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Finding Na,K-ATPase II - From fluxes to ion movements

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Abstract. After identification of the Na,K-ATPase as active ion transporter that maintains the Na^+ and K^+ concentration gradient across the membrane of virtually all animal cells, a long history of mechanistic studies began in which enzyme activity and ion-transport were intensively investigated. A basis for detailed understanding was laid in the so-called Post-Albers pump cycle. Developing new experimental techniques allowed the determination of different flux modes, the analysis of the kinetics of enzyme phosphorylation and dephosphorylation as well as of the transport of Na^+ and K^+ ions across the membrane. The accumulation of results from transport studies allowed the proposal of the gated channel concept that turned out to be a successful approach to explain the transport-related experimental findings. Eventually, it found its counterpart in the high-resolution structure of the ion pump. Recently it turned out that simple mutations of the Na,K-ATPase are the cause of several diseases.

Keywords. Ion transport, enzyme activity, flux modes, structure-function relation, electrogenicity, gated-channel concept, pump-related diseases.

Dedicated to the late Prof. David C. Gadsby (1947-2019), a brilliant physiologist and biophysicist

I. DEVELOPMENT OF A FUNCTIONAL CONCEPT

In the 1950s the need for active ion transport through membranes was recognized. A number of concepts of the molecular mechanism of active transport had been proposed and discussed before the identification of the Na,K-ATPase. During that time James F. Danielli reviewed five possible mechanism that summarized the ideas.[1] They were adaptations of the carrier mechanism, which at that time had already been introduced as concept for passive ion transport. To perform active transport contractile proteins were coupled to the carrier to enable appropriately directed substrate transport. A different approach was proposed in 1957 by Peter Mitchell. His idea was substrate binding in a transporter to specific sites that experience translocation across the membrane by a rocking mechanism.[2] This proposal was published the same year as when Jens P. Skou identified the Na,K-ATPase as protein in crab nerve cell membranes.[3]

II. THE POST-ALBERS CYCLE

The consequence of Skou's identification of the Na,K-ATPase and the fact that the ion pump could be selectively inhibited by ouabain (or other cardiac steroids) led directly to numerous target-oriented studies that provided a wealth of characteristical details.[4] A first proposal of the pump mechanism was published in 1963 by R. Wayne Albers and colleagues.[5] They discussed, as a possible pump mechanism of the "adenosine phosphatase" in the electrophorus electric organ Na^+-ATPase, a transphosphorylation in which phosphates were transferred along a chain of sites for phosphorylation from the cytoplasmic to extracellular side. Na^+ transport was suggested to be a by-product of the oriented transphosphorylation by acting as counter ion to the phosphate. K^+ transport might have been coupled with phosphate uptake.

Compilation of the continuously increasing experimental findings led Robert L. Post and Amar K. Sen in 1965 to a first guess of a reaction cycle with seven states.[6] In 1967 it was followed by the presentation of a reaction cycle by Albers and collaborators that described the enzymatic reactions, in which phosphorylation by ATP required the presence of Na^+ and dephosphorylation required K^+.[7] The four steps of the cycle are shown in Fig. 1A. In its E_1 forms the enzyme had inwardly oriented cation sites of high Na^+ affinity. The E_2 forms were characterized by outwardly oriented cation sites of high K^+ affinity.

While Albers and collaborators focused their view on the enzyme activity of the ion pump, Post et al. included five years later detailed information on the ion transport and presented the first pump cycle that assigned enzyme and transport activity together in a unified reaction cycle.[8] His proposal accounted not only for the physiological Na,K-ATPase function but also for Na-ATPase activity observed under (two) unphysiological conditions (Fig. 1B). This scheme, the so-called Post-Albers cycle, has become the prototypical reaction cycle of all P-type ATPases studied so far. Two basic features of the pump mechanism of the Na,K-ATPase are captured in this scheme: (1) The transport is performed in a consecutive (or "Ping-Pong") mode, which means that at first one ion species is translocated in one direction, then, after an exchange of ions, the second species is conveyed in the opposite direction. Since it was impossible to establish that Na^+ and K^+ were bound to the ion pump at the same time, it was suggested that they bind alternatingly to the same spatial sites which exhibit different binding affinities when accessible from one or the other side of the membrane. (2) Na^+ transport is connected to enzyme phosphorylation by ATP, K^+ transport takes place when the enzyme runs through the dephosphorylation half cycle. Two non-physiological pump modes, included in Post's proposal, were identified when the substrate conditions were modified appropriately: The first was observed when K^+ was removed from the extracellular medium. Nevertheless, a ouabain-sensitive but significantly reduced ATPase activity was detected. This finding led to the suggestion of a ATPase that is able to transport Na^+ out of the cell without a counter-transport of K^+, named "Na-ATPase". The second modi-

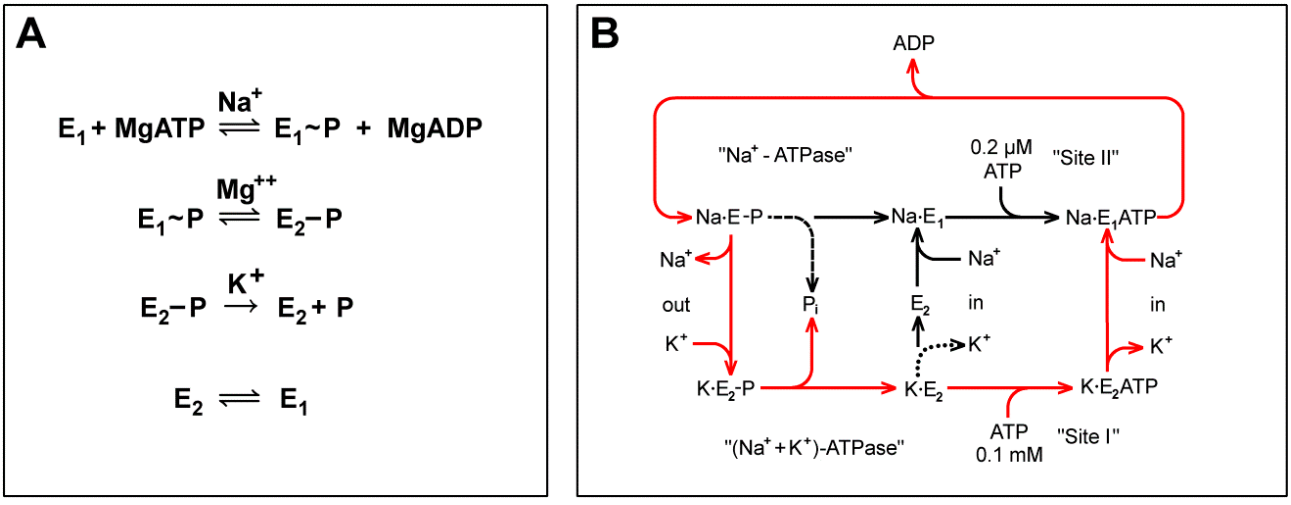


Figure 1. First representation of the pump cycle of the Na,K-ATPase. **A:** Reaction cycle of the enzyme activity with the ion substrates needed to enable the respective reaction step, published in 1967 by Albers and collaborators.[7] **B:** Reaction cycle with merged enzyme and transport functions, the so-called Post-Albers cycle, introduced 1972 by Post and collaborators.[8] The cycle marked in red represents the physiological mode.

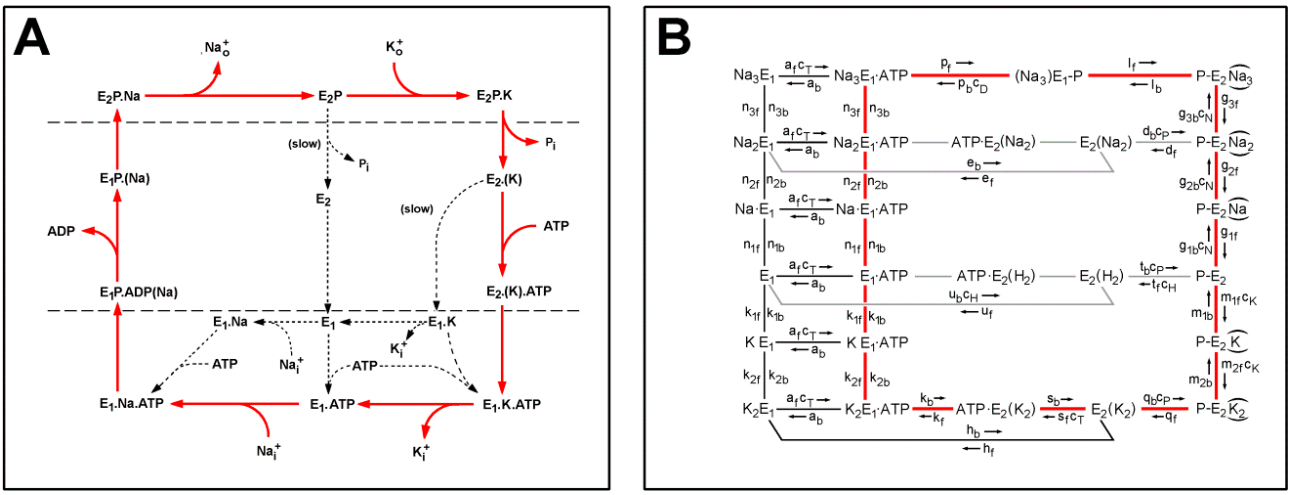


Figure 2: Development of the Post-Albers cycle with enhanced complexity provoked by increasing experimental insights. **A:** Inclusion of Na^+ and K^+ occluded states, indicated by framing the ions in parentheses, (Na) and (K). This scheme was adapted from Karlish and collaborators.[11] **B:** Pump scheme composed of all reaction steps that were determined experimentally from rabbit kidney ATPase until 1994, and which was used for successful numerical simulations of the experimental results.[12] The cycles drawn in red represent the physiological pump mode.

fication of the pump mechanism was found when the ATP concentration in the cell was reduced to values far below the physiological millimolar range. The dependence of the ATPase activity on the ATP concentration revealed the existence of two distinct binding affinities, "low affinity binding" in the range above 10 µM, and a high affinity binding in the 100 nM range. The low-affinity binding was associated with the Rb^+ (or K^+) bound E_2 conformation. Binding of ATP accelerated the transition from the E_2 to the E_1 conformation and deocclusion of the ion sites. But this process was not accompanied by phosphorylation of the enzyme.[8] High affinity binding of ATP occurred in the E_1 conformation and the presence of Na^+.[9] It was also shown that the ATP-concentration dependence revealed both low and high affinity binding of the nucleotide in the presence of Na^+ and K^+. In the absence of K^+ only high-affinity binding was detected[10] that took place in the Na^+ exporting half cycle, which was passed through in both pump modes.

In the years after 1972 extensive series of kinetic studies were published by several authors, in which a whole range of research activities were covered, such as studies on conformation transitions, enzymatic and transport activities. Based on these findings Karlish and collaborators presented in 1978 an extended Post-Albers scheme (Fig. 2A) which summarized all observed functional properties known at that time.[11] In their pump cycle an additional fundamental characteristic of the transport process, ion occlusion, was included: In the E_1 conformation the ion-binding sites were accessible from the cytoplasm, but after 3 Na^+ ions were bound, the enzyme became phosphorylated by ATP, and simultaneously the access to the ion-binding sites was locked and the ions were trapped inside the protein. Only thereafter the access of the ion sites to the extracellular side was unlocked, a process related to the conformation transition from E_1 to E_2, and the Na^+ ions were released. With respect to the K^+-transporting half cycle a corresponding reaction sequence occurred: Enzyme dephosphorylation caused occlusion of the K^+-loaded ion sites, and release of the K^+ ions to the cytoplasm only happened after deocclusion which was coupled to the conformation transition from E_2 to E_1.

Systematic and particularly time-resolved kinetic measurements led to a further extended Post-Albers scheme that allowed a successful simulation of those experiments.[12] The pump scheme shown in Fig. 2B is adapted from Heyse et al. and includes all six known flux modes of the Na,K-ATPase (see below). In this reaction scheme those reaction steps which have been identified in experiments with rabbit-kidney Na,K-ATPase are labeled with rate constants. The rate constants were either directly measured, determined from experiments or calculated from theoretical constraints. The reaction cycle shown in red represents in clockwise direction the Post-Albers cycle under physiological conditions. The counterclockwise reaction sequence describes the performance of the Na,K-ATPase as ATP synthase.

III. FLUX MODES

Under diverse specific substrate conditions at least six additional transport modes ("non-canonical flux modes") were detected besides the physiological transport mode in which 3 Na^+ were removed from the cytoplasm in exchange against 2 K^+ taken up from the extracellular medium.[13,14] These flux modes are:

(1) Pump reversal, which can be observed at high intracellular concentrations of K^+, ADP and inorganic phosphate, P_i as well as low concentration of ATP, high extracellular concentration of Na^+ and in the absence of K^+.[15,16] In this substrate condition the pump cycle is run through backwards and ATP in synthesized.

(2) Isostoichiometric exchange of Na^+ across the cell membrane was found to have taken place in the absence of K^+ and the presence of cytoplasmic ADP. In this mode the Na,K-ATPase acted as Na^+ shuttle by which the Na^+-translocating half cycle was executed forward and backward. First, 3 Na^+ were transferred out of the cell under consumption of ATP, then the 3 Na^+ were exchanged on the outside and transported back into the cell while ATP was produced from ADP and P_i, i.e. no net consumption of ATP took place in this mode.[17]

(3) Isostoichiometric exchange of K^+ operated also as shuttle service in which the K^+-translocating half cycle was executed forward and backward. 2 K^+ were bound extracellularly and transported into the cell via enzyme dephosphorylation and binding of ATP. In the absence of intracellular Na^+ and the presence of P_i the physiological process was reversed by K^+ binding from the cytoplasmic side, release of the bound ATP and enzyme phosphorylation by P_i. ATP was bound but not hydrolyzed.[18] ATP was required only to promote the E_2/E_1 conformation transition.

(4) Uncoupled Na^+ efflux consuming ATP could be measured when neither Na^+ nor K^+ were present extracellularly.[19,20] In this mode it was assumed for a long time that after external release of Na^+ the pump cycle was completed by a return from the E_2-P to the E_1 conformation with empty binding sites. Not so long ago it was revealed, however, that this rather small flux (compared to the Na^+,K^+ mode) was only apparently uncoupled, but a Na^+,H^+ exchange in which protons were transported into the cell as K^+ congeners much less effectively but with the standard stoichiometry of 3 Na^+/2 H^+/ATP.[21]

(5) Na^+ exchange consuming ATP was detected in the absence of external K^+ but in the presence of Na^+ on both sides of the membrane.[22,23] This mode evolved from the uncoupled Na^+ efflux with increasing external Na^+ concentration.[14] An obvious mechanistic explanation for this flux mode was that the extracellular Na^+ acted as (less well fitting) congener of K^+ with a stoichiometry of 3 Na^+/2 Na^+/ATP.[10]

(6) Finally, an uncoupled K^+ efflux from red blood cells was found in the absence of extracellular Na^+ and K^+ that did not require the presence of ATP.[24] In the light of H^+ acting as congener of K^+ this flux mode may be explained also as shuttle mechanism exchanging K^+ and H^+ in homology to mode (3). This concept would avoid the necessity of an energetically less favorable return of the pump from state E_2-P to E_1 with empty ion-binding sites, as proposed in the originally published mechanism.[24]

IV. ELECTROGENICITY

An important feature of the Na,K-ATPase (and of biological ion transporters in general) is the transfer of ions from one side of the membrane to the other, because ions are charged particles and well soluble in water but not in the membrane. A main task of the cell membrane is to exactly prevent unfacilitated permeation of ions between different compartments of the cell.

Therefore, structure and properties of biological membranes are optimized to reduce diffusion of ions through the membrane to a minimum. Repulsive electrostatic interactions are the predominant reason for this effect.[25] The charge of an ion is the origin of an electric field that influences the surrounding matter by attracting charges of opposite sign, repelling charges of the same sign, and reorienting electric dipoles. The energy needed to promote these responses in matter is provided by the kinetic energy of the moving ion. Because of the long range of electrostatic interactions a considerable portion of the surrounding matter is involved, and the amount of energy needed is dependent on a property of the matter called polarizability. The higher the energy is to displace a charge or reorient a dipole and make way for ion movement, the less probable it is that an ion will be able to permeate through the matter. In Fig. 3A a schematic representation of the energy profile of a lipid membrane is shown. The rise of the potential energy close to the water-membrane interface indicates the amount of energy needed to transfer the ion from the water into the hydrophobic and "apolar" core of the membrane formed by the fatty acids of the lipid molecules. This amount is large compared to the thermal energy of the ions. Therefore, a common property

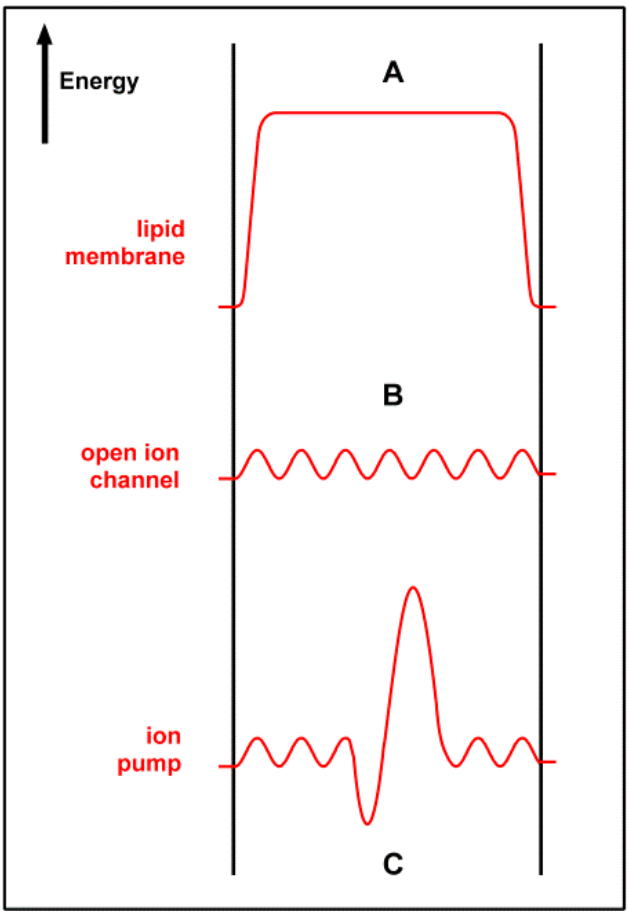


Figure 3. Schematic representation of potential energy profiles as detected by an ion along a pathway across a membrane in three different cases: (A) a simple lipid membrane, in which specific effects of the membrane-water interface are neglected, (B) an ideal ion channel without binding sites, and (C) an ion pump with an internal ion-binding site accessible from the left side and an energy barrier preventing propagation to the right-hand aqueous phase.

of all ion transporters in membranes has to be that they provide a pathway through the membrane that requires only a low amount of energy to be passed by an ion. This is accomplished by a pathway with a diameter that exceeds an ion-species dependent minimum and a lining of the pathway by molecules or parts of molecules such as amino-acid side chains that are easily polarizable (or "polar"). In Fig. 3B the energy profile of an ideal ion channel without ion-binding sites is depicted. Thus, the energy expenditure is low enough to allow an easy diffusion of ions along the pathway.

That can be implemented, as one extreme, by a wide, water-filled corridor or, as the other extreme, by a narrow channel lined with polar groups. An example for the latter is the gramicidin channel with a diameter of 4 Å in which carbonyl groups mimic the hydration shell which the passing monovalent cations have to leave behind for the most part at the entrance into the channel.[26] Numerous variations of channel shapes were found in between both extremes throughout the "channel kingdom". In the case of ion pumps, a pathway must exist that allows ion movement at low energy cost, however, it may not be continuous between both sides of the membrane because that would create a counterproductive bypass for ions. A promising proposal, the gated channel concept, which will be discussed in the subsequent chapter, is outlined in one of its states in Fig. 3C. Here an ion-binding site, indicated by a dimple in the energy profile, is accessible from the left side. A discharge of the ion from its binding site to the right side of the membrane is prevented by a high energy barrier.

The fact that a biological membrane consists primarily of a core of hydrophobic, apolar and insulating matter enclosed between conducting aqueous phases, allows the representation of a membrane as physical (plate) capacitor, which has in principle exactly this composition. Since the layer between both conductive plates of a capacitor is named "dielectric", the layer of the membrane formed by fatty acids is called membrane dielectric. It is characterized by a "dielectric constant", ε, that is low in the case of apolar matter (e.g. lipids, ε = 3-4) and high in polar phases (water, ε = 80). It controls the membrane capacity, $C = \varepsilon \cdot A/d$, where A is the membrane area and d its thickness. A fundamental consequence of this membrane property is that the transfer of an ion across the membrane is an "electrogenic" process.

Electrogenic transport is defined as the movement of electric charge through a medium with a low dielectric constant such as a biological membrane.[27,28] Electrogenic transport is characterized by two basic properties that were and are exploited constantly to study details of ion transport in the Na,K-ATPase and other ion transporters. The first impact of electrogenicity is that ion transport through the membrane dielectric produces an electric current and affects the electric membrane potential, V_m (Fig. 4). Therefore, electrogenic ion pumps act as current generators, and charge movements can be detected as current signals with an external measuring device.[27]

The second impact is that the activity of an electrogenic transporter is affected by the membrane potential. When charges are moved inside the membrane in the course of voltage-dependent reaction steps, they move 'uphill' (as in Fig. 4) or 'downhill' on the electric membrane potential, Δφ. This generates an additional energy term for the process, $\Delta E = \Delta q \cdot \Delta\varphi$, which in turn modifies the rate constant of this reaction step and can be detected as altered kinetic behavior. In consequence,

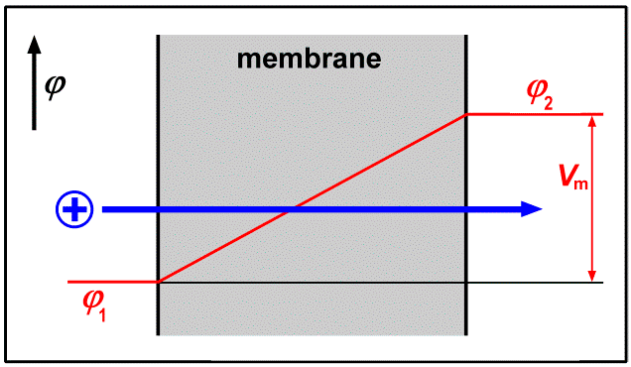


Figure 4. Schematic representation of electrogenic transport. The red line indicates the course of the electric membrane potential, here in case of a homogeneous membrane dielectric. The difference of the electric potentials on both sides, $\varphi_1 - \varphi_2$, is the membrane potential V_m. In cells it is always inside negative. According to basic principles of electrostatics, the movement of a charge, Δq, from one side of a capacitor to the other alters the electric potential difference, $\Delta V_m = C_m \cdot \Delta q$, proportionally to the membrane capacitance, C_m.

the externally measured pump current becomes voltage dependent.[29]

When the electrogenicity of an ion pump is investigated one may find an overall electrogenic behavior as in the case of the Na,K-ATPase or the sarcoplasmic reticulum (SR) Ca-ATPase, when after a complete pump cycle net charge is transferred across the membrane. In the case of the H,K-ATPase, two K^+ are exchanged against two H^+, therefore, no net charge is transferred after a pump cycle, the overall transport is electroneutral. But when the pump cycle is subdivided into single reaction steps, in some of these partial reactions charge is moved within the membrane dielectric, and these steps show electrogenic behavior.[30] The experimental concept to confine the activity of the Na,K-ATPase to specific partial reactions by appropriate experimental conditions, has turned out to be a powerful approach to identify electrogenic reaction steps in the pump cycle and to analyze their kinetic behavior.[12]

V. THE GATED CHANNEL CONCEPT

As indicated above, the initial ideas of the molecular mechanism of ion translocation across the membrane by ion pumps were influenced by the carrier concept[1] or a rocking mechanism.[2] In both cases ions bind in a first step to sites provided by the protein and then these sites, imbedding the ion in a cage, are moved through the membrane. Already in 1957 Clifford S. Patlak introduced another mechanistic proposal on a more general level, the "gate type non-carrier mechanism".[31] He assumed that the transporter had a substrate-chelating moiety that was not physically displaced during pumping. Initially, it was accessible only from one side of the membrane at a time (Fig. 5A). Then the transporter "closed" on the approachable side and "opened" subsequently on the opposite side, where the substrate was released. After the site is empty, the conformational arrangement is reversed and the transporter returned to its initial state.

In 1979 Peter Läuger published an enhancement of this concept as "channel mechanism for electrogenic ion pumps"[28] in which he assumed an ion channel traversing the membrane inside the transport protein with varying energy barriers that were able to separate the ion-binding site from the external aqueous phases. He applied this approach first to the light-driven proton pump bacteriorhodopsin. A few years later he introduced this concept to ATPases (Fig. 5B),[32] and used it to provide a detailed microscopic model to analyze the current-voltage behavior of the Na,K-ATPase.[27,33] In this concept the ion pump was represented by a channel which consisted of a sequence of shallow energy dimples and two barriers that were able to change their height when the pumps ran through its multiple conformational states (Fig. 5B). Those variable barriers correspond to the gates of the channel.

There is a variety of designs possible to construct the ion pathway in the gated channel concept. The ion-binding sites can be arranged asymmetrically, i.e. close to one interface of the ion pump with the aqueous outside, or symmetrically buried deep inside the hydrophobic core of the protein. The implications would be the existence of one or two access channel, respectively, through which the ions have to move. For the sake of the gating mechanism, which is needed on either side of the binding sites, these must not be located on the protein's surface. Access channels may, however, differ in their shape. Two principal cases have to be distinguished, a narrow, even ion-selective ion channel (or "ion well") in contrast to a wide funnel (or "vestibule") that is filled with water molecules and various ions. In case of a narrow channel, the ions moving through it may be partly stripped of their hydration shell and interact with the wall-forming amino-acid side chains. The diffusion of ions through this structure resembles the process taking place in a typical ion channel. An important feature in this case is that part of the transmembrane voltage drops along the length of the channel and this action would be electrogenic (see above).[27] In the case of a wide open vestibule ion movement, it occurs more or less as free diffusion in a solution, and correspondingly, the electric conductance in this environment is high. This fact entails that

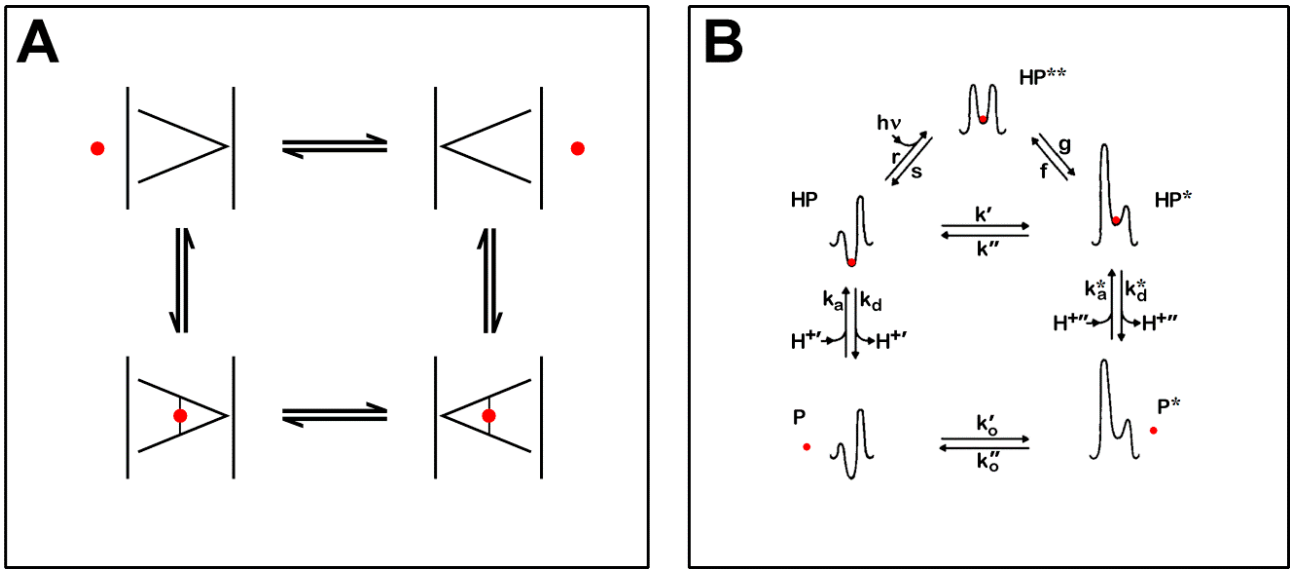


Figure 5. Original mechanistic concepts in which the ion-binding sites are not displaced during the transport process rather than the protein structure that controls access to these sites. **A:** The first proposal was the so-called gate type non-carrier mechanism by C.S. Patlak[31] in which two different conformational states of the protein generate alternatingly access to immobile ion sites from either side of the membrane. (Scheme adapted from Ref. 31). **B:** The second proposal is a channel mechanism in which ions diffuse through a low-resistance access channel (or 'ion well') to a binding site inside the membrane domain of the transporter, which is framed by mobile barriers on either side that control access from the outside. Here it is applied to the light-driven proton pump bacteriorhodopsin. In this representation the energy profiles of the access channels were omitted on both sides.(Scheme adapted from Ref. 32).

electric-field strength is low in such a vestibule, and no (significant) drop of the transmembrane voltage occurs. Correspondingly, both appearances were described in the literature as high-field and low-field access channels, respectively.

Based on the assumption that in the Na,K-ATPase the transported ions have to pass through access channels on either side of the ion-binding sites, the transfer of the ions from one aqueous phase to the other can be subdivided into at least four different reaction steps which are ion binding from one side, ion occlusion, deocclusion on the opposite side of the membrane, and ion release, as depicted in Fig. 6 for the Na^+-translocating half cycle. For K^+ transport a corresponding series of transport steps is valid.

In principle, all partial reactions indicated in color in the simplified Post-Albers cycle (Fig. 6A) may be accompanied by ion movements within (or through) the Na,K-ATPase. When, as in this case, a Na^+ ion is at the beginning in the cytoplasm, it resides at the electric potential, V_m, of the cell. At the end of the transport process, it is located outside the cell, where the level of the electric potential is 0 (per definition). Therefore, at each of the indicated reaction steps the ion may move through a fraction of the membrane potential, V_m. In the cartoon of Fig. 6B, these fractions of V_m were indicated by the parameters α', β', β", and α". For each ion that traverses V_m completely the condition, α' + β' + β" + α" = 1 must hold. These parameters were termed as "dielectric coefficients".[33] By determining their magnitude experimentally, important information can be achieved on the molecular mechanism of the ion transport. If the dielectric coefficient is zero, no charge is moved through the electric field within the membrane domain of the Na,K-ATPase. This has to be expected if the ion moves in a wide water-filled vestibule or if it is sterically fixed within the protein, e.g. in an immobile binding site. A high dielectric coefficient of a specific reaction step indicates a movement through a narrow channel. As will be shown later in detail, in case of the Na,K-ATPase the coefficients β' and β" were zero (or not significantly different from zero) which means that during enzyme phosphorylation and ion occlusion as well as during the conformation transition and ion deocclusion the ions were not shifted within the ion pump, their binding sites were sterically immobile.[34,35]

VI. EXPERIMENTAL APPROACHES

After the identification of the Na,K-ATPase the first functional study was restricted to monitoring of the

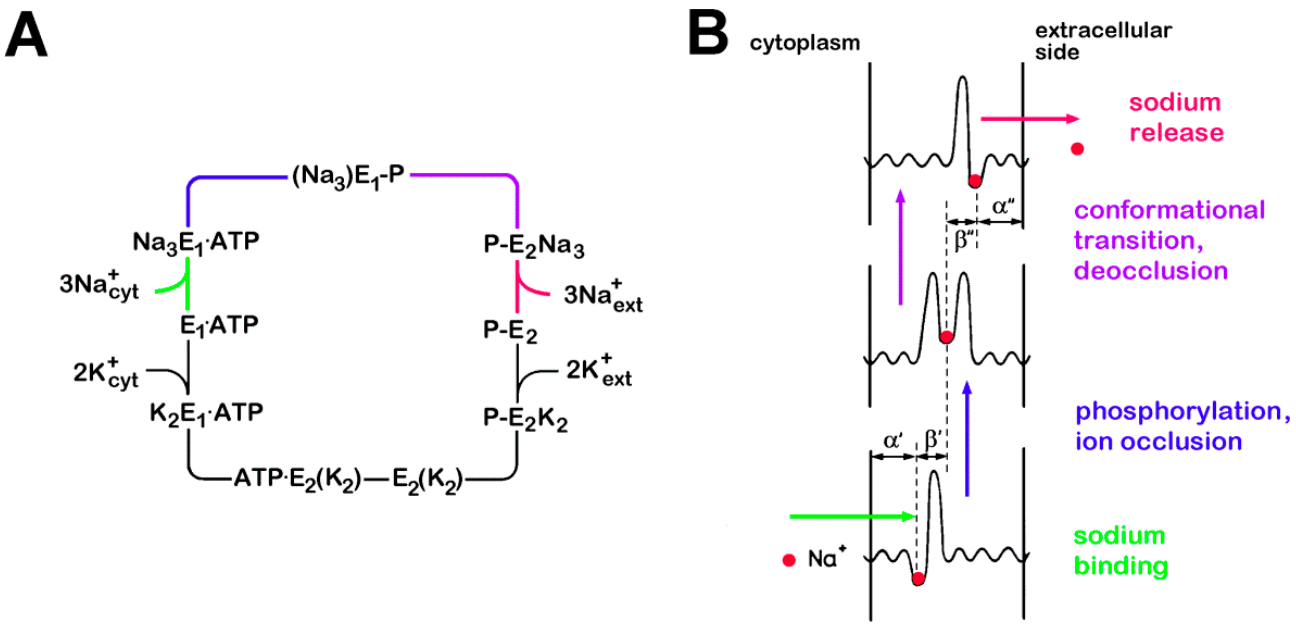


Figure 6. A: Post-Albers cycle of the Na,K-ATPase under physiological conditions. The Na^+-transporting half cycle is subdivided in four partial reactions, Na^+ binding (green), enzyme phosphorylation by ATP, occlusion of 3 Na^+, and release of ADP (blue), conformation transition, E_1-P to P-E_2, and deocclusion of the binding sites to the extracellular side (magenta), and release of the Na^+ ions (red). **B:** Sequence of schematic energy profiles as detected by the ions during Na^+-transport in three consecutive conformations as indicated in the pump cycle (**A**). The height of the energy barriers is schematic. This representation shall indicate that the high barriers are virtually impregnable for the ions with their available (thermal) energy. The quantities α', α", β', and β" are so-called dielectric coefficients that describe the fraction of the electric potential traversed by an ion in the respective reaction step (for details see text).

enzymatic activity because open membrane preparations from crab nerve were used, which did not separate both aqueous compartments that are needed to detect ion transport.[3] ATPase activity was measured as amount of inorganic phosphate released per volume of "enzyme solution." P_i was determined by the colorimetric method introduced by Fiske and Subbarow in 1925,[36] and in later years by variations derived therefrom.[37,38] When Post studied broken erythrocytes he calculated a specific enzyme activity "per mg dry weight".[39] After introduction of a method to isolate and purify active Na,K-ATPase from kidney medulla preparations by Peter Jørgensen in 1969,[40] two methods were used to determine the amount of the enzyme in an assay, a micro-Kjeldahl method that quantifies the nitrogen content in a solution,[41] and the Lowry method (1951)[42] which became the standard method of protein determination in the years following. In 1978 a modified assay was introduced by Markwell et al. that allowed protein determination also in membranes without prior solubilization of the membrane-bound proteins.[43] An elegant method to determine the ATPase activity was introduced by Schwartz and collaborators in 1971.[44] A coupled pyruvate kinase/lactate dehydrogenase assay allowed 'real time' monitoring of ATP consumption by the Na,K-ATPase (or other ATPases) in buffers within a reasonable range around physiological conditions, which was and is widely used. Post et al. published in 1965 a study with enzyme isolated from guinea-pig kidneys in which they used radioactive [γ-^{32}P]ATP as further technique to study enzyme phosphorylation and dephosphorylation.[45] Measuring bound and released radioactive P_i for a long time became the 'gold standard' to investigate enzyme phosphorylation and dephosphorylation. A thorough and comprehensive review on enzymatic properties of the Na,K-ATPase was published by Ian M. Glynn in 1985.[14]

The first ion-transport studies of the Na,K-ATPase were performed with intact erythrocytes by Post and Jolly in 1957 who measured changes of Na^+ and K^+ in the cells by flame photometry and determined a transport ratio of 2 K^+/3 Na^+ for the strophanthin sensitive flux.[46] This method, at that time was state of the art, but was improved about ten years later by Garrahan and Glynn who introduced the use of a radioactive sodium isotope, ^{24}Na, to measure Na^+ transport in red cells.[20] In 1970 Paul De Weer applied tracer ions in experiments with squid giant axons. He measured $^{22}Na^+$ and $^{24}Na^+$ fluxes, determined rate constants and studied substrate dependencies,[47] followed by the first direct measurement of the electrogenicity of the Na,K-ATPase in axons with

electrodes in 1973.[48] Rhoda Blostein introduced in 1976 the use of inverted erythrocytes[49] to combine [γ^{32}P]ATP phosphorylation studies with ^{22}Na and ^{42}K fluxes in order to identify the sidedness as well as interaction of fluxes and enzymatic activities.[50] Important contributions could be provided also by the use of human resealed red cell ghosts.[51-53] Another chapter on transport studies with cells was opened by David Gadsby in 1979 who used Purkinje fibers from dog hearts and performed voltage clamp experiments with a microelectrode set-up.[54] He determined strophanthidin sensitive outward currents through the membranes of these cells. A further development was the whole-cell patch clamp with isolated cells from guinea pig ventricles that allowed the determination of the current-voltage dependence to the Na,K-ATPase.[55] In 1994 Don Hilgemann supplemented the set of electrophysiological techniques by the giant membrane patch method.[56] Applied to guinea pig myocytes he obtained in kinetic experiments, a time resolution of 4 µs, and analyzed external Na^+ binding. Six years later electrophysiological equipment was even further developed so that high-speed voltage jump experiments could be performed with squid giant axons, and time-resolved Na^+ release in the P-E_2 conformation was measured at a 3 µs time resolution.[57]

A different electrophysiological approach was introduced in 1988 by Bob Rakowski and Cheryl Paxson.[58] They were able to measure the current-voltage dependence of the Na,K-ATPase in *Xenopus laevis* oocytes in a membrane potential range between -120 mV and +60 mV by a conventional two-microelectrode voltage-clamp circuit. These cells were of interest in several ways: In the maturation state, in which they typically were used, they had low passive membrane conductance and a significantly reduced set of ion transporters compared to other cells. They were easy to investigate with electrophysiological techniques, and most of all, they were very suitable for heterologous expressions of Na,K-ATPase mutants.[59] Since then this technique has been used in numerous projects such as to study the interaction mechanism with ouabain[59], the role of glycolysation[60] or the properties of disease-inducing mutations.[61]

When transport activity is tracked by ion fluxes and overall electrogenicity in cellular systems, other ion transporters present in the membrane have to be accounted for. This is typically achieved by performing two identical experiments successively, once in the absence and once in the presence of a saturating concentration of a Na,K-ATPase-specific inhibitor, mostly ouabain. The difference of both recorded currents (or fluxes) is the contribution of the Na,K-ATPase. In 1974 a new approach was introduced when Stanley Goldin and Siu Tong reconstituted purified Na,K-ATPase from canine kidney in lipid vesicles.[62] They demonstrated that it was possible to incorporate active ion pumps into the lipid membrane by a dialysis method, and at least a fraction of pumps was oriented in a way that they could be activated by externally added ATP. Active and passive fluxes could be monitored by the use of tracer ions, $^{22}Na^+$, $^{42}K^+$, and $^{36}Cl^-$. At almost the same time, Lowell E. Hokin and collaborators published a study on purified shark enzyme reconstituted in vesicles. They showed that ouabain inhibited the pump activity only from inside the vesicles when ATP was added on the outside and confirmed pump-mediated Na-Na exchange as it was found in erythrocytes.[63] Later on, Beatrice Anner and collaborators used reconstituted vesicles to measure ^{22}Na uptake and ^{86}Rb export (as congener of K^+, more suitable because of its appropriately longer radioactive half-life time), and determined a transport ratio of approximately 3 Na^+ against 2 Rb^+.[64] In 1980 Elisabeth Skriver and collaborators published an electron-microscopical study in which they reported vesicle diameters of 90 ± 20 nm with randomly oriented intramembranous particles which were assigned as Na,K-ATPase molecules.[65] A few years later, Bliss Forbush introduced a rapid sampling technique of tracer fluxes across vesicle membranes that allowed a determination of rate constants in the order of below 10 ms.[66] This approach was very successfully applied to analyze the kinetics of $^{86}Rb^+$ or $^{42}K^+$ release from the occluded E_2P conformation of the Na,K-ATPase in the presence of other substrates and inhibitors.[67,68] In 1985, an alternative method to the use of radioactive substrates was introduced by Apell and collaborators when the electrogenicity of the transport was exploited by a membrane-potential sensitive fluorescence dye, DiIC1(5), that was used together with valinomycin, to determine K^+ fluxes out of the vesicles upon pump activation by addition of ATP.[69] Two years later a further voltage sensitive fluorescence dye, oxonol VI, was introduced by Apell and Bersch which became a frequently used fluorescent probe to directly record the electrogenic pump activity of reconstituted Na,K-ATPase.[70] The detection mechanism was analyzed and it was shown that this technique may be used to measure a significant part of the current-voltage curve of the reconstituted ion pumps in a single experiment.[71]

A potent tool to gain access to details of the transport kinetics of the Na,K-ATPase was provided by Jack Kaplan and collaborators in 1978 when they introduced "caged ATP" that allowed triggering of the ATPase activity by production of an ATP-concentration jump.[72] Caged ATP is a photolabile 2-nitrobenzyl derivative of ATP that cannot be metabolized. By a short intensive UV flash in

the nano- to microsecond time range photolysis is activated and ATP released in millisecond time range.[73] Based on this concept of the synchronized activation of the Na,K-ATPase, Peter Läuger proposed an assay of adsorbing Na,K-ATPase-containing open "membrane fragments" onto an artificial lipid bilayer (BLM), creating a capacitive coupling between both membranes and then trigger the pumping process by a UV-flash induced release of ATP from its caged precursor in the buffer. Thus current transients may be detected in an external current pickup system by electrodes on both sides of the BLM. A first implication of this technique was published by Klaus Fendler and collaborators in 1985.[74] They verified that current transients could be recorded by this method and information on the kinetics of the Na^+ translocation through the Na,K-ATPase may be determined from the current transients. Two years later Borlinghaus et al. provided a detailed mechanistic analysis of the compound membrane system and the current transients activated by the ATP-concentration jumps.[34,75,76] They showed that enzyme phosphorylation, ion occlusion and the conformation transition, E_1-P to P-E_2, were not electrogenic. The time resolution of this technique was, however, limited by the photochemistry of ATP release with a pH-dependent limit of about 4 ms (at pH 7.2).[73] To overcome the pH-dependent limitations a modified caged ATP was introduced with a (pH-independent) ATP release rate of $>10^5$ s^{-1}, and it could be successfully applied to experiments with the Na,K-ATPase.[77,78] Since it turned out that in the pump cycle much faster reaction steps follow the rate-limiting conformation transition, a modified technique was developed that allowed the use of the compound membrane system to obtain kinetic parameters of those fast Na^+-moving reaction steps. In 1995, this charge-pulse technique was applied to measure the kinetics of Na^+ release in the E_2P conformation and the rate constants in the submillisecond time range could be determined.[35] Further modifications of the compound membrane techniques were used to determine Na^+ binding and release on the cytoplasmic side by monitoring membrane-capacitance changes,[79,80] and by correlation of capacity changes and RH421 fluorescence signals (see below).[81] The problem of the fragility of the BLM was circumvented by the introduction of so-called solid supported membranes onto which Na,K-ATPase containing membrane fragments were adsorbed. These very robust compound membranes allowed fast buffer exchange. The possibility to freely choose buffer compositions had the advantage that ion-concentration changes could be performed in both directions.[82-84]

Since 1976, fluorescence techniques have been introduced to gain detailed information on the kinetics of conformation transitions in the Na,K-ATPase. Steven Karlish established several approaches with different collaborators. In stopped-flow experiments he used intrinsic tryptophan fluorescence to monitor and analyze the rate of the conformational transition $E_2(K) \rightarrow E_1Na$ and its dependence on ATP.[85] Formycin triphosphate, a fluorescent analog of ATP, was used to detect binding and dissociation of the nucleotide at different states of the pump cycle and the substrate dependence of these reactions.[11,86,87] While the enzymatic activities of the Na,K-ATPase were not or not significantly affected by these two techniques, a third assay, labeling the enzyme with fluorescein[88] or with fluorescein isothiocyanate (FITC),[89] confined the possibilities to investigate functional properties significantly. Labeling of the enzyme with these fluorescent compounds occurred close or in the nucleotide binding site, therefore, ATPase activity, phosphorylation by ATP, and nucleotide binding were abolished, but phosphorylation from inorganic phosphate and K-phosphatase activity were only partially inactivated. Advantage of these fluorescent labels was that they reported transitions between the E_1 and E_2 conformation.[89-91] FITC-labeled enzymes showed high fluorescence levels in E_1 and lower in E_2. FITC was found to bind covalently to a specific lysine of the cytoplasmic domain related to ATP binding, and was correspondingly affected by ATP (if present).[92] The molecular mechanism of the conformational sensitivity was that fluorescein is a pH sensitive dye and conformation transitions of the Na,K-ATPase include spatial rearrangements of the N domain with its nucleotide binding site, and thus minor local pH changes in the binding-site environment modulated the detected fluorescence (Stürmer & Apell, unpublished data). In 1982 a different fluorescein derivative, 5'-isothiocyanate fluorescein (5-IAF), was introduced by Kapakos and Steinberg.[93] This dye bound covalently to Cys457 on the cytoplasmic surface of the protein without inhibiting the enzyme activity.[94] It was used to study conformational changes, especially, $E_2 \rightleftarrows E_1$,[95] as well as Na^+-binding and ATP-induced partial reactions.[12,96]

Another conformation-sensitive fluorescent dye was eosin, whose application was introduced by Skou and Esmann in 1981.[97] They demonstrated that eosin bound reversibly to the Na,K-ATPase, with low affinity in the presence of K^+ and showed the same fluorescence emission as the free form in solution. In the presence of Na^+ it bound with high affinity and exhibited enhanced fluorescence. Its competition with ATP indicated that it bound to the ATP site. Eosin was used to monitor changes of enzyme conformations in the presence of a wide variety of substrate conditions.[97-99]

In the 1980s electrochromic styryl dyes[100] were introduced to gain further access to details of the ion-transport mechanism of the Na,K-ATPase. Originally these dyes were used to detect changes of the membrane potential of nerve cells in brain tissue.[101] The extremely hydrophobic compounds insert into the lipid phase of membranes, parallel to the fatty acids with their polar head facing the interface to the water phase. Because of an electrochromic effect their fluorescence properties change with the electric field in the hydrophobic part of the membrane. Due to this mechanism the response time upon changes of electric field was µs or faster. In 1988 Klodos and Forbush applied the dye RH160 to Na,K-ATPase containing membrane fragments and detected fluorescence changes upon addition of various substrates, although no transmembrane voltage was able to build up across the open membrane fragments.[102] They could not discriminate whether the detected response was caused by changes of local electric fields or due to interaction between dye and protein. The Läuger lab started in 1989 comparable studies with the dye RH421. They provided evidence that local electric fields induced by ions within the membrane domain of the Na,K-ATPase were the predominant cause of the observed fluorescence changes and presented a detection mechanism that correlated ion movement into and out of the membrane domain of the ion pump with the fluorescence changes.[103,104] The advantage of an easy application of styryl dyes with ion pumps in membrane fragments led to a frequent use of this technique by several groups.[105-110] It allowed the investigation of electrogenic and rate-limiting reaction steps around the Post-Albers cycle. The initial limitation that this technique required membrane fragments with a high density of ion pumps was conquered by its adaption to single Na,K-ATPase complexes solubilized in lipid/detergent micelles. This allowed an extension of its use even to recombinant Na,K-ATPase expressed in yeast which could be solubilized only as single enzyme molecules and not be isolated in form of purified membrane fragments.[111]

VII. ENZYME FUNCTION

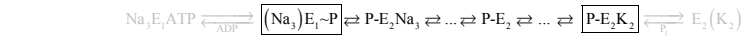
As mentioned above, ATP hydrolysis was the very feature to identify the enzyme.[3] It was found that Na^+, K^+, and Mg^{2+} were essential cofactors to control the enzyme activity and assumed that it was involved in the active extrusion of Na^+ from the cell. [3] Three years later, in 1960, an ADP-ATP exchange was detected with ^{32}P-labeled ADP, which was phosphorylated by an enzyme that was phosphorylated beforehand. The (β-^{32}P) labeled ATP was formed although no adenylate kinase was present.[112] Since 1962 it was known that the γ-phosphate of ATP forms an acid-stable phosphoenzyme.[113] In 1963 it was shown by the use of ^{32}P-labeled ATP that the enzyme is phosphorylated in the presence of Na^+, and dephosphorylated subsequently by addition of K^+, the latter reaction was inhibited by the presence of ouabain.[114] In 1965, it was demonstrated that an acyl phosphate was formed by ^{32}P-labeled ATP.[115,116] At the same time 'E1P' and 'E2P' were discussed as different conformations with respect to dephosphorylation,[7,117,118] as well as their decomposition by K^+,[45,115,119] or ADP.[120,121] In 1967 Garrahan and Glyn reported for the first time a backward running enzyme by formation of ATP from ADP and inorganic phosphate.[15] Three years later it was shown that the MgATP complex was the effective compound needed for enzyme activity,[122] although free ATP could bind and subsequent addition of Mg^{2+} was able to start the reaction.[9] In 1972 it was found that ATP accelerated at high concentrations (> 400 µM) the conformation transition $E_2K \rightarrow E_1K$ in a non-phosphorylating fashion.[8] In 1973 it could be shown that an aspartyl side chain was phosphorylated by ATP.[123,124] It lasted until 1985, when the first amino-acid sequence became available, that the phosphorylated aspartate was identified in the large cytoplasmic loop between the fourth and fifth transmembrane helix.[125] This aspartate formed together with the next three amino acids a characteristic motif, Asp-Lys-Thr-Gly, that turned out to be invariant in the phosphorylation site of all P-type ATPases.[126]

The striking observation that the phosphorylated enzyme could be dephosphorylated by ADP or K^+ attracted a lot of interest and was considered to be a useful property to gain more insight into the molecular mechanism of the enzymatic machinery. The first concept to describe the enzymatic behavior was that of a two-pool model of the phosphorylated enzyme, E_1~P and E_2P. The first pool was filled upon binding of cytoplasmic Na^+ and phosphorylation from ATP. Enzyme in this pool had the Na^+ ions occluded[127,128] and could be dephosphorylated by ADP ("ADP-sensitive EP"). This pool was discharged by spontaneous conformation transition into the second pool, in which the ion-binding sites were extracellularly accessible. Therefore, Na^+ was released, and subsequently the enzyme could be dephosphorylated upon addition of K^+ ("K-sensitive EP"). The experimentally observed bi-phasic time course of the dephosphorylation of both pools and its dependence of the Na^+ concentration could, however, not be explained by this simple model.[129] In particular, experiments showing that the amount of enzyme dephosphorylat-

ed by either ADP or K^+ together was larger than 100% made the two-pool model obsolete.

The consequence was the introduction of an extended concept, a three-pool model that included an additional pool intercalated between E_1~P and E_2P.[130] The additional pool was thought to be drained via both dephosphorylation modes, by K^+ and ADP. Extensive experiments and discussion of Na^+, K^+ and ADP dependences as well as the sizes of the proposed three pools under various substrate conditions led to different concepts according to whether the pool 'in the middle' might "effectively be both ADP- and K-sensitive"[131] or might be "not sensitive to both ADP and K^+ but has to be converted to E_a~P, the first pool, which is E_1-P."[132]

When in retrospect the concept of enzyme dephosphorylation is revisited in terms of the detailed Post-Albers pump cycle available nowadays, the (linear) sequence of phosphorylated states of the Na,K-ATPase consists of (derived from Fig. 2B):



Only the (boxed) first and last state in the respective reaction sequence may be dephosphorylated directly. All states in between are inert to dephosphorylation. They are E_2-P states in which the ion-binding sites are accessible from the extracellular side of the membrane and are able to bind or release ions and are occupied by 1 to 3 Na^+, by 1 K^+ or no ion. The occupancy of these states rapidly achieves a steady state distribution in a diffusion controlled manner, depending on the current ion concentrations in the extracellular medium. The low Na^+ affinity of the ion-binding sites in the P-E_2 conformation and high affinity for K^+ leads under physiological conditions preferentially to dephosphorylation upon binding of a second K^+, P-E_2K_2 → $E_2(K_2)$ + P_i. Only at high Na^+ concentrations and very low (or no) K^+ a considerable amount of enzyme will undergo the (backward) conformation transition, P-E_2 Na_3 → $(Na_3)E_1$~P, and the resulting state can be dephosphorylated in the presence of ADP, $(Na_3)E_1$~P + ADP → Na_3E_1ATP.[53,133] This approach to a mechanistic description makes the introduction of pools of phosphorylated states unnecessary to explain the various dephosphorylation experiments.

In 1965 Post and Sen showed that it was possible to produce a K^+ influx into cells in the absence of ATP but in the presence of Mg^{2+} and inorganic phosphate[6] which indicated binding of P_i to the unphosphorylated enzyme, a reaction step later called 'backdoor phosphorylation'. Only two years later Glynn and Garrahan demonstrated that the thermodynamic requirement that the enzyme runs backwards could be fulfilled experimentally under appropriate substrate condition.[15] In the presence of K^+, Mg^{2+} and P_i, addition of ouabain induced rapid backdoor phosphorylation.[134] In the absence of ouabain less steady-state phosphorylation was obtained because of K^+ promoted dephosphorylation.[135] The identical proteolytic digestion pattern obtained from the P_i-induced and ATP-induced phosphoenzyme was understood as strong indication that both phosphoenzymes were the same.[136,137]

Another interesting question was the nucleotide specificity of the enzyme. In 1968 Matsui and Schwartz studied the effect of nucleotides other than ATP, namely CTP, ITP, GTP, UTP.[138] Subsequently, their dissociation constants were determined.[9] After the method was introduced to reconstitute enzyme functionally in lipid vesicles, active transport of Na^+ and K^+ energized by CTP (almost as effective as ATP) und UTP (relatively ineffective) was reported.[63] From the Na^+ transport with various nucleotides and two synthetic ATP analogs a correlation was found between the proton-accepting properties of the nucleotides and their ability to provide active transport.[139]

Considerable attention was paid to the role of Mg^{2+} for the enzyme activity after it was noticed from the very beginning that this ion was indispensable for function.[13,112] Detailed studies revealed that the MgATP complex was the activating substrate of the Na,K-ATPase.[122,140] This is not really surprising since physicochemical investigations yielded equilibrium dissociation constant of the MgATP complex in a pH-dependent manner between 1.5 μM (pH 8) and 10 μM (pH 6)[141], while the free Mg^{2+} concentration in cells typically is in the order of 200 μM[142]. This implies that under physiological conditions more than 95% of total ATP is present as Mg complex. Free Mg^{2+} was reported to bind to a low-affinity site where it caused inhibition of the enzyme activity.[143] It was shown that Mg^{2+} is released from the enzyme only after its dephosphorylation in the E_2 conformation.[144] Therefore, it could be considered to be a product inhibitor when high Mg^{2+} concentrations in the buffer impeded dissociation from its site and thus affected the E_2 → E_1 transition.[145] This concept was confirmed by Forbush[67,68] and complemented by the proposal that only one site for Mg^{2+} per enzyme was required for both phosphorylation by ATP and enzyme inhibition by stabilizing the E_2 conformation. Binding of ATP at the low-affinity site in E_2 promoted Mg^{2+} release and the site was reoccupied only after enzyme phosphorylation in E_1 by MgATP. In 2000, a conserved segment in the P domain of the α subunit was identified in which Asp710 contributed to the coordination of Mg^{2+}.[146]

Another tool to enlarge the insight into enzyme functions were various inhibitors that allowed the arrest

of the Na,K-ATPase in defined states or a restriction of possible reaction sequences to specific parts of the pump cycle. A review presenting a comprehensive survey was published by Glynn in 1985.[14] The most important group of inhibitors is that of cardiac steroids. Compounds in which a sugar is attached to the steroid are so-called cardiac glycosides (CGs), of which the most prominent is ouabain. Although it was clear that CGs interact with the extracellular side of the Na,K-ATPase, the molecular mechanism of inhibition was unknown until the 1990s. With the progress of molecular-biological methods mutagenesis of numerous (and 'suspect') amino acids was performed and the effect of the mutations and their resistance against different CGs was investigated to identify the binding site of the inhibitor.[147,148] Crucial amino acids were found in transmembrane and extracellular domains. At the same time, in 1996, functional studies revealed that K^+ accelerated enzyme dephosphorylation and thus antagonized ouabain binding. In the presence of high concentrations of ouabain (in the mM range), however, ouabain was able to bind even when 2 Rb^+ (as congeners of K^+) were bound, E_2Rb_2, and the inhibitor stabilized this state.[149] Major progress in mechanistic understanding was made when detailed structural information became available. In 2009, a first crystal structure of Na,K-ATPase at 2.8 Å resolution was published with a low-affinity bound ouabain in a state analogous to P-E_2K_2.[150] Ouabain was deeply inserted into the transmembrane domain with the lactone ring close to both K^+ ions bound to their sites. Most of the mutagenesis data, obtained with high-affinity bound ouabain, could be explained by this arrangement, which suggested that the CG binding site should be essentially the same in both conditions. Two and then four years later structures with high-affinity bound ouabain became available with a resolution of 4.6 Å and 3.4 Å.[151,152] These structures made visible that ouabain was bound to a site in the α subunit formed by transmembrane segments M1 to M6 and thus blocked the ion pathway from the extracellular side to the ion-binding sites. In the structure with the higher resolution it was found that a Mg^{2+} ion was present in the cation transport site II when ouabain was bound with high-affinity. Comparison of the position of ouabain in the low- and high-affinity bound state showed that both were indeed mostly not significantly different. Prominent was only a difference in the location of the lactone ring of the inhibitor in the Mg^{2+}-bound and K^+-occluded condition. Altogether, functional and structural findings allowed a consistent explanation of the inhibition mechanism by preventing the conformation transition from E_2 to E_1 while clogging the extracellular access channel of the Na,K-ATPase. The well-known antagonistic effect of K^+ on ouabain (or any other cardiac glycoside) binding could be attributed to K^+-induced low-affinity ouabain binding.[152]

When the effect of CGs was studied with enzyme from many animals, typical binding affinities of the Na,K-ATPase were found in the range of µM.[153] Such a high affinity raised the question of why the enzyme should have evolved such a specific binding site for an exogenous compound and whether there were endogenous inhibitors aimed at this site. John M. Hamlyn and collaborators reported in 1982 the existence of a circulating inhibitor of the Na,K-ATPase,[154] in 1991 they identified a ouabain-like factor,[155] in 1999 it was confirmed that it was ouabain,[156] and in 2000 Wilhelm Schoner introduced ouabain as new steroid hormone.[157] In a recent review the story of discovery, advances and controversies of endogenous ouabain was published.[158]

Ouabain also plays a role as a signal messenger. Regulatory effects of the Na,K-ATPase inhibition by ouabain were initially assigned to changes in intracellular Na^+ and K^+ concentrations.[159] From research in cardiac hypertrophy crucial information was collected over a couple of years and it was established that ouabain stimulated myocyte growth and protein synthesis, comprised the induction of a number of early response proto-oncogenes and activated transcription factors already at low, nontoxic concentrations. Finally, experimental observations were published that ouabain binding activated signaling cascades.[160] The ouabain-stimulated signal transduction was mediated by the Na,K-ATPase but was apparently independent of ion transport function.[161] The signaling function of Na/K-ATPase controlled by CGs has been gradually appreciated over the last 20 years as can be followed in several reviews.[161-164]

Another potent inhibitor of the Na,K-ATPase (and all other P-type ATPases) is orthovanadate, VO_4^{3-}. It was identified 1977 by Lewis C. Cantley and collaborators as a potent inhibitor of the sodium pump with a K_I of 40 nM. Inhibition was reversed to 100% by millimolar additions of norepinephrine. Vanadate was initially found as contamination in commercial "Sigma grade" ATP, isolated from horse muscle. From its tetrahedral structure it was concluded that it may bind to "a phosphate site".[165] From their study of interaction with the Na,K-ATPase Cantley concluded that "the unusually high affinity for vanadate is due to its ability to form trigonal bipyramidal structure analogous to the transition state for phosphate hydrolysis."[166] Mg^{2+} was required as cofactor for inhibition. Its inhibitory action can be attributed to its high-affinity binding to the phosphate binding site, a condition in which it stabilizes the E_2 conformation of the enzyme.

One further inhibitor should be mentioned in the framework of this presentation, oligomycin, which was found to inhibit the mitochondrial ATP synthase. It is an antibiotic originally isolated from *Streptomyces*. In 1962, first reports were published that this macrolide also inhibited the Na,K-ATPase.[167,168] It was demonstrated that oligomycin is a potent inhibitor, however, it did not inhibit the enzyme completely, e.g. ADP-ATP exchange was unaffected. Eventually, all experimental findings were explained by the mechanistic concept that oligomycin blocked the conformation transition E_1-P → P-E_2.[14,169] This was supported by findings that the inhibitor shifted the equilibrium from a Na^+-deoccluded form to a Na^+-occluded form,[128] and stabilized Na^+ occlusion but not K^+ occlusion.[170] In 2013 a structure of the Na,K-ATPase complex with 3 Na^+ and an oligomycin molecule was published at a resolution of 2.8 Å.[171] Therein the inhibitor was bound close to helix 1' on the cytoplasmic side adjacent to the membrane surface.

VIII. TRANSPORT FUNCTION

As can be seen from Figure 2, ion transport is a complex process even when restricted to the pump cycle under physiological conditions. By means of experimental studies at least ten reaction steps were identified that form the complete pump cycle and embrace the interplay of enzyme and transport functions (Fig. 2A). In the following, the focus will be set on molecular processes investigated in experimental studies to enlighten the transport mechanism of both ion species across the membrane. (An earlier review was published in 2004.[172]) To discuss the transport function of the Na,K-ATPase in detail, the pump cycle will be divided into four partial reactions: (1) Cytoplasmic ion exchange, (2) access transfer from the cytoplasmic to the extracellular side, (3) extracellular ion exchange, and (4) access transfer from the extracellular to the cytoplasmic side.

(1) $K_2E_1{\cdot}ATP \rightleftarrows E_1{\cdot}ATP \rightleftarrows Na_3E_1{\cdot}ATP$

The cytoplasmic ion exchange occurs in the E_1 conformation of the Na,K-ATPase. Under physiological conditions, in which the cytoplasmic K^+ concentration is high, Na^+ low, and the binding affinity for K^+ is higher than for Na^+, a significant fraction of the enzyme is found in the $K_2E_1{\cdot}ATP$ state.[173] Consequently, under steady-state conditions only a small fraction of the Na,K-ATPase populates $Na_3E_1{\cdot}ATP$, the state which is the one capable of being phosphorylated. Despite this unfavorable displacement of the occupation of the states in this reaction sequence, the pump is obviously able to perform its task of extruding Na^+ from the cytoplasm. This fact has to be assigned to the finding that the exchange of ions between binding sites and aqueous bulk phase is fast (and for the most part diffusion controlled) compared to the subsequent phosphorylation step so that a quasi-equilibrium distribution between the differently occupied states may be assumed. Therefore, the drain of the $Na_3E_1{\cdot}ATP$ state by the phosphorylation step is instantaneously compensated. It is known, however, that the Na,K-ATPase runs way below their kinetically possible maximum turnover due to this limiting condition.

When initial studies of ion binding and release in the E_1 conformation were performed, it was observed that K^+ release and binding of the first two Na^+ ions were not electrogenic.[174,175] This observation was interpreted as indication that both binding sites were negatively charged and located close to the cytoplasmic surface in a wide, water filled vestibule. This electroneutrality turned out to be, however, only an apparent effect[176]. In the E_1 conformation, the affinity of both binding sites for protons is so high that at physiological pH and in the absence of Na^+ and K^+ the sites are mostly protonated. Therefore, binding of both K^+ ions and of the first two Na^+ ions in titration experiments was effectively an electroneutral exchange against bound protons. At unphysiologically high pH, the electrogenicity of K^+ and Na^+ binding became very well visible in titration experiments when beginning with a cation-free electrolyte[176].

While K^+ and the first two Na^+ ions compete for the same sites in the pump, binding of the third Na^+ occurs to an exclusively Na^+-specific site in the E_1 state. This process was found to be electrogenic and the dielectric coefficient was shown to be in the order of 0.25.[12,35,83,177] It was demonstrated that electrogenic binding of the third Na^+ could be detected by styryl dye RH421 and simultaneously by a directly measured charge movement with identical results.[81] That means that Na^+ traverses 25% of the electric-potential drop across membrane to reach its binding site from the cytoplasm. (Which does not necessarily imply that the spatial distance is also 25 % of the membrane thickness.[27]) A study of cytoplasmic Na^+ binding and detailed analysis of the binding affinities revealed that the third Na^+ binds to a site with a higher affinity for Na^+ than the second site.[173] Such an observation could be explained by the assumption that the third binding site became available only after the first two sites were already occupied by Na^+. A possible mechanism was a conformational rearrangement in the transmembrane helices of the membrane domain upon binding of the second Na^+ which then assembled the third Na^+ site or opened access to it. Furthermore, bind-

ing of the third Na^+ was monitored also with a fluorescence change of the conformation-sensitive label FITC, which was linked to a highly conserved lysine in the nucleotide-binding site at the cytoplasmic N domain.[175] The accordance of results from the FITC and RH421 experiments was a strong indication that binding of the third Na^+ and conformational rearrangement at the cytoplasmic N domain of the protein were concurrent events.

In the composition of a mechanistic concept it may be concluded that binding of only the third sodium triggers a rearrangement of the cytoplasmic N domain with an appropriately bound MgATP, and thus arms the protein to make way for enzyme phosphorylation at the specific aspartate in the cytoplasmic P domain. Such a mechanism would also be in agreement with the finding that binding of the third Na^+ needed significantly higher activation energy (63.4 kJ/mol) than binding of the first two Na^+.[178] An activation energy of such a high magnitude points as well to a conformational rearrangement related to binding/release of the third Na^+.

An alternative proposal was suggested by Kanai et al., based on their structure of the Na,K-ATPase in an E_1 conformation with 3 Na^+ occluded.[171] Their concept was that the first Na^+ is bound to the Na^+-specific site III followed by occupation of sites I (in the middle) and II (outermost). They assumed from their crystal structure, which represented a Na^+-occluded phosphorylated intermediate, that there is just one access pathway to all three sites and the "innermost" site III can be reached only through (empty) sites I and II. If so, a possible reconciliation with the results from functional studies could be a single-file push-on mechanism in which sites I and II are occupied first, but with the arrival of a third Na^+ both ions in sites I and II are moved forward into sites III and I to make way for binding of the third ion into site II. To clarify the actual mechanism a crystal structure of the pump in the antecedent, non-occluded (and preferably only partly occupied) state would be very helpful.

(2) $Na_3E_1{\cdot}ATP \rightleftarrows (Na_3)E_1\text{-}P \rightleftarrows P\text{-}E_2Na_3$

When all three ion sites are occupied by Na^+ and MgATP is bound to the nucleotide binding site, the pump is able to perform auto-phosphorylation associated with a simultaneous occlusion of the ions. During this reaction step no charge movements were detected.[34] This observation indicated that the three ions in their binding sites are not displaced (at least not perpendicular to the membrane plane). This behavior is in agreement with the observation that the ion-binding sites of the closely related SR Ca-ATPase also are not significantly relocated throughout the complete pump cycle as can be established by comparison of the crystal structures obtained in numerous different states of the pump.[179] In the absence of oligomycin the gained occluded state, $(Na_3)E_1\text{-}P$, is transient and followed by a spontaneous transition to the $P\text{-}E_2$ conformation with deoccluded ion binding sites. This step is the rate-limiting process in the Na^+-translocating half cycle,[12,35,57,78] with a rate constant of about 22 s^{-1} at 20 °C, and has the highest activation energy of all reactions of the pump cycle. With purified membrane preparations from rabbit kidney a value in the order of 115 kJ/mol was determined.[180]

The conformation transition showed only a minor dielectric coefficient (0 – 0.1),[35,57] and it could not be determined whether this was caused by ion movements or (more probably) movements of charged side chains in the helices of the membrane domain which underwent considerable reorientations during the transition. Besides unclasping the access channel between binding sites and extracellular aqueous phase, another major functional consequence of the transition was the reduction of the binding affinity for Na^+ by a factor of about 500.[12] This dramatic change was caused by minor movements of transmembrane helices which in turn modified the coordinating interactions between amino-acid sidechains and the Na^+ ions.

(3) $P\text{-}E_2Na_3 \rightleftarrows P\text{-}E_2 \rightleftarrows P\text{-}E_2K_2$

The extracellular sodium release is the best investigated partial reaction of the Na,K-ATPase pump cycle.[12,35,56,57,181-183] Release of the three Na^+ occurred sequentially and with different kinetic and electrogenic properties, which allowed an assignment to the respective reaction steps. The first Na^+ released had the highest dielectric coefficient of the whole pump cycle. The ion traversed 65 – 70 % of the electric potential in the membrane, and its release process had the lowest rate constant of the three ions in the order of 1000 s^{-1} at 20 °C.[35,57,183] The activation energy of this partial reaction was found to be about 80 kJ/mol.[184] Dissociation of ions from a binding site and diffusion through a narrow pore-like structure typically would have activation energies below 20 kJ/mol. The observed high activation energy was, therefore, an indication of a conformational rearrangement of the participating membrane domain. The commonly accepted release mechanism is an initial rate-limiting deocclusion process for the first ion as associated consequence of the conformation transition from $E_1\text{-}P$ to $P\text{-}E_2$. The high dielectric coefficient of the first Na^+ released has to be explained by an ion

migration through a narrow access channel. Thereafter, another conformational relaxation was required before the second and third Na^+ ion exited the membrane domain, since the electrogenicity of these reaction steps was found to be just 10 – 20 % even though the distance between the ion binding sites was small. Because it is expected that the binding sites are not (significantly) dislocated during this partial reaction, a rearrangement of the α helices in the protein's membrane domain has to take place in a way that they form a wide access structure being filled with water molecules, as was proposed.[35,185,186] Such a vestibule would remodel the dielectric shape of the protein so that the bound ions would be able to reach the polar aqueous phase within a short "dielectric" distance of < 20 %.[35,57] The existence of such a structural rearrangement was supported by the high activation energy of 70 kJ/mol that was determined for this partial reaction.[57] The subsequent release of the second Na^+ was found to be fast with a rate constant in the order of 10,000 s^{-1}.[57] Thereafter, the release of the last Na^+ occurred with a similarly low dielectric coefficient, and with a rate so fast ($\geq 10^6$ s^{-1})[57] that it could not be resolved with the experimental techniques available.

In the resulting P-E_2 state the ion-binding sites had a significantly lower binding affinity for protons than in the E_1 conformation[176] and were virtually empty at low Na^+ concentrations and in the absence of K^+. The following K^+ binding and transport into the cell have been studied extensively.[12,187-191] Sequential binding of K^+ (or its congener Rb^+) was resolved and a mechanism described as "flickering gate model" was introduced which implied that the first K^+ is slowly bound (or released) while the second K^+ bound was able to exchange fast with the aqueous phase[187]. The equilibrium dissociation constants for the first and second K^+ differed by a factor of 5 to 6 at a level in the sub-millimolar range. Besides K^+, congeneric monovalent cations were also able to be transported, such as Rb^+, Cs^+, Tl^+, NH_4^+, H^+ or even Na^+. Quaternary organic amines, which are large monovalent cations of different size, were used to probe the extracellular access channel and, in addition, they could be used as inhibitors of the Na,K-ATPase.[186,192-195]

(4) P-E_2K_2 ⇄ *ATP*·$E_2(K_2)$ ⇄ K_2E_1·*ATP*

While K^+ binding (or release) on either side of the membrane was electrogenic, occlusion, conformation transition and deocclusion on the opposite side of the membrane were electrically silent.[104,196,197] Release of K^+ to the cytoplasmic aqueous phase was actually a K^+/Na^+ exchange or a K^+/H^+ exchange in the absence of Na^+ (see above), and therefore, also only apparently electroneutral.[176]

When the second K^+ ion was embedded in its binding site in the P-E_2 state, a spontaneous conformational rearrangement occurred that caused ion occlusion and dephosphorylation of the enzyme, resulting in state $E_2(K_2)$.[187] The available experimental evidence indicated that dephosphorylation and occlusion go hand in hand. In 1988, the rate constant could only be estimated, and Forbush reported that it has to be much larger than 100 s^{-1}.[198] A few years later fits to kinetic data led to values of $>10^3$ s^{-1} (all at room temperature).[12] For the reverse reaction, the backdoor phosphorylation, rate constants of $> 10^5$ $M^{-1}s^{-1}$ were determined.[68,199] Under physiological conditions, the dephosphorylated $E_2(K_2)$ state was only transient. From there, the pump cycle could advance in two different ways, depending on the ATP concentration present (Fig. 2).

At physiological ATP concentrations ("high ATP"), low-affinity binding of the nucleotide occurred,[8] and the resulting state, *ATP*·$E_2(K_2)$, underwent an accelerated transition to the E_1 conformation, K_2E_1·*ATP*. Rate constants of about 60 s^{-1} were obtained for this reaction step at saturating [ATP] and room temperature.[200,201] Nevertheless, this reaction step was rate-limiting in the K^+-transporting half cycle. In analogy to the E_1/E_2 transition, it was linked up with a deocclusion of the ion sites, then accessible from the cytoplasmic side. In the presence of low ATP (< 100 nM), the occluded $E_2(K_2)$ state was able also to perform a transition to the E_1 conformation, $E_2(K_2) \rightarrow K_2E_1$, with a dramatically lower rate constant of < 0.3 s^{-1} at room temperature.[67,201] The rate constant of the reverse step was much larger (290 s^{-1})[89] so that in the absence of ATP and presence of K^+ the equilibrium of both conformational states was strongly shifted to the E_2 conformation when two ions occluded.

Summarizing all these findings, the pump mechanism can be represented schematically by the cartoon shown in Figure 7. It is based on the gated channel concept, in which the ion sites are embedded deep inside the membrane domain of the Na,K-ATPase. The ion sites are accessible only from one side at the same time. The observation that electrogenic ion movements were found only in one of both access channels at the same time may be interpreted as indication that the respective other channel is completely blocked. Only reaction steps in which ions are taken up from the aqueous phase or released from their binding sites to the outside of the protein are electrogenic and produce a detectable electric signal. Ion movements in the access channels are diffusion controlled, which leads to the consequence that under physiological conditions the exchange of both

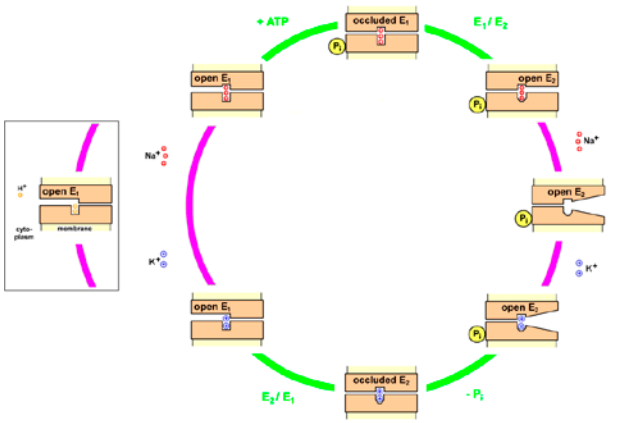


Figure 7. Schematic representation of the functional behavior of the Na,K-ATPase membrane domain (the cytoplasmic domain is omitted for clarity) in characteristic states of the pump cycle. The ion binding sites are located almost in the center of the membrane domain and are accessible from the cytoplasm in the E_1 conformation and from the extracellular aqueous phase in P-E_2. The open states in both conformations are separated by occluded states in which no ions may move within the access channels. In the P-E_2 conformation the initially narrow access channel widens up after release of the first Na^+. The pink fractions of the cycle indicate electrogenic (ion uptake and release), the green ones represent electroneutral processes (phosphorylation/dephosphorylation, conformation transition). While in the P-E_2 conformation the ion-binding sites may be empty, in the E_1 conformation the binding sites are occupied by protons in the absence of other monovalent cations (due to the high affinity for protons in E_1) as insinuated by the inset.

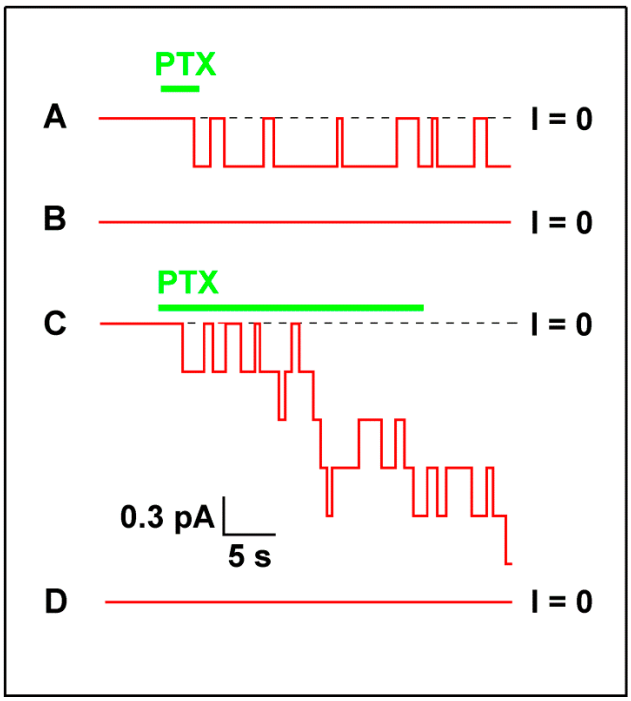


Figure 8. Schematically represented palytoxin (PTX) induced ion-channel behaviour of the Na,K-ATPase. **A.** In the presence of Na^+ and Mg·ATP and 25 pM PTX for a few seconds typical opening and closing of a single cation-selective channel were observed. **B.** After washout of PTX no longer channel events could be recorded and ion-pump activity was restored. **C.** Upon prolonged exposure to 25 pM PTX more and more Na,K-ATPase molecules were transformed into ion channels. **D.** In the presence of high concentrations of ouabain channel activity was completely suppressed. Figure adapted from Artigas & Gadsby[209].

K^+ against (the first) two Na^+ and correspondingly the reverse reaction occur so fast that the respective electric current contributions cancel each other. Therefore, it was assumed for some time that K^+ transport by the Na,K-ATPase was electroneutral until experiments were performed under K^+-limiting conditions.[176,188,191]

In the open P-E_2 conformation two different conformational arrangements of the access were found. Initially after the transition from E_1 a narrow channel with high electrogenicity was formed, and after the release of the first Na^+ a wide water-filled funnel developed and induced low electrogenicity. In the E_1 conformation, no such significant changes were found. The question whether the third Na^+ that binds to the Na^+-specific site, enters through an access different from that of the first two Na^+ or both K^+ ions could not be answered so far.

IX. TRANSMEMBRANE CHANNEL FORMATION

The gated channel concept received convincing experimental support when the molecular mechanism of the interaction of palytoxin with the Na,K-ATPase was investigated. Palytoxin is a lethal marine toxin extracted from polyps of the genus Palythoa.[202] It was found that addition of palytoxin to mammalian cells caused the occurrence of rather nonselective cation channels with a single-channel conductance of about 10 pS.[203,204] Scrutinizing the membranes led to the discovery that those ion channels were formed by the Na,K-ATPase.[205-208] An important step forward was obtained when Artigas and Gadsby used outside-out or inside-out excised membrane patches to detect the effect of palytoxin on the level of single Na,K-ATPase molecules.[209,210] They recorded typical single-channel events upon addition of palytoxin with conductance of 7-10 pS[210] (Fig. 8), and proposed that palytoxin modified in the P-E_2 conformation the gate between the cytoplasm and the ion-binding sites, when the access channel to the extracellular side was already open. Thus a continuous pathway was established that formed a relatively non-selective cation channel. At a low palytoxin concentration, when only one or a few Na,K-

ATPase molecules were modified, a characteristic toggling between conducting and non-conducting states of the channel could be observed (Fig. 8). Therefore, it was concluded that the effect of palytoxin is reversible. In addition, when the toxin was washed out, the channel activity ceased. The fast reversibility of the open-channel formation suggested that no major conformational reorganization or even denaturation of the protein occurred but a simultaneously open condition of both occlusion gates was induced.[209] This concept was supported by the fact that common blockers of the access channels were able to clog the continuous ion pathway on both sides of the Na,K-ATPase.[211] By mutation of more than sixty amino acids in transmembrane helices TM1 to TM6, those could be identified by which the conductance of the ion channel could be affected, and a comparison with the crystal structure of the Na,K-ATPase allowed the proposal of the channel's shape and position.[212,213]

X. COUPLING OF ENZYME AND TRANSPORT ACTIVITYIES

One of the major unresolved issues of the function of the Na,K-ATPase is the mechanism of energy conversion by the Na,K-ATPase (or any other P-type ATPase). From basic thermodynamic principles it is known that hydrolysis of ATP in the presence of known concentrations of ATP, ADP and P_i provides under physiological conditions a Gibbs free energy in the order of -55 kJ/mol.[27] This energy is transferred to the ion pump by a chemical reaction, the phosphorylation of the specific aspartate in the P domain. Terrell L. Hill showed that energy transduction in molecular machines does not occur in a single reaction step of the reaction cycle (here: the pump cycle of the Na,K-ATPase) but is distributed over the whole cycle.[214,215] Therefore, to analyze the energetics of the Na,K-ATPase, it has to be determined to what extent single reaction steps contribute to the storage and consumption of the system's free energy in terms of changes of the so-called "basic free energy levels".[27,180] It was found that there was indeed no single "power stroke" reaction step in the pump cycle. Under physiological conditions many steps were even close to thermodynamic equilibrium such as the Na^+ binding and release steps. Extracellular K^+ binding and ATP binding in the $E_2(K_2)$ state were distinct "down-hill" steps in which energy was dissipated, where in contrast, release of both K^+ to the cytoplasm were the most prominent energy consuming steps. The overall energy consumption during a pump cycle could be calculated from the definition of the electrochemical potential gradients of Na^+ and K^+ across the membrane using the known concentrations of both ion species on either side of the membrane, and the electrical membrane potential.[27] At typical values of mammalian cells it was calculated that about 80 % of the energy provided by ATP hydrolysis was utilized in ion transport.[180] Compared to macroscopic machines such a yield is impressive.

While it is possible to calculate basic free energy levels, these numbers do not provide insight into the molecular processes of how energy is transferred from the initial "high energy phosphate" in state $(Na_3)E_1$-P to other moieties of the protein with the result that ions are eventually transferred from one side of the membrane to the other, a vectorial process. It can be assumed that the provided energy is distributed over the protein by rearrangements of amino-acid side chains in response to the coordination of the high-energy phosphate, thus creating changes in spatial alignments, mechanical tension and torque of helices, modified electrostatic interaction and dipole movements. Such transiently enhanced potential energy is buffered in various subdomains of the protein structure. Subsequently, it may drive a meticulously concerted sequence of relaxation processes that perform ion pumping by promoting specific reactions such as ion binding, occlusion and release to the opposite side of the membrane. So far the whole process did not, however, advance from the level of hand-waving arguments. Perhaps, additional structural details with atomic resolution of closely neighboring states of the pump cycle will produce fuel for thoughts, or an inspiring idea may be triggered by revisiting the available wealth of experimental data. Here, but not only here, an exciting terra incognita in the world of the Na,K-ATPase is waiting for exploration.

XI. PUMP-RELATED DISEASES

A final chapter shall reveal and summarize how the knowledge on the location of single amino acids and their role in the functional context provided understanding of specific diseases. Small mutations on the molecular level of the Na,K-ATPase were found to significantly affect pump functions with far-reaching organismic impact. As in the case of ion-channel induced pathology, it was found also for the Na,K-ATPase that errors in the genetic code may provoke malfunctions of the ion pump that lead to phenotypes of explicit diseases.

From early studies of the Na,K-ATPase it has been known already that cardiac glycosides inhibit this enzyme.[216] Because these compounds were applied to treat congestive heart failure and cardiac arrhythmias,

it stood to reason that improper regulation of the Na,K-ATPase activity may correlate with various clinical conditions. Until the year 2000, the focus has been commonly set onto investigations with alteration of endogenous or xenobiotic factors. The cause of several diverging diseases such as cardiovascular, neurological, metabolic or renal disorders were traced back to a dysfunction in salt and water homeostasis of cells that is controlled by the Na,K-ATPase.[217]

In 2004, a specific mutation in the α2 isoform of the Na,K-ATPase was found to cause familial hemiplegic migraine,[218] and in the years that followed, further mutations were discovered to provoke various forms of migraine.[219] More recently it has been reported that mutations in the neuron-specific Na,K-ATPase α3 subunit are linked to rapid-onset dystonia Parkinsonism[220] and that a mutated α3 subunit may play a role in the neurodegeneration of Alzheimer patients.[221] A further disease, primary aldosteronism, was also attributed – among other causes – to malfunction of the Na,K-ATPase. Modifications of pump activity caused secondary hypertension by overproduction of aldosterone, which is initiated by single mutations of the α1 isoform of the Na,K-ATPase in adenomas within the zona glomerulosa of the adrenal cortex. Each of at least five single mutations in the α1 subunit has been found to induce overproduction of aldosterone.[222-224] Very recently, it was reported that the CAPOS (cerebellar ataxia, areflexia, pes cavus, optic atrophy, and sensorineural hearing loss) syndrome is caused by the single mutation, E818K, of the α3-isoform of Na,K-ATPase.[225] Mutations in the gene ATP1A1, which encodes the α1 subunit of the Na,K-ATPase, were identified as a cause of autosomal-dominant Charcot-Marie-Tooth Type 2 disease. A missense change was found that induced loss-of-function defects, resulting in peripheral motor and sensory neuropathies.[226] A missense mutation of the α2 subunit of the Na,K-ATPase was found in a patient with hypokalemic periodic paralysis and CNS symptoms.[227] An informative review on structure and function of the Na,K-ATPase isoforms in health and disease that contains an overview of the currently known disease-causing mutations was published by Clausen et al. in 2017.[228] In this rapidly developing field, the abundance of experimental methods and mechanistic studies collected in recent decades will surely promote further progress and provide invaluable benefits.

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REFERENCES

1. J. F. Danielli; *Symp. Soc. Exp. Biol.,* **1954**, *8*, 502.
2. P. Mitchell; *Nature,* **1957**, *180*, 134.
3. J. C. Skou; *Biochim. Biophys. Acta,* **1957**, *23*, 394.
4. H.-J. Apell; *Substantia,* **2018**, *2*, 17.
5. R. W. Albers, S. Fahn, G. J. Koval; *Proc. Natl. Acad Sci U. S. A,* **1963**, *50*, 474.
6. R. L. Post, A. K. Sen; *J. Histochem. Cytochem.,* **1965**, *13*, 105.
7. R. W. Albers; *Ann. Rev. Biochem.,* **1967**, *36*, 727.
8. R. L. Post, C. Hegyvary, S. Kume; *J. Biol. Chem.,* **1972**, *247*, 6530.
9. C. Hegyvary, R. L. Post; *J. Biol. Chem.,* **1971**, *246*, 5234.
10. H.-J. Apell, V. Häring, M. Roudna; *Biochim. Biophys. Acta,* **1990**, *1023*, 81.
11. S. J. D. Karlish, D. W. Yates, I. M. Glynn; *Biochim. Biophys. Acta,* **1978**, *525*, 230.
12. S. Heyse, I. Wuddel, H.-J. Apell, W. Stürmer; *J. Gen. Physiol.,* **1994**, *104*, 197.
13. I. M. Glynn, S. J. D. Karlish; *Annu. Rev. Physiol.,* **1975**, *37*, 13.
14. Glynn, I. M., in *Membrane Transport* (Martonosi, A. N., Ed.), Plenum Press, New York, **1985**, p. 35.
15. P. J. Garrahan, I. M. Glynn; *J. Physiol.,* **1967**, *192*, 237.
16. P. De Weer, R. F. Rakowski; *Nature,* **1984**, *309*, 450.
17. J. D. Cavieres, I. M. Glynn; *J. Physiol.,* **1979**, *297*, 637.
18. T. J. B. Simons; *J. Physiol.,* **1974**, *237*, 123.
19. P. J. Garrahan, I. M. Glynn; *J. Physiol,* **1967**, *192*, 175.
20. P. J. Garrahan, I. M. Glynn; *J. Physiol,* **1967**, *192*, 159.
21. H.-J. Apell, G. Benz, D. Sauerbrunn; *Biochemistry,* **2011**, *50*, 409.
22. K. H. Lee, R. Blostein; *Nature,* **1980**, *285*, 338.
23. M. Forgac, G. Chin; *J. Biol. Chem.,* **1982**, *257*, 5652.
24. J. R. Sachs; *J. Physiol.,* **1986**, *374*, 221.
25. A. Warshel, S. T. Russell; *Q. Rev. Biophys.,* **1984**, *17*, 283.
26. B. A. Wallace; *Annu. Rev. Biophys. Biophys. Chem.,* **1990**, *19*, 127.
27. P. Läuger, *Electrogenic Ion Pumps*, Sinauer Associates, Inc., Sunderland, MA, **1991**, p. 313.
28. P. Läuger; *Biochim. Biophys. Acta,* **1979**, *552*, 143.
29. P. Läuger, H.-J. Apell; *Biochim. Biophys. Acta,* **1988**, *945*, 1.
30. P. Läuger, H.-J. Apell; *Biochim. Biophys. Acta,* **1988**, *944*, 451.
31. C. S. Patlak; *Bull. Math. Biophys.,* **1957**, *19*, 209.

32. P. Läuger; *Biochim. Biophys. Acta,* **1984**, *779*, 307.
33. P. Läuger, H.-J. Apell; *Eur. Biophys. J.,* **1986**, *13*, 309.
34. R. Borlinghaus, H.-J. Apell, P. Läuger; *J. Membr. Biol.,* **1987**, *97*, 161.
35. I. Wuddel, H.-J. Apell; *Biophys. J.,* **1995**, *69*, 909.
36. C. H. Fiske, Y. Subbarrow; *J. Biol. Chem.,* **1925**, *66*, 375.
37. L. F. Leloir, C. E. Cardini; *Meth. Enzymol.,* **1957**, *1*, 840.
38. O. Vagin, S. Denevich, K. Munson, G. Sachs; *Biochemistry,* **2002**, *41*, 12755.
39. R. L. Post, C. R. Merritt, C. R. Kinsolving, C. D. Albright; *J. Biol. Chem.,* **1960**, *235*, 1796.
40. P. L. Jørgensen, J. C. Skou; *Biochem. Biophys. Res. Commun.,* **1969**, *37*, 39.
41. J. Shaