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Kirk L. Hamilton
Daniel C. Devor *Editors*

Studies of Epithelial Transporters and Ion Channels

Ion Channels and Transporters of Epithelia in Health and Disease - Vol. 3

Second Edition

american physiological society

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We dedicate this second edition to our families . . . Judy, Nathan, and Emma for KLH, and Cathy, Caitlin, Emily, and Daniel for DCD.

Preface to Second Edition—Volume 3

Our ultimate goal for the first edition of *Ion Channels and Transporters of Epithelia in Health and Disease* was to provide a comprehensive and authoritative volume that encapsulated the most recent research findings in the basic molecular physiology of epithelial ion channels and transporters of molecular diseases from the laboratory bench top to the bedside. Additionally, we envisioned that the book would be very exciting and useful to a range of readers from undergraduate and postgraduate students, to postdoctoral fellows, and to research and clinical scientists providing a wealth of up-to-date research information in the field of epithelial ion channels and transporters in health and disease. We firmly believe that the first edition fulfilled a niche that was crucially required. We have been informed that the first edition of the book has proven to be the best performing APS/Springer book based on downloaded chapters, to date. This is a direct testament to the world-class scientists and clinicians who contributed excellent chapters to that edition. Of course, there were many epithelial ion channels and transporters which were not included in the first edition, but certainly warranted inclusion.

With our second edition, we have superseded our original expectations by increasing the number of chapters from 29 in the first edition to a 3-volume second edition including 54 chapters; resulting in 25 new chapters. All of the original chapters have been expanded. Again, we were very fortunate to recruit "key" outstanding scientists and clinicians who contributed excellent chapters, some who were unable to commit to the first edition. In the end, the second edition has a total of 128 authors from 13 countries across four continents and both hemispheres. We truly believe that this book series represents a worldwide collaboration of outstanding international scientists and clinicians.

Volume 3: Studies of Epithelial Transporters and Ion Channels

This is the third of three volumes highlighting the importance of epithelial ion channels and transporters in the basic physiology and pathophysiology of human diseases. This volume has been expanded for the second edition. Volume 3 consists of 30 chapters, including 11 new chapters, and 2 original chapters with new authors, written by experts of ion transporter and ion channel families. Additionally, this volume contains chapters from experts in the pharmacology/pharmaceutical world who have contributed chapters on the most recent preclinical drug discovery efforts, culminating in what they have learned from clinical trials. Chapter topics include the Na^+/K^+-ATPase, $Na^+/K^+/2Cl^-$ cotransporter, Na^+-Cl^- cotransporter, NBCe 1 bicarbonate cotransporter, Na^+-glucose cotransporters (including GLUT transporters), Na^+/H^+ exchangers, amino acid transporters, Na^+-Ca^{2+} exchanger, urea transporters, H^+:K^+-ATPase, zinc transporters, SLC26 transporters, CLC-2 chloride channel, Cl^-/H^+ antiporter CLC-5, CFTR, TMEM16 proteins (anoctamins), ENaC, ROMK, and Kir4.1. In addition, there are chapters on KCa3.1 and BK channels, including on their pharmacology. There is a chapter on the KCNE regulation of KCNQ channels, as well as chapters on Orai channels, TRP channels, P2X receptors, polycystins (PC1 and PC2), and aquaporins. This volume provides vast background information about these transport proteins, structure and function of specific ion transporters and ion channels, and normal physiology and pathophysiology of these transport proteins in disease.

It is our intent that the second edition continues to be the comprehensive and authoritative work that captures the recent research on basic molecular physiology of epithelial ion channels and transporters of molecular diseases. We hope this new edition will be the "go-to" compendium that provides significant detailed research results about specific epithelial ion channels and transporters, and how these proteins play roles in molecular disease in epithelial tissues.

As stated in the preface of the first edition, the massive undertaking of a book of this enormity would certainly be an "Everest" of work. We want to sincerely thank all of our authors, and their families, who have spared time from their very busy work and non-work schedules to provide exciting and dynamic chapters, which provide depth of knowledge, informative description, and coverage of the basic physiology and pathophysiology of the topic of their individual chapters.

We want to, again, thank Dr. Dee Silverthorn who planted the "initial seed" that developed into the first edition; which stemmed from a Featured Topic session entitled "Ion Channels in Health and Disease" held during the Experimental Biology meetings in Boston in April 2013 (chaired by KLH). Then, based on the performance of that edition, Dee "twisted" our arms, with love, to attempt a second edition in 2017. We, once again, want to extend our huge thanks, gratitude, and appreciation to the members of the American Physiology Society Book Committee for their continued faith in us to pursue such a monumental second edition.

As with the first edition, this 3-volume second edition would not have been possible without the excellent partnership between the American Physiological Society and Springer Nature and the publishing team in Heidelberg, Germany. Many thanks to Markus Spaeth, Associate Editor (Life Science and Books), and Dr. Andrea Schlitzberger, Project Coordinator (Book Production Germany and Asia), who guided us on our second book publication journey never dreaming that this edition would be a 3-volume book bonanza.

We extend special thanks to Anand Venkatachalam (Project Coordinator, Books, Chennai, India) at SPi Global who answered unending questions during the production process. We thank his production team who assisted us through the many stages of the publication of the second edition. We also thank Nancey Biswas (Project Management, SPi Content Solution, Puducherry, India), Nedounsejiane Narmadha (Production General, SPi Technologies, Puducherry, India), and Mahalakshmi Rajendran (Project Manager, SPi Technologies, Chennai, India) at Spi Global for their assistance for overseeing the production of the chapters during the final print and online file stages of the second edition.

We want to thank our mentors Douglas C. Eaton and the late Dale J. Benos for KLH; Michael E. Duffey and Raymond A. Frizzell for DCD; and our colleagues who guided us over the years to be able to undertake this book project.

Finally, and most importantly, we want to thank our families: Judy, Nathan, and Emma for KLH, and Cathy, Caitlin, Emily, and Daniel for DCD for all your love and support during this 8-year journey.

