in a given direction). Yet, a study at the macroscopic level in a homogenized preparation where pumps point in all directions masked their intrinsic vectoriality. (**d**) In a cell membrane where all Na^+,K^+-ATPase pumps are aligned, vectoriality is recovered. (**e**) J.Ch Skou found Na^+,K^+-ATPase in the plasma membrane of an extract of crab nerve. The enzyme simultaneously pumps three Na^+ and $2K^+$ (albeit in different directions) per molecule of ATP hydrolyzed. (**f**) Koefoed-Johnsen and Ussing (1958) proposed that the Na^+,K^+-ATPase proposed by Skou is distributed in a polarized manner in the epithelial cells of frog skin. O.c.m., outer cell membrane, which is permeable to Na^+. I.c.m., inner cell membrane, impermeable to Na^+ but passively permeable to K^+. *P* place where the Na^+,K^+-ATPase is needed to act as an ion pump. (**g**) The pumping of Na^+ out of the cell and toward the extracellular space creates an electrochemical potential difference that drives most co- and counter-transporters, which act as "secondary pumps;" however, these are not considered to be active transporters because they are not driven directly by the chemical energy generated by ATP hydrolysis

metabolism. This suggestion was also rejected on the basis that it would be in violation of Curie's principle: *phenomena of different tensorial order cannot be coupled*. In plain words, metabolism is the sum of chemical processes, which were assumed to be *scalar* phenomena (no particular direction in space), implying that metabolism would never sustain net fluxes, which are clearly *vectorial* processes. Eventually, it was argued that chemical phenomena, such as the splitting of ATP by Na^+,K^+-ATPase, are vectorial at the microscopic level but lose vectoriality at macroscopic levels. Figure 8.1c illustrates this paradox as well as the solution provided (Fig. 8.1d).

Classic thermodynamics assumed that each flow (e.g., of Na^+, K^+, H^+, etc.) was powered by its specific chemical potential, i.e., Na^+ flux was assumed to be driven by the concentration gradient of Na^+ but not by that of other ion species or by differences in the temperature or pressure between two points. It took a major conceptual change to show that, in principle, a flux can be driven by *any* of the forces present in the system, a concept for which Lars Onsager (Skou 1957; Onsager 1967) was awarded the Nobel Prize in 1968. Following this, de Donder and Rysselberghe (de Donder and Van Rysselberghe 1936; Cereijido et al. 1978; Boulan and Sabatini 1978) demonstrated that even chemical reactions can be represented as fluxes driven by chemical affinity. Therefore, taking both groups of considerations into account, fluxes such as those of Na^+ and K^+ can be formally attributed to the hydrolysis of ATP, and therefore, ion pumping can be carried out by Na^+,K^+-ATPase. Eventually, Kedem and Essig (Csaky and Thale 1960; Crane et al. 1961, 1965; Kedem and Essig 1965; Schultz and Curran 1970) gauged ion flux through the amount of Na^+ transported and chemical reactions through the amount of oxygen consumed and were able to show that epithelia can in fact transport ions using metabolic energy. Ironically, this was a "permission to occur" of a process that had already been experimentally demonstrated. Nevertheless, this is the essence of science: to rest assured that the body of knowledge is self-consistent, theories and experimental observations should not disagree.

8.3 Why Do Cells Need Ion Pumps?

Pumps create an asymmetric distribution of ions between the cytoplasm and the external bathing solution that offers some physiological advantages to the cell. (1) The asymmetric distribution enables the plasma membrane to act as an electric capacitor that can, in a given moment, spark an action potential used by muscle fibers to contract and neurons to communicate with each other. (2) The principle of electroneutrality requires that the amount of negative charges in a given volume exactly matches the amount of positive ones, implying that mobile ions should penetrate into the cell to neutralize fixed electric charges from proteins and other molecules trapped inside the cytoplasm. However, simple coulombic considerations (*see* Cereijido and Rotunno 1970; Shoshani et al. 2005; Padilla-Benavides et al. 2010) show that these charges will be perennially neutralized by protons (H^+) because their negligible radius enables their positively charged nuclei to closely approach and bind to fixed negative charges in the cytoplasm with a force far stronger than between the negative charge and any other mobile cation, thereby provoking a molecular debacle in the cell. Hence, cells protect themselves from this disaster by expressing pumps and counter-transporters in their plasma membrane, which, by extruding H^+ toward the extracellular space, maintain such low concentrations of H^+ (in the nanomolar range) that protons are unable to compete with other mobile cations (in the millimolar range). Ca^{2+} faces the same situation because even though its radius compares to that of Na^+ and K^+, calcium has two