We dedicate this second edition to our families.

Dunedin, New Zealand — Kirk L. Hamilton
Pittsburgh, PA — Daniel C. Devor
July 2020

Preface

Ion channels and transporters play critical roles both in the homeostasis of normal function of the human body and during the disease process. Indeed, as of 2005, 16% of all Food and Drug Administration-approved drugs targeted ion channel and transporters, highlighting their importance in the disease process. Further, the Human Genome Project provided a wealth of genetic information that has since been utilized, and will again in the future, to describe the molecular pathophysiology of many human diseases. Over the years, our understanding of the pathophysiology of many diseases has been realized. The next great "step" is a combined scientific effort in basic, clinical, and pharmaceutical sciences to advance treatments of molecular diseases.

A number of unique ion channels and transporters are located within epithelial tissues of various organs including the kidney, intestine, pancreas, and respiratory tract, and all play crucial roles in various transport processes responsible for maintaining homeostasis. Ultimately, understanding the fundamentals of ion channels and transporters, in terms of function, modeling, regulation, molecular biology, trafficking, structure, and pharmacology, will shed light on the importance of ion channels and transporters in the basic physiology and pathophysiology of human diseases.

This book contains chapters written by notable world-leading scientists and clinicians in their respective research fields. The book consists of four sections. The first part of the book is entitled "Basic Epithelial Ion Transport Principles and Function" (Chaps. 1, 2, 3, 4, 5, 6, 7 and 8) and spans the broad fundamentals of chloride, sodium, potassium, and bicarbonate transepithelial ion transport, the most recent developments in cell volume regulation, the mathematical modeling of these processes, the mechanisms by which these membrane proteins are correctly sorted to the apical and basolateral membranes, and protein folding of ion channels and transporters. The chapters in Part 1 provide the foundation of the molecular "participants" and epithelial cell models that play key roles in transepithelial ion transport function of epithelia detailed throughout the rest of this volume.

The second part is entitled "Epithelial Ion Channels and Transporters" and contains seventeen chapters (Chaps. 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24 and 25) in which authors have concentrated their discussion on a particular ion channel or transporter ranging from chloride channels to the Na^+/K^+-ATPase, for example. Generally, the authors have initially provided a broad perspective of the physiology/biology of a particular ion channel or transporter in epithelial tissues, followed by a focused in-depth discussion of the latest physiology, cell biology, and molecular biology of the ion channel/transporter and then finish their discussion on aspects of pathophysiology and disease.

It will be appreciated following the discussion of the various ion channels and transporters that many of these transport proteins are potential pharmacological targets for possible treatment of disease. Therefore, the third part is entitled "Pharmacology of Potassium Channels" that consists of two chapters (Chaps. 26 and 27) that provide the latest developments on the pharmacology of calcium-activated potassium channels and small-molecule pharmacology of inward rectified potassium channels. It should be noted, however, that pharmacological information about various ion channels and transporters is also provided in some of the chapters found within Part II of this volume.

Finally, the last part in this book is entitled "Diseases in Epithelia" and consists of two chapters (Chaps. 28 and 29). These chapters are designed to bridge the basic cellular models and epithelial transport functions discussed throughout this volume with a compelling clinical perspective: from bench to bedside. In these chapters, Dr. Whitcomb discusses the role of ion channels and transporters in pancreatic disease, while Dr. Ameen and her colleagues similarly provide insight into the secretory diarrheas.

Our utmost goal, with this book, was to provide a comprehensive and authoritative volume that encapsulates the most recent research findings in the basic physiology of ion channels and transporters of molecular diseases from the laboratory bench top to the bedside. Additionally, we hope that the book will be very exciting and useful to a range of readers from students to research scientists providing a wealth of up-to-date research information in the field of epithelial ion channels and transporters in health and disease.

The undertaking of a book of this scale would always be a "mountain" of work. We want to give our heartfelt thanks to all of our authors who have taken time from their very busy work and non-work schedules to provide excellent chapters, which provided depth of knowledge, informative description, and coverage of the basic physiology and pathophysiology of the topic of their particular chapters.

We want to thank Dr. Dee Silverthorn who planted the "seed" that developed into this volume; which stemmed from a Featured Topic session entitled "Ion Channels in Health and Disease" held during the Experimental Biology meetings in Boston in April 2013 (chaired by KLH). We thank the members of the American Physiology Society (APS) Book Committee who had faith in us to pursue such an exciting book.

As with any book, this volume would not have been possible without the excellent partnership between the APS and Springer-Verlag and the publishing team at Heidelberg, Germany (Britta Mueller, Springer Editor, and Jutta Lindenborn,

Project Coordinator). We wish to thank Portia Wong, our Developmental Editor at Springer+Business Media (San Mateo, CA) and her team who assisted with the early stages of the publishing process that greatly added to this contribution. Finally, special thanks to Shanthi Ramamoorthy (Production Editor, Books) and Ramya Prakash (Project Manager) of Publishing—Springer, SP1 Content Solutions—Spi Global and their production team who assisted us through the final stages of the publication of our book.

Finally, we want to thank our mentors Douglas C. Eaton and the late Dale J. Benos for KLH; Michael E. Duffey and Raymond A. Frizzell for DCD; and our colleagues who guided us over the years to be able to undertake this volume.

Dunedin, New Zealand — Kirk L. Hamilton
Pittsburgh, PA — Daniel C. Devor
June 2015

Contents

About the Editors

Kirk L. Hamilton was born in Baltimore, Maryland, in 1953. He gained his undergraduate (biology/chemistry) and M.Sc. (ecology) degrees from the University of Texas at Arlington. He obtained his Ph.D. at Utah State University under the tutelage of Dr. James A. Gessaman, where he studied incubation physiology of Barn owls. His first postdoctoral position was at the University of Texas Medical Branch in Galveston, Texas, under the mentorship of Dr. Douglas C. Eaton where he studied epithelial ion transport, specifically the epithelial sodium channel (ENaC). He then moved to the Department of Physiology at the University of Alabama, Birmingham, for additional postdoctoral training under the supervision of the late Dr. Dale J. Benos where he further studied ENaC and nonspecific cation channels. He took his first academic post

in the Department of Biology at Xavier University of Louisiana in New Orleans (1990–1994). He then joined the Department of Physiology at the University of Otago in 1994, and he is currently an Associate Professor. He has focused his research on the molecular physiology and trafficking of potassium channels (specifically KCa3.1). He has published more than 60 papers and book chapters. His research work has been funded by the NIH, American Heart Association, Cystic Fibrosis Foundation, and Lottery Health Board New Zealand. Dr. Devor and he have been collaborators since 1999. When not working, he enjoys playing guitar (blues and jazz) and volleyball. Kirk is married to Judith Rodda, a recent Ph.D. graduate in spatial ecology. They have two children, Nathan (b. 1995) and Emma (b. 1998).

Daniel C. Devor was born in Vandercook Lake, Michigan, in 1961. His education took him through the Southampton College of Long Island University, where he studied marine biology, before entering SUNY Buffalo for his Ph.D., under the guidance of Dr. Michael E. Duffey. During this time, he studied the role of basolateral potassium channels in regulating transepithelial ion transport. He subsequently did his postdoctoral work at the University of Alabama, Birmingham, under the mentorship of Dr. Raymond A. Frizzell, where he studied both apical CFTR and basolateral KCa3.1

in intestinal and airway epithelia. He joined the University of Pittsburgh faculty in 1995 where he is currently a Professor of Cell Biology. During this time, he has continued to study the regulation, gating, and trafficking of KCa3.1 as well as the related family member, KCa2.3, publishing more than 50 papers on these topics. These studies have been funded by the NIH, Cystic Fibrosis Foundation, American Heart Association, and pharmaceutical industry. When not in the lab, he enjoys photography and growing exotic plants. Dan is married to Catherine Seluga, an elementary school teacher. They have 3 children, Caitlin (b. 1990), Emily (b. 1993), and Daniel (b. 1997).

Chapter 1
Na^+/K^+-ATPase Drives Most Asymmetric Transports and Modulates the Phenotype of Epithelial Cells

Isabel Larre, Marcelino Cereijido, Omar Paez, Liora Shoshani, and Arturo Ponce

Abstract Usually, the history of an enzyme is the narrative of the works to isolate and purify it, measure its molecular weight, determine its crystal configuration, measure its activity, and so on, along years of research. The history of the Na^+/K^+-ATPase is instead a tortuous road full of pitfalls, skirmishes with physical chemistry, thermodynamics, and even philosophy. Fortunately, it has a happy ending, because it was the first known molecule to produce vectorial movement of ions, at the expense of chemical energy, cyclically modifying its selectivity. Later on, its role has evolved to act as a self-adhesion molecule at cell–cell contacts, to act as a receptor of the hormone ouabain, whose main physiological role is to modulate cell contacts, to generate a Na^+ gradient that enables co- and anti-transporters to transport net amount of ions, sugars, amino acids, i.e., to act as secondary pumps. It is enough to say that one of its crucial properties, i.e., to be expressed in a polarized manner at the intercellular membrane of transporting epithelial cells involves the β-subunit of the pump, that happens to be an adhesion molecule which plays a crucial role in that polarization mechanism.

Keywords Active transport · Na^+/K^+-ATPase · Ouabain · Epithelia phenotype · Cell–cell contacts

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1.1 Introduction

As recently as 1807, Humphry Davy was the first person to differentiate Na^+ from K^+. An ironic situation indeed, because if he was an average-sized person, he must have had 3.72×10^{13} cells (Bianconi et al. 2013), each one discriminating those cations along their whole life. Nevertheless, science would take another century and a half to start learning how cells identify certain ion species and handle each one differently, but from the very beginning, it was clear that, in order to explain ion distributions and movements in biological systems, the known laws of physical chemistry would not suffice. No wonder the history of the Na^+/K^+-ATPase can be compared to a double-sided step ladder, one climbed by biologists that investigated its structure, subunits and intrinsic mechanisms and the other by accomplished physicists who denounced (in a first step) but helped to solve (in a second step) formidable theoretical obstacles in the road toward understanding active transport. In a given moment discrepancies were so sharp that led to fear that life would never be explained in terms of physical and chemical principles (Cereijido et al. 2003, 2004).

Thus, for a long while, the peculiar composition of the cytoplasm was attributed to an alleged membrane impermeability to Na^+ because, if this ion were not able to penetrate to neutralize negative charges bound to macromolecules inside the cell, some other cations would have to do it to satisfy the principle of electroneutrality; hence the high K^+ content in the cell was accounted for by the claimed membrane impermeability to Na^+. This alternative was disproved 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 throughout the cytoplasm. This rekindled the question more emphatically: If Na^+ can readily penetrate into the cytoplasm, why does it remain at a concentration much lower (~5 mM) than in the extracellular water (~140 mM)?

1.2 It All Started with Émile Du Bois Raymond

In the second half of the nineteenth century, Émile Du Bois Raymond observed that a frog skin (Fig. 1.1a, *green bar*) maintains an electrical potential difference between its inner and its outer sides (i.e., an asymmetry). Galeotti (1904) studied this electrical potential as a function of the ionic composition of the solutions and proposed that it would be accounted for if the flux of sodium from the outside toward the inside (*influx*) were larger than the *outflux* in the opposite direction (Fig. 1.1b, *black arrows*). His proposal was rejected on the basis that it would be in violation of the laws of thermodynamics. Thus, in a *Gedankenexperiment*, a frog skin mounted in a doughnut-shaped chamber containing saline solution, an asymmetric permeability would increase the concentration of Na^+ on the right-hand side of the epithelium (*small magenta dots*), and the accumulation of Na^+ will start the diffusion of this ion down its gradient (counterclockwise), thus establishing a

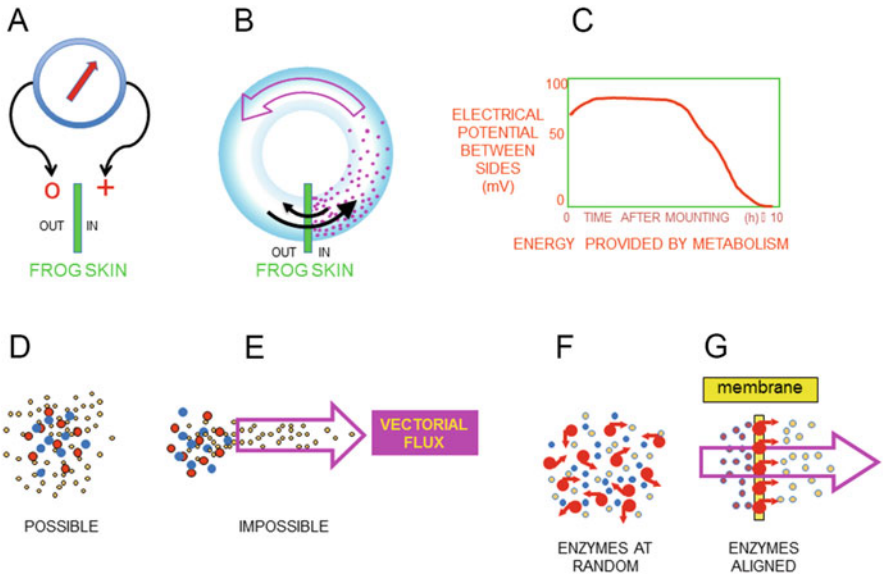

Fig. 1.1 Antecedents and roles of Na^+/K^+-ATPase. (**a**) Du Bois Raymond discovered that a frog skin can develop and maintain an electrical potential difference. (**b**) In 1905, Galeotti proposed that such potential would be accounted for if the frog skin were more permeable to Na^+ in one direction than in the opposite one. The proposal was easily refuted because it would originate a perpetuum mobile, i.e., Na^+ would rotate forever without a corresponding expenditure of free energy. (**c**) The electrical potential is not actually perpetual but lasts as long as the skin is alive, and energy is provided by metabolism. (**d**–**e**) Yet, according to the Curie principle, chemical reactions could not drive a unidirectional flux because this is a vectorial process. (**f**) Enzymes functioning as pumps are vectorial at the microscopic level, but when studied macroscopically, it becomes unnoticeable. (**g**). Pumps ordered as in a plasma membrane exhibit macroscopic vectoriality

perpetuum mobile (*magenta arrow*), that would in fact perform a work without the corresponding dissipation of energy. Yet biologists observed that a frog skin dissected and mounted between two chambers dies in a few hours and the electrical potential (Fig. 1.1c, *red curve*) (Ussing and Zerahn 1951) vanishes; in other words, far from being *perpetuum*, the potential lasts as long as the skin is alive. "Perhaps, the energy is afforded by metabolic energy" they proposed. Yet, given that metabolism is the sum of chemical reactions, physicists claimed that physiologists were now violating Curie's Principle: "Chemical reactions are scalar phenomena (again: a wrong assumption): they occur regardless of the orientation of the reacting molecules, and the resulting chemical products cannot diffuse in a particular direction" (Fig. 1.1d); therefore, chemical reactions would never originate a vectorial flux across a frog skin as in Fig. 1.1e (another incorrect supposition).

Biologists argued that each molecule (Fig. 1.1f, *red*) may be asymmetric, but since there are millions of molecules oriented at random, asymmetry cannot be observed at a macroscopic scale. However, if they were ordered in a membrane as suggested in Fig. 1.1g, the asymmetry would be recovered, and a macroscopic flux

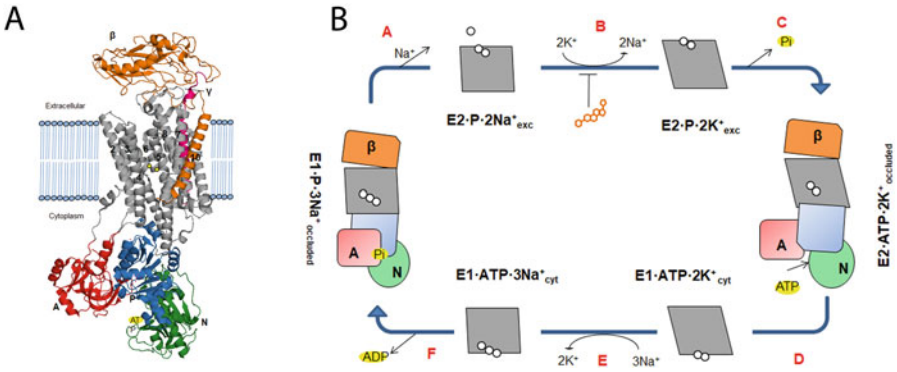

Fig. 1.2 (**a**) Ribbon model of the crystal structure of shark Na^+/K^+-ATPase ([Shinoda et al. 2009; PDB code: 2ZXE) indicating alpha subunit transmembrane domains (TM1–9, gray) and cytoplasmic domains N, P, and A in green, blue, and red, respectively. β-subunit is colored in orange. γ-subunit is colored in magenta. TM 1 and TM 9 are not visible from this projection. The two K^+ (yellow) are occluded in the crystal structure. In the N domain, the ATP binding site is also indicated. (**b**) Schematic depiction of the catalytic cycle of Na^+/K^+-ATPase. A structural rearrangement, especially in domain A (red), 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. (1) Outward transport of three Na^+ ions (white circles) is coupled to the E1 to E1 transition. (2) Two K^+ (red circles) bind at binding sites oriented to the extracellular space. (3) Extracellularly bound K^+ activates dephosphorylation which in turn results in ion occlusion. Ouabain binds at this conformational state stopping the cycle. (4) ATP with a low affinity triggers the acceleration of inward transport of K^+ through the pore as the pump enters the E1 conformational transition with a low affinity for K^+. (5) Three Na^+ bind the intracellularly oriented sites. (6) Phosphorylation from ATP occurs and Na^+ are occluded again. Several transitional substates exist; nevertheless, they are not depicted for simplification

would take place. 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 products? Around 1955–1959 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 (1957) was able to inhibit the ATP splitting activity of his extract by adding *ouabain*, a substance of vegetal origin that, a few years earlier was found to inhibit ion pumping when added from the outer, but not from the inner side of the cell membrane. By performing the K^+/Na^+ 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." Today, thanks to high-resolution crystallography (Shinoda et al. 2009), the molecular structure of the Na^+/K^+-ATPase is known in great detail (Fig. 1.2a), and its cyclical reactions are described in Fig. 1.2b.

1.3 Polarized Distribution of Na^+/K^+-ATPase in Epithelial Cells

A unicellular organism in the ocean may consume nutrients and eliminate metabolic wastes without risking neither an exhaustion of nutrients nor pollution of the sea. In a metazoan, the ocean is represented by a narrow extracellular space that would be rapidly exhausted of nutrients and spoiled with wastes, were it not for a circulatory apparatus that moves fluids to and from tissues to transporting epithelia, where the true exchange with the external milieu takes place. Thanks to the circulation of blood, in spite of being so small, this intercellular "ocean" behaves as a constant and reliable reservoir (homeostasis). Figure 1.3a depicts the model developed by Koefoed-Johnsen and Ussing (1958) in which the pump, represented by Na^+/K^+-ATPase, is assumed to be located on the basal side of the epithelial cell, and this asymmetric distribution, together with the specific Na-permeability of the outer cell membrane (*Ocm*) and the specific K-permeability of the inner facing membrane (*Icm*) are responsible for net movement of Na^+. This model served as a blueprint to understand the net transport of important biological substances across most epithelia.

Notice that the Na^+/K^+-ATPase is *primarily* responsible for the pumping of Na^+ and K^+, but the concentration gradients that it generates result in the *secondary* transport of amino acids, sugars, and ions other than Na^+ and K^+. Since these transports occur in a net amount and can be inhibited with ouabain, for a while this was taken as a proof of the existence of glucose pumps, as well as other pumps for diverse amino acid species (Fig. 1.3b). Yet, eventually, it was demonstrated that carriers for sugars and for amino acids are not pumps, as they are not *directly* coupled to metabolism, but their affinity for sugars and amino acids drastically increases when loaded with Na^+. Today we are so used to talking about co- and

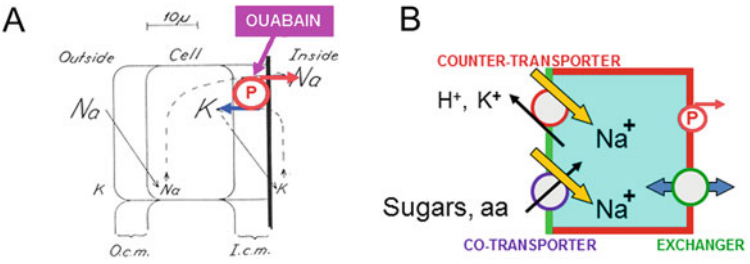

Fig. 1.3 Model proposed by Koefoed-Johnsen and Ussing (1958) to account for the net pond-to-blood transport of Na^+. (**a**) An outer cell membrane (O.c.m.) with a high selectivity for Na^+ and low selectivity for K^+ and an inner (I.c.m.) whose ion selectivity is high for K^+ and low for Na^+. A pump (P, red circle) located on the basal side takes Na^+ from the cytoplasm and transports it toward the blood (inner) side and takes K^+ from the inside and transports it toward the cytoplasm. This pump can be inhibited by ouabain added from the blood side. (**b**) The pump keeps the concentration of Na^+ in the cytoplasm at a level lower than in the extracellular fluid; this ion flows passively (yellow arrow) toward the cytoplasm, driving a cation in the opposite direction or an anion in the same direction. Furthermore, the penetration of Na^+ can also drive sugars and amino acids from the outer bathing solution to the cytoplasm

anti-transport that we forget it caused another famous, but easy to understand squirmish between biologists and physicists. Notice that co- and counter-transport assumed that the electrochemical potential difference for Na^+ can be used to move sugars and amino acids as depicted in Fig. 1.3b. Although we will not make a digression to explain how this important theoretical problem was solved, it is worth mentioning that for making a revolution in the Thermodynamics of Irreversible Processes, Lars Onsager was awarded the Nobel Prize in 1968. But since then, it was realized that, in principle, the flux of a substance (sugar, amino acid, chloride, etc.) can be moved by the energy arisen from any driving force present in the system. When Na^+/K^+-ATPase is inhibited with ouabain the concentration of sodium in the cytoplasm rises and its concentration gradient across the apical membrane vanishes. In summary, the Na^+/K^+-ATPase is the *primum movens*, responsible for the exchange of substances between metazoan and the environment across transporting epithelia, as well as for net exchange between the internal milieu and the cytoplasm.

1.3.1 Why Do Epithelial Cells Express Na^+/K^+-ATPase in a Polarized Manner?

Single cells, e.g., a leukocyte, a bacterium, or an amoeba, have Na^+/K^+-ATPase distributed at random all over the plasma membrane. The cells of transporting epithelia instead have Na^+/K^+-ATPase distributed in a peculiar way; in the model of Koefoed-Johnsen and Ussing (1958), for instance (Fig. 1.3a), they are assumed to be expressed mainly on the basal membrane. For a while, the apical/basolateral polarity of epithelial cells was erroneously attributed to the tight junction (TJ). This mistaken idea was based on the demonstration that the chelation of Ca^{2+} with EDTA or EGTA not only opens the TJs but destroys the apical/basolateral polarity, as well. However, TJs have no sorting mechanisms and, at most, maintain the apical/basolateral polarization that some molecular species have obtained by other mechanisms (e.g., lipids). This was clearly demonstrated by studies of Contreras et al. (1989), who found that MDCK (Madin–Darby canine kidney) cells adopt a spherical shape after harvesting and resuspension and a large fraction of plasma membrane is endocytosed along with a large amount of Na^+/K^+-ATPase units inserted on it, whereas those remaining at the surface lose their polarity and become randomly distributed. When cells are seeded again, and subject to the "calcium switch protocol" (Gonzalez-Mariscal et al. 1990; Contreras et al. 1992), TJ forms so quickly that catches Na^+/K^+-ATPase still randomized, but in spite of the presence of the barrier constituted by the already sealed TJ, the enzyme bypasses this structure and polarizes correctly. Hence, to explain the nonrandom distribution of molecules in the plasma membrane, it was necessary to study the intracellular routes and sorting compartments involved, such as the endoplasmic reticulum (ER) and the trans-Golgi network (TGN), and different carrier vesicles for delivery to their corresponding plasma membrane domains (Weisz and Rodriguez-Boulan 2009; Rodriguez-Boulan

and Macara 2014). For each of these routes, polarity depends critically on the existence of specific signals (motifs) encoded within the membrane proteins themselves. The basolateral sorting signals are short peptide sequences most often found within the cytoplasmic domain of the protein. Some basolateral sorting signals resemble endocytic signals, e.g., variations of the canonical endocytic dileucine, YXXΦ, where Φ is any hydrophobic amino acid, and NPXY motifs. Other basolateral signals are unrelated to endocytic signals, e.g., the tyrosine motifs in the low-density lipoprotein receptor and the G-protein of the vesicular stomatitis virus (VSV). Early studies demonstrated that the Na^+/K^+-ATPase, comprising 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 MDCK cells (Caplan et al. 1986; Mays et al. 1995). Therefore, a basolateral signal was assumed to exist in the α-subunit of the Na^+/K^+-ATPase. The Na^+/K^+-ATPase and H^+/K^+-ATPase are highly homologous ion pumps, yet in LLC-PK1 cells, they are polarized to the basolateral and apical domain, 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 (Blostein et al. 1993). An apical sorting motif was identified 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 still unknown, noncanonical molecular signal seems to be 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. It has been shown that the adaptor involved in basolateral protein sorting is the epithelial cell-specific AP-1B (adaptor protein 1B). Nevertheless, the correct polarization of Na^+/K^+-ATPase is not significantly affected by knocking down clathrin expression. It also remains correctly polarized in both the μ1B-deficient cell line LLC-PK1 (Ohka et al. 2001) and in MDCK cells in which μ1B expression had been suppressed via RNAi (Duffield et al. 2004). By taking advantage of the SNAP tag system to reveal the trafficking itinerary of the newly synthesized Na^+/K^+-ATPase, it was shown that basolateral delivery of the Na^+/K^+-ATPase is very fast 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 (Farr et al. 2009). 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 (Zimmermann 2006). 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 the adhesion of its β1-subunits with those of neighboring cells. Readers interested in apical and basolateral sorting of transport proteins are directed to Chap. 5 of Volume 1.

The β-subunit functions as a molecular chaperone of the catalytic α-subunit. It facilitates the correct membrane integration and packing of the newly synthesized catalytic α-subunit, which is necessary for its protection against cellular degradation, acquisition of functional properties, and routing to the plasma membrane (Geering 2008). In addition to its chaperone function, β-subunits influence the transport properties of mature Na^+/K^+-ATPase. α-Subunits associated with different β-isoforms exhibit different apparent potassium affinities, and the β-structure influences the apparent sodium affinity of Na^+/K^+-ATPase (Geering 2001). During the catalytic cycle, there is a conformational rearrangement between α- and β-subunits (Dempski et al. 2005).

1.3.2 Cues Leading to a Model of Na^+/K^+-ATPase Polarity

As with most transporting epithelia, the monolayer of MDCK expresses Na^+/K^+-ATPase in a polarized manner toward the basolateral side (Cereijido et al. 1980). Figure 10.4a shows a monolayer of MDCK cells with nuclei stained with propidium iodide (*red*) and Na^+/K^+-ATPase (*green*). Contrary to the assumption of Koefoed-Johnsen and Ussing (Koefoed-Johnsen and Ussing 1958), the pump is not located on the *basal* domain of the plasma membrane, but only in the *lateral* domain (*first cue,* Fig. 1.4a). Although green lines in Fig. 1.4a, b appear as a single, undivided green line, upon treating the monolayer with EGTA to sequester Ca^{2+}, the apparent single green line splits into two (Fig. 1.4c), indicating that, in order to express Na^+/K^+-ATPase at a cell–cell contact, both neighboring cells have to contribute part of the enzyme (*second cue*). In order to express the Na^+/K^+-ATPase at a given lateral border, both contributing neighboring cells should be homotypic, for instance, MDCK/MDCK (dog/dog) but not heterotypic: MDCK/Ma104 (dog/monkey) (Shoshani et al. 2005), as depicted in Fig. 1.4d (*third cue*).

Figure 1.4e depicts the position and arrangement of Na^+/K^+-ATPase obtained by crystallography, showing the positioning of this trimer: α-subunit (*violet*), β-subunit (*green*), and γ-subunit (*red*). The β-subunit has the typical structure of a cell-attachment protein (Geering 2008). Karlish's group has confirmed that the β-subunit ectodomain contains an immunoglobulin-like structure that would be responsible for its adhesive properties (Bab-Dinitz et al. 2009). Accordingly, the β-subunit has a short cytoplasmic tail, a single transmembrane segment, and a long extracellular segment that is heavily glycosylated. We also showed that β1-subunit immobilized on Ni beads could specifically bind to the soluble extracellular domain of β1-subunits of the same animal species (dog; Padilla-Benavides et al. 2010). To test this property and to see if it works in MDCK cells, we transfected a gene coding for the β-subunit of the dog (remember that MDCK cells are also derived from a dog) into MDCK cells, and demonstrated that this considerably increases cell–cell adhesion (Fig. 1.4f). This constituted our *fourth cue*. We also examined this property by transfecting other cell types with dog β-subunit, that is, the same experiment as the one in Fig. 1.4d, except that this time the "other" cell (CHO cell from Chinese

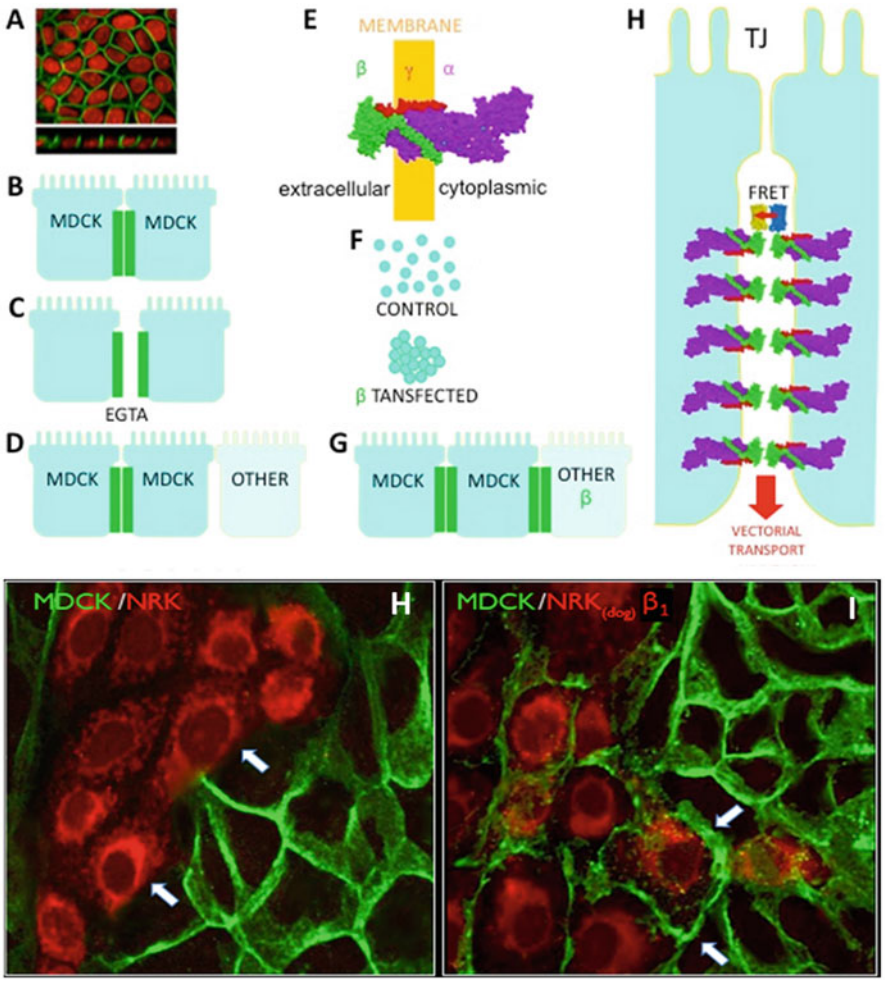

Fig. 1.4 Cues for the polarized distribution of Na^+/K^+-ATPase. (**a**) Monolayer of MDCK cells. Na^+/K^+-ATPase (green) and nuclei (red). The enzyme is expressed on the lateral membrane of the cells. (**b**, **c**). Although the image of the enzyme appears as a single green line, the use of EGTA splits the line, demonstrating that each cell contributes its own Na^+/K^+-ATPase. (**d**) In a monolayer prepared with a mixture of MDCK cells and epithelial cells of a different animal species (Chinese hamster ovary, CHO), the MDCK cell in the center only expresses its Na^+/K^+-ATPase on the side contacting another MDCK cell, but not on the side contacting the epithelial cell of a different animal species. Confocal image of a mixture of MDCK and NRK (normal rat kidney) cells is depicted in (**h**). Arrows indicate heterotypic borders lacking Na^+/K^+-ATPase. (**e**) The α-, β-, and γ-subunits of Na^+/K^+-ATPase as expressed in a cell membrane: most of the β-subunit (green) is exposed on the intercellular space (**f**) (above). Suspension of CHO cells (light blue) with a poor attachment to each other below CHO cells transfected with dog β-subunits tend to attach to each other and form aggregates, thereby demonstrating the self-adhesion tendency of this subunit. (**g**) A study essentially identical to the one described in (**d**), except that this time the third cell on the right is a CHO transfected with β-subunits of dog. MDCK cell at the center now expresses its β-subunits on both lateral sides. (**i**) A confocal image showing a mixture of MDCK and NRK cells transfected with dog β-subunit. Arrows indicate the presence of Na^+/K^+-ATPase at heterotypic borders. (**j**) Scheme showing the intercellular space of two neighbor MDCK cells in monolayer. Na^+/K^+-ATPase units

Hamster Ovary) is transfected with *dog* β-subunit. Notice that an MDCK and a CHO cell transfected with a dog β-subunit, offer the image of a single line, as in Fig. 10.4b. To complement the description of this cartoon, Fig. 1.4h, i show two types of mixed monolayers, one with MDCK/NRK cells (NRK, from rat kidney) and the other with NRK transfected with dog β-subunit, showing, again, that MDCK cells only expose Na^+/K^+-ATPase, when the neighboring cell exposes β-subunit from the same animal species. Finally, Fig. 1.4j, shows that the Na^+/K^+-ATPase expressed at the lateral border of the cell can only pump Na^+ into the intercellular space and, given that this space is sealed toward the outer side of the epithelium by the tight junctions (Fig. 1.4j), this ion can only flow vectorially toward the blood side.

Of course, the first question that arises is whether two matching β-subunits from different cells would get close enough to be able to span the intercellular space and interact as proposed. To answer this question, we prepared monolayers with a mixed population of MDCK cells transfected with a β-subunit fused to a cyan fluorescent protein (*blue*), with MDCK cells transfected with a β-subunit fused to yellow fluorescent protein, showing that in a fluorescence resonance energy transfer (FRET) analysis energy can be transferred from the first to the second cell type (Fig. 1.4j) (Padilla-Benavides et al. 2010). In other words, two β-subunits can interact directly at <10 nm, thereby anchoring the whole enzyme at the cell membrane facing the intercellular space (Padilla-Benavides et al. 2010). This basic mechanism seems to be strengthened through interactions with extracellular ligands and intracellular scaffolds such as cytoskeletal elements or arrays of PDZ domain-containing proteins. β_1-subunit has three conserved N-glycosylation sites. Remarkably, N-glycans stabilize the amino acid-mediated interactions between β1 subunits (Tokhtaeva et al. 2010). Moreover, intercellular adhesion can be regulated via N-glycan-mediated modulation of β1–β1 interactions. This modulation occurs because of cell-adhesion-dependent regulation of the level of the specific glycosyltransferases that control the degree of N-glycan branching of the Na^+/K^+-ATPase β_1 subunit (Tokhtaeva et al. 2011; Vagin et al. 2008). In this respect, several studies have shown that lateral targeting of the pump in transporting epithelial cells is related to the expression of the β_1 isoform, while apical targeting is related to expression of the β_2 isoform. Furthermore, this differential polarization is directly related to the variation of N-glycosylation between these two isoforms (Vagin et al. 2007a, b).

Fig. 1.4 (continued) are expressed at the lateral border, where they are anchored by the β-subunits. Notice that these pumps can only inject Na^+ into the intercellular space. Since this space is sealed toward the outer bathing solution by the tight junction (TJ), Na^+ can only diffuse toward the inner (blood) side. In summary, this cartoon depicts these two attributes whose mechanism of synthesis, assembly, and sealing, as well as the peculiar polarized distribution, are detailed elsewhere (Cereijido et al. 2008, 2012)

1.4 Structural Insights into the Na^+/K^+-ATPase Adhesion Mechanism

The crystal structure of the shark's Na^+/K^+-ATPase 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] (Shinoda et al. 2009). The extracellular C-terminal domain of the protein folds into an Ig-like β-sheet sandwich as predicted in silico (Bab-Dinitz et al. 2009). Actually, the deletion of this C-terminal domain abolishes the β_1 adhesion capacity (unpublished observations). However, a large number of adhesion and non-adhesion proteins contain domains with an immunoglobulin-like topology [CATH database]. Structural alignments of the β_1 subunit extracellular domain against other well-studied cell adhesion molecules reveal no structural homolog 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 that of a typical Ig domain (unpublished observations). Furthermore, the β_1 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 Fig. 1.4f. 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 (Padilla-Benavides et al. 2010). Based on this difference, Tokhtaeva et al. (2012) found that this difference in affinity between rat and dog is caused by the presence of different residues between the two species, all of them within 198–207 segment. Such a segment constitutes one of the characteristic protruding loops in the connecting β-strands B and C of the β1-subunit extracellular fold. The majority of the ectodomain surface-exposed residues located most distal from the membrane resides within loops interconnecting β-strands, some of which must be involved in the dimer interface in conjunction with segment 198–207. We have performed protein docking and molecular dynamics simulation to identify putative amino acids that participate in β1–β1 interaction. Our model suggests that Na^+/K^+-ATPase β1-subunit extracellular domain is a stable structure and that species-specific residues 198–207 interact with segment 223–229 on the associating β1-subunit protein [unpublished results].

1.5 Cardiac Steroids

Cardiac steroids (CS) became a medical tool since William Withering (1741–1799) observed that patients with a weak heart improved when drinking a herb tea of foxglove (*Digitalis purpurea)*. He was emulated by European physicians using tisane of plant species from other regions, such as *Digitalis lanata* and West African plant (*Strophanthus gratus*) that led to coining of the name Strophanthin (ouabain). In 1953, Hans J. Schatzmann observed that ouabain inhibits active potassium and sodium transport in the erythrocyte membrane (Schatzmann 1953). Later, Jens Christian Skou prepared an extract from crab nerves that contained an ATPase that hydrolyzed ATP into ADP and Pi. Since the activity of this extract was enhanced when exposed to a certain combination of Na^+, $K^{+,}$ and Mg^{2+}, Skou hypothesized that the enzyme involved might be related to the active movement of sodium and potassium across the plasma membrane, and properly named it Na^+/K^+-ATPase. Furthermore, this enzyme was inhibited by ouabain, an observation that reinforced the suspicion that there must be an endogenous analog (Skou 1957).

The remarkable affinity of cardiac steroids for Na^+/K^+-ATPase in mammalian organisms stressed the possibility that there was an endogenous substance analogous to the one extracted from plants. Hamlyn et al. (1991) and Mathews et al. (1991) demonstrated the presence of a substance in plasma that could not be distinguished from ouabain of vegetal origin, a finding confirmed with more advanced methods such as 1H-NMR and mass ionization spectrometry. There are other analog substances in human blood (Kawamura et al. 1999; Komiyama et al. 2005; Takahashi 2000; Tymiak et al. 1993) and in other animal species, such as digoxin-like factor (Goto et al. 1990; Qazzaz et al. 2004) marinobufagenin (Bagrov et al. 1998); telocinobufagenin (Komiyama et al. 2005), 19-nobufalin (Lichtstein et al. 1993), and proscillaridin A (Hilton et al. 1996; Li et al. 1997). Endogenous ouabain is synthesized and secreted by the hypothalamus (Ludens et al. 1992; el-Masri et al. 2002) and the adrenocortical gland (Doris et al. 1996; Laredo et al. 1994; Qazzaz et al. 2004; Schreiber and Stepan 1986). The status of ouabain as an endogenous hormone was unambiguously recognized when it was demonstrated that it increases during exercise (Bauer et al. 2005), salty meals (Fedorova et al. 2005a, b, 2000), and pathological conditions such as arterial hypertension and myocardial infarction (Gottlieb et al. 1992; Manunta et al. 2009). Once ouabain was accepted as a hormone, it became of interest to find its physiological role. Our laboratory showed that ouabain binding to the Na^+/K^+-ATPase modulates epithelial cell adhesion (Larre et al. 2011a).