positive charges instead of one. Again, the combined work of the Na^+,K^+-ATPase and Na^+/Ca^{2+} co-transporters, as well as the endoplasmic reticulum's pumps and chelating proteins, keeps the activity of Ca^{2+} very low in the cell water (Bois-Reymond 1848; Berridge et al. 2003). Therefore, in spite of the fact that hydrogen and calcium are two strong binders to fixed negative charges, the work of the Na^+, K^+-ATPase keeps them at sufficiently low concentrations to insure physiological exchanges among protons, calcium, and monovalent cations. (3) Counter-transporters present in the plasma membrane, such as those of Na^+/glucose, have an affinity for glucose proportional to the Na^+ concentration. Therefore, when facing the extracellular solution, the high Na^+ concentration makes the counter-transporter develop a high affinity for glucose and load itself with this sugar; however, due to the low concentration of Na^+ in the cytoplasm, as soon as the carrier faces the inside of the cell, it releases its Na^+, loses its affinity for glucose, does not combine with the sugar on this side, and returns empty to the outer bathing solution. Notice that this "secondary transport" generates a *net* transport of the sugar and works as long as Na^+,K^+-ATPase maintains the Na^+ asymmetry across the plasma membrane. An analogous mechanism is observed in Na^+-amino acid co-transporters.

It is generally assumed that the water/ion composition of the cytoplasm reflects the composition that the primeval ocean had the day a droplet was trapped inside a vesicle surrounded by a lipid bilayer (Galeotti 1904; Griffiths 2007). Without discarding this possibility, the considerations made in the previous paragraph indicate that the nature of cellular content is the consequence of having a plasma membrane studded with proteins, i.e., synthesized and installed with mechanisms coded in the genome.

8.4 An "Ocean" Less Than a Micron Thick

The intense exchange of a myriad of different substances across the cell membrane of a unicellular organism does not exhaust the nutrients or pollute the ocean (*external milieu*); rather, this external milieu acts as a reservoir (Fig. 8.2a). However, when the cell belongs to a metazoan, say a neuron in the brain (Fig. 8.2b), the "ocean" is replaced by an extremely narrow extracellular space (Fig. 8.2c, *black*), less than a micron thick, that would be quickly exhausted and spoiled were it not for a circulatory apparatus that shuttles its extracellular fluid to and from "transporting epithelia," where the exchange with the outer milieu takes place. To illustrate the amount of epithelia involved in this exchange with the environment, the area of several of them is scaled with the silhouette of a man (Fig. 8.2d). In turn, the exchange between blood and cells proceeds across endothelia, which have basically the same cellular phenotype as epithelia but occupy an even larger area (Fig. 8.2e, *pink*). Figure 8.2f represents the transporting epithelial phenotype of a kidney tube, which is a continuous layer of cells surrounding the lumen, showing its two fundamental features: TJs and apical/basolateral polarity. Interestingly, in spite of

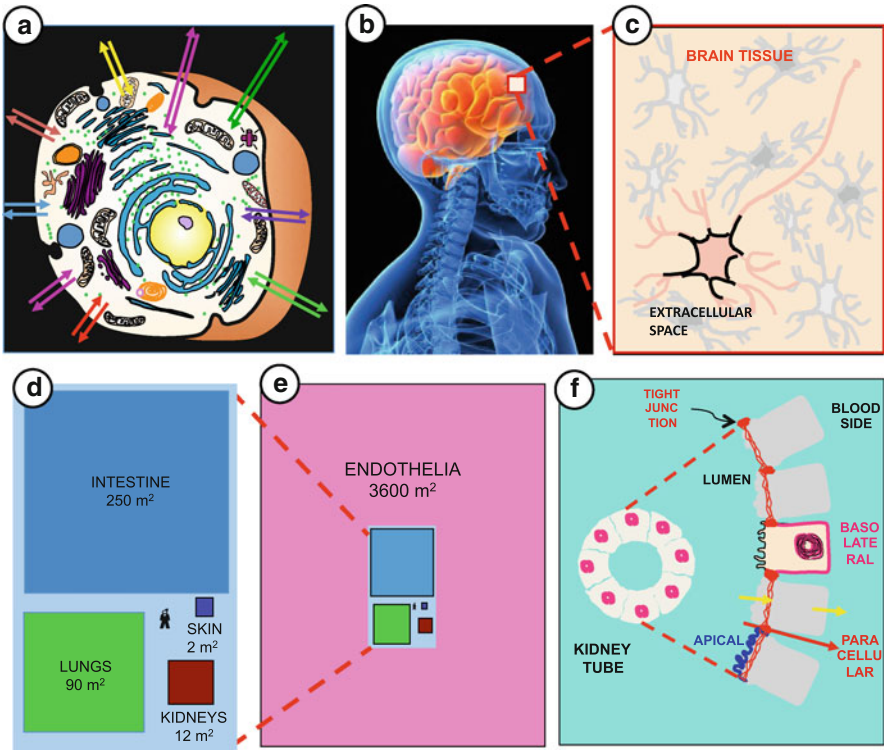

Fig. 8.2 Transporting epithelial phenotype. (**a**) A unicellular organism in the primitive ocean that acts as a reservoir; hence, it cannot be exhausted nor spoiled as a consequence of exchanges with the cell. (**b**) A cell in a metazoan has the "ocean" reduced to a narrow extracellular space (**c**, *black*) that, nevertheless, is able to act as a reservoir due to a circulatory apparatus that takes its fluid back and forth to and from transporting epithelia where the exchange with the environment takes place. (**d**) A few transporting epithelia scaled with the figure of a man to give an idea of the large area devoted to the exchange of substances with the environment. (**e**) Represents (**d**) at a smaller scale to compare the area of epithelia with those of endothelia (*pink*). (**f**) Schematic representation of five epithelial cells forming a segment of the wall of a kidney tube, showing the two fundamental differentiated features: *tight junctions* (*red*) that seal the intercellular pace and *apical/basolateral polarity*. The apical domain of the cell membrane (*blue*) is in contact with the lumen and the basolateral one (*magenta*) with the interstitial fluid on the blood side

this lumen running at the central axis of the tube, the tube is considered to be full of *external* milieu because it connects with the urinary tract, which interacts with the external milieu bathing the animal.

Therefore, the phenotype composed of a sealing element, the TJs, and an apical/basolateral polarity is the preferred structure implemented by higher metazoans to seclude a large portion of their bodies, e.g., the gastrointestinal space, the renal space, or the space within the genitalia, blood, and lymphatic fluids, or even to create a "castle-within-the-castle" effect as seen with the brain and its surrounding

extracellular fluid. This suggests that polarization became an important requirement in the evolution from single cells to metazoans. Let's consider this suggestion.

Shortly after J. Ch. Skou introduced Na^+,K^+-ATPase (Skou 1957) (Fig. 8.1e), V. Koefoed-Johnsen and H. H. Ussing (Koefoed-Johnsen and Ussing 1958) (KJU) used this pump to develop a fertile working model that acted as a blueprint to explain active transport across most epithelia (Fig. 8.1f). Interestingly, KJU transformed a common cell into one of the transporting epithelia by proposing that the pump is expressed in a polarized manner on the basal domain of the plasma membrane (Fig. 8.1f). Significantly, further adaptations of the KJU model to other epithelia proposed that co- and counter-transporters are also expressed in a polarized manner (Fig. 8.1g).

8.5 The Polarized Distribution of Proteins in the Plasma Membrane

The generation of cell surface polarity for most membrane proteins involves sorting signals encoded in their amino acid sequence, trafficking routes that include apical or basolateral recycling endosomes, and interactions with epithelial-specific protein complexes such as AP-1B and clathrin, which can be regulated by small GTPases (Cereijido et al. 2003; Duffield et al. 2008; Bryant and Mostov 2008; Mellman and Nelson 2008). Early studies demonstrated that the Na^+-K^+-ATPase, composed of a catalytic subunit (α) and an accessory subunit (β), is assembled in the endoplasmic reticulum, sorted in the trans-Golgi network, and delivered directly to the basolateral membrane of epithelial cells (Caplan et al. 1986; Gottardi and Caplan 1993; Zurzolo and Rodríguez-Boulan 1993). Therefore, a basolateral signal was assumed to exist in the α-subunit of the Na^+-K^+-ATPase (Muth et al. 1998). Na^+-K^+-ATPase and H^+-K^+-ATPase are highly homologous ion pumps; yet in LLC-PK1 cells, the former is polarized to the basolateral domain, whereas the latter is localized to the apical plasma membrane. To identify the sorting signals of these ion pumps, the polarized expression of chimeric constructs of the α-subunit of the H^+-K^+-ATPase and the Na^+-K^+-ATPase was studied (Muth et al. 1998). Apical sorting information was recognized within the fourth transmembrane domain of the α-subunit of the H^+-K^+-ATPase that is sufficient to redirect the Na^+-K^+-ATPase from the basolateral to the apical surface of these cells (Dunbar et al. 2000). However, it remains unclear whether basolateral sorting information exists in the fourth transmembrane domain of the α-subunit of the Na^+-K^+-ATPase; thus, a nonconventional signal could be involved in the basolateral targeting of this pump (Dunbar and Caplan 2001). Moreover, efforts to elucidate the trafficking mechanism of newly synthesized Na^+-K^+-ATPase revealed that it is independent of AP-1B because the pump localizes to the basolateral surface in the μ1B-deficient cell line LLC-PK1 (Duffield et al. 2004) and in MDCK cells in which μ1B expression is suppressed via RNA interference (Gravotta et al. 2007).

Although the manner in which the Na^+-K^+-ATPase achieves polarized distribution remains mysterious, several clues have emerged on how this is accomplished. The first clue came from the observation that in MDCK cells, the pump is not expressed in the apical or basal domains but only at the lateral membrane (Fig. 8.3a) (Hammerton et al. 1991; Contreras et al. 1995a; Shoshani et al. 2005). The second clue was obtained when monolayers of MDCK cells were treated with EDTA to chelate Ca^{2+}. The cells detached from each other and took their own Na^+-K^+-ATPase pumps with them (Fig. 8.3c) (Contreras et al. 1995b), indicating that the fluorescent mark observed under control conditions (Fig. 8.3b) was formed by pumps on both neighboring cells (Cereijido et al. 2000). The third clue was that monolayers formed with cells belonging to different epithelia and even different animal species formed sealed TJs but did not express Na^+-K^+-ATPase at a given lateral border, unless both epithelial cells belonged to the same animal species (Fig. 8.3d) (Contreras et al. 1995a; Shoshani et al. 2005). The fourth clue was that the β-subunit of the Na^+-K^+-ATPase resembles an adhesion molecule: it has a short cytoplasmic domain, a single transmembrane domain, and a long and heavily glycosylated extracellular domain. Likewise, experiments implemented by the Schachner group (Antonicek et al. 1987; Antonicek and Schachner 1988) revealed that the adhesion molecule on glia (AMOG) is an isoform of the β-subunit of the Na^+-K^+-ATPase (Gloor et al. 1990). Finally, the structure of the Na^+-K^+-ATPase, with its three subunits, has been resolved by X-ray crystallography (Fig. 8.3e) (Morth et al. 2007; Ogawa et al. 2009). These clues indicated that the β-subunit is an adhesion molecule. In accordance with this attribute of the β-subunit, we have demonstrated that Chinese hamster ovary cells transfected with the canine β_1-subunit of Na^+-K^+-ATPase (CHO-β) increase their tendency to form aggregates (Fig. 8.3f) (Shoshani et al. 2005). Using cocultures of MDCK and CHO-β cells, we showed that the Na^+-K^+-ATPase of MDCK cells was now polarized to the lateral border even when the adjacent cell was of another species (Fig. 8.3g) (Shoshani et al. 2005). We also showed by a pulldown assay that the dog β_1-subunit could specifically bind to the soluble extracellular domain of the β_1-subunit of the same animal species and that β_1-subunits of neighboring epithelial cells interact directly with each other in vivo (FRET and Co-IP essays). In the crystal structure of the Na^+, K^+-ATPase, the β-subunit is mostly exposed toward the intercellular space (Morth et al. 2007; Ogawa et al. 2009). This position of the β-subunit would favor the β-β association between Na^+-K^+-ATPases of adjoining cells at the intercellular space (Fig. 8.3h).

In this respect, several studies in mammals have shown that polarized targeting of the Na^+-K^+-ATPase in transporting epithelial cells is related to the expression of specific β-isoforms. Basolateral targeting is related to the expression of the β_1- and β_3-isoforms, while apical targeting is related to the expression of the β_2-isoform (Burrow et al. 1999). Accordingly, it has been shown that in the human gastric adenocarcinoma cell line (HGT-1), the pump is localized to the apical membrane domain and constitutes the β_2-isoform. When the β_1-isoform is expressed in this cell line, the pump is delivered to the basolateral domain (Vagin et al. 2005). Furthermore, there is a wealth of information suggesting that the N-glycans of the

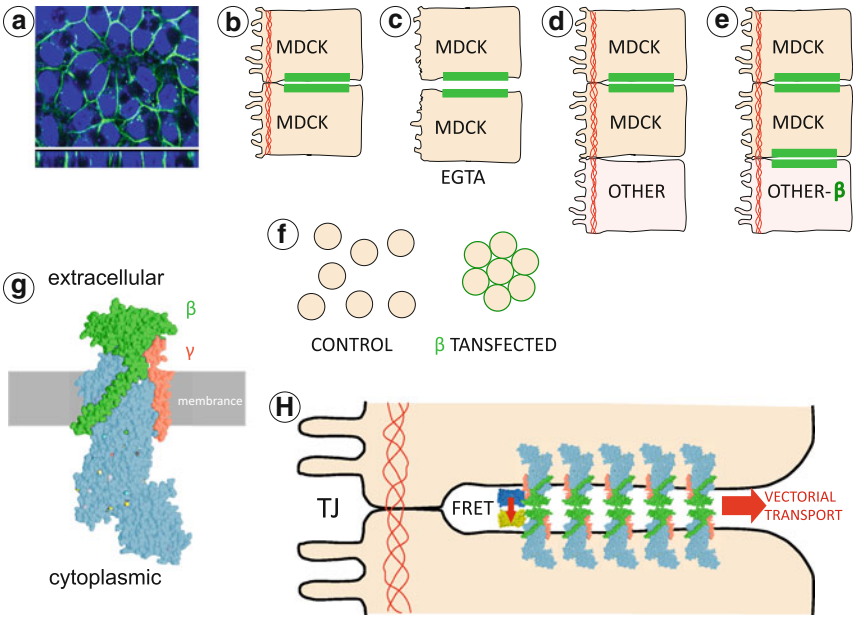

Fig. 8.3 Role of the β-subunit in the polarized distribution of Na^+,K^+-ATPase. (**a**) Normal (*upper panel*) and lateral (*lower panel*) view of a monolayer of MDCK cells, with the β-subunit stained in *green* and nuclei in *blue*, showing that the enzyme only occupies the lateral membrane. (**b**) Two neighboring cells expressing lateral Na^+,K^+-ATPase (*green*). (**c**) Ca^{2+} removal shows that detached cells separate, hauling their own enzyme. (**d**) A monolayer formed with a mixed population of MDCK cells plus "other" cell types (Ma104, LLC-PK_1, or CHO) expresses Na^+, K^+-ATPase on homotypic but not heterotypic lateral borders. (**e**) Mixed monolayers of MDCK and CHO cells transfected with the dog β-subunit (other-β) express this subunit in homotypic and heterotypic contacts. (**f**) The β-subunit of Na^+,K^+-ATPase has the characteristics of an adhesion molecule. Transfecting this subunit in CHO fibroblasts (β-transfected) confers adhesion properties. (**g**). Crystallography indicates that the enzyme on the plasma membrane exposes its α- (*light blue*), β- (*green*), and γ- (*orange*) subunits as depicted. The C-terminal lobe of the β-subunit is exposed to the intercellular space and shares structural similarities with proteins from the IgG-like superfamily. (**h**) Monolayers prepared with a mixed population of MDCK cells transfected with a β-subunit fused to a cyan fluorescent protein (*blue silhouette*) and MDCK cells transfected with a β-subunit fused to yellow fluorescent protein (*yellow silhouette*), as depicted in the first pair of Na^+,K^+-ATPases, show in a FRET assay that energy can be transferred from one β-subunit to the other, indicating that two β-subunits can interact directly from a distance less than 10 nm, thereby anchoring the whole enzyme at the cell membrane facing the intercellular space. Combining the crystal silhouette in G with the position of the enzyme in epifluorescence (**a**) shows that Na^+,K^+-ATPases can pump Na^+ toward the intercellular space. Given that this space is closed by TJs at its outermost end, Na^+ can only diffuse toward the basal (blood) side, in a net (vectorial) manner (*red arrow*)

β-subunit play an important role in the polarized sorting and trafficking of Na^+ pumps. Studies in which N-glycosylation of the Na^+,K^+-ATPase β-subunit was modified by either pharmacological or site-directed mutagenesis have shown that

the β_1-isoform contains basolateral sorting information (Lian et al. 2006), while the β_2-isoform contains apical polarization information (Vagin et al. 2005, 2007).

Studies in *Drosophila* have also shown that the β-subunit is a key determinant of the subcellular localization and function of the Na^+-K^+-ATPase. Of the three Na^+-K^+-ATPase β-subunits, the Nrv1 and Nrv2 isoforms are localized to the epithelia, while Nrv3 is expressed in the nervous system. Interestingly, Nrv1 is localized to the basolateral domain of almost all epithelial cells; by contrast, Nrv2 is expressed at septate junctions (SJs) (the insects' analog of the vertebrate TJ) and co-localizes with the SJ marker coracle. Moreover, it has been shown that Nrv2 controls, by its extracellular domain, the functionality of SJ and the tracheal tube size in a pump-independent function (Paul and Palladino 2007).

The basic mechanism of lateral polarization is reinforced through interactions with extracellular ligands or with intracellular scaffolds such as cytoskeletal elements or arrays of PDZ domain-containing proteins (Mays et al. 1995; Cohen et al. 1998). Na^+-K^+-ATPase has been shown to be retained at the basolateral membrane domain by binding to the ankyrin-fodrin cytoskeleton (Hammerton et al. 1991). As demonstrated with FRET analysis, the two external moieties belonging to neighboring cells achieve a close proximity (<10 nm) (Padilla-Benavides et al. 2010). The fact that the extracellular moieties of β-subunits and that the α- and β-subunits have a high affinity for each other results in the anchoring of the Na^+-K^+-ATPase of both neighboring cells to the lateral border. This has profound functional consequences for the overall transport of Na^+ in the inward direction because Na^+ pumped into the intercellular space can only diffuse toward the blood side because the outermost end of this space is sealed by a TJ.

8.6 Apical Distribution of the Na^+,K^+-ATPase

In contrast to most epithelia, in the choroid plexus and the retinal pigment epithelium (RPE), both with neuroepithelial origins, the Na+,K(+)-ATPase is localized to the apical plasma membrane domain. The retinal pigment epithelium (RPE) separates the photoreceptors (rods and cones) from the choroid generating a proper ionic environment for the photoreceptor's function in the subretinal space. The apical domain of RPE is in intimate contact with the distal segments of rods and cones, generating a subretinal space. The subretinal K^+ and Na^+ concentrations that generate the dark current necessary for vision are under the control of the RPE cells. This crucial task requires that the Na^+,K^+-ATPase localize to the apical surface (Bok 1982; Frambach and Misfeldt 1983). In addition, RPE also transports a net amount of fluid in the apical to basal direction, creating a negative pressure that helps attach the neural retina to the RPE (Adijanto et al. 2009) in coordination with apical and basolateral bicarbonates and Cl^- transporters. Cl^- and Na^+ transport from the apical to the basolateral compartments drives fluid transport, which can be facilitated by aquaporin 1 (Strauss 2005). The retinal activity drastically changes the levels of Na^+, K^+, and CO_2 in the subretinal space, which are balanced

by changes in the activity of various Na^+, K^+, Cl^-, and bicarbonate transporters, including the Na^+,K^+-ATPase. The retina produces a large amount of lactate that RPE cells transport through proton-coupled monocarboxylate transporters (MCTs). Thus, lactate is captured through the apical MCT1 and excluded from the basolateral MCT3 (Philp et al. 1998), resulting in the swelling of RPE cells (Philp et al. 1998; Hamann et al. 2003; Adijanto et al. 2009).

The choroid plexus epithelium (CPE) produces 600 ml of cerebrospinal fluid per day (Wright 1978), which is required for mechanical support, for communication, and as a pathway for waste removal and nutrient supply to the brain. The apical membrane of the CPE contacts the ventricle space and the basolateral surface, a highly vascularized compartment that provides a high blood supply. Na^+, Cl^- and HCO_3^- transports drive apical fluid secretion from the blood to the ventricles (Brown et al. 2004). This transport is possible due to the exquisite polarized structure of the choroid plexus epithelial cells that includes the expression of Na^+,K^+-ATPase in the apical membrane (Masuzawa et al. 1984; Siegel et al. 1984), apical and basolateral bicarbonate transporters, and active intracellular carbonic anhydrases that promote the intracellular formation of bicarbonate (Johanson et al. 2011), as in the kidney proximal tubule, Na^+, and bicarbonate gradients that constitute the driving force for the movement of fluid, which is facilitated by the aquaporin AQP1 (Wolburg and Paulus 2010).

The cellular mechanism responsible for the apical polarization of the Na^+,K^+-ATPase in both RPE and CPE is far from being elucidated. Nevertheless, as neuroepithelial cells, both tissues are expected to express the β_2-isoform that contains an apical signal and therefore would deliver the $\alpha\beta_2$ dimer to this domain. Evidently, the Na^+,K^+-ATPase does not carry a simple or classic basolateral sorting determinant. Moreover, plasma membrane proteins composed of two or more subunits, such as Na^+,K^+-ATPase, are interesting to study in terms of sorting mechanisms, as sorting signals could be present in one or more subunits that act hierarchically. Which subunit dominates the sorting of the Na^+,K^+-ATPase heterodimer is still not well understood. Remarkably, sorting signals can be interpreted depending on the cell type and be recognized by different components of the cellular sorting machinery (Philp et al. 2011; Castorino et al. 2011).

8.7 Na^+,K^+-ATPase Acts as a Receptor for the Hormone Ouabain Due to Its Polarized Distribution in the Plasma Membrane

It has recently been demonstrated that Na^+,K^+-ATPase is a receptor for the hormone ouabain. This hormone modulates signaling routes, regulating the survival of the cell in stressful situations, proliferation, and differentiation (Aizman and Aperia 2003; Xie 2006; Aperia 2007; Liu and Xie 2010).

We have recently demonstrated that ouabain regulates the two basic features of transporting epithelia: TJs and polarity (Larre et al. 2010, 2011). This was tested in monolayers of MDCK cells where the Na^+,K^+-ATPase is expressed at the plasma membrane in contact with the intercellular space. We observed that ouabain only acts when added from the basal side, i.e., the side that a hormone will reach in a living animal. Keep in mind that this is also the side where the enzyme is needed as a pump. Once it reaches the binding site, ouabain can elicit the following effects as a consequence of the polarized expression of the enzyme:

(a) Hormonal effects of ouabain on the TJ.
The effect of ouabain consists of an increase in the degree of tightness as gauged by TEER (transepithelial electrical resistance) and is mediated by the individual expression of specific claudin isoforms through specific signaling pathways. Thus, while the cell content of cln-1 is modulated through a route involving c-Src and ERK1/2, cln-4 is regulated through ERK1/2 but not c-Src. This specificity is also reflected in the modulation of specific permeabilities. Thus, while the ion flux through the TJ is controlled by c-Src and partially through ERK1/2, the flux of neutral 3 kDa dextran is regulated through ERK1/2 but not c-Src (Larre et al. 2010).

(b) Hormonal effects of ouabain on polarity.
The development of polarity was gauged through the development of a cilium in the middle of the apical domain of MDCK cells. Ciliogenesis is stimulated by ouabain through ERK1/2, provided cell proliferation is arrested. This can even be observed in single cells whose proliferation is prevented by plating a mixture of 5 % MDCK cells and 95 % NRK cells at saturating densities. Under this condition, single MDCK cells can be found completely surrounded by NRK cells. Because the expression of E-cadherin occurs at homotypic MDCK/MDCK contacts, but not in heterotypic ones, this molecule is absent when an MDCK cell is completely surrounded by NRK cells. Interestingly, in spite of the fact that under this situation these MDCK cells do not express E-cadherin, they nevertheless undergo ciliogenesis (Larre et al. 2011).

Another interesting case of ouabain-controlled polarity is the reduction in the expression of the apical Na^+/H^+ exchanger isoform 3 in proximal tubular epithelial cells (Cai et al. 2008). The hormone also reduces the basolateral expression of the pump itself and therefore the transepithelial transport of Na^+. This mechanism seems to operate in the proximal tubular epithelial cells and is impaired in salt-sensitive hypertension (Liu et al. 2011).

(c) Ciliar expression of claudin-2.
This isoform of claudin is an important component of the TJ, where it confers permeability to Na^+. However, we have observed that MDCK cells express claudin-2 at the cilium as well, where it cannot possibly play a role in permeation. Given that claudin-2 has an exquisite sensitivity to Na^+, it is conceivable that it could act as a sensor of Na^+ concentration in the fluid bathing the apical domain of the cell. The expression of claudin isoforms at the TJ and at the cilium follows independent kinetics, but both are modulated through ERK1/2 (Larre et al. 2010).

In summary, ouabain modulates the two fundamental differentiated features of transporting epithelial cells, TJs and polarity, thereby playing a crucial role in metazoan life. Ouabain binds to Na^+,K^+-ATPase once the cell stops proliferation and engages in differentiation, and therefore, it accesses its receptor from the basolateral domain of epithelial cells.

8.8 Na^+,K^+-ATPase Polarity and the Emergence of Metazoan Life

The ocean and the internal milieu act as reservoirs, the first because of its enormous size and the second because, in spite of having a very small size, its composition is kept constant by the extremely efficient quickness of the circulatory apparatus and the multitude of organs that participate in maintaining its homeostasis. All cells have to devote part of their efforts to housekeeping, a task that is enormously simplified when the internal milieu is constant (same pH, availability of nutrients, clearance of catabolites, etc.). That is why the basolateral domain of the plasma membrane of all cells in multicellular organisms is virtually identical. The apical domain, however, has to adapt to wildly different external milieus (gastric juice, bile, intestinal flora, tears, sea water, and glomerular filtrate that is progressively becoming urine), whose composition, pH, content of hormones, and other properties vary drastically throughout the day, with the diet, and has to coordinate peristalsis and movement of flagella. One may say that, having the house secured (i.e., the constancy of the internal milieu), epithelial cells can indulge in differentiation in much the same way as gene duplication, when one gene fulfills an indispensable fixed requirement and the other is "free" to diverge and explore other possibilities. This promoted the expression of diverse cell phenotypes, which enabled metazoans to progress in a mindboggling range of environments.

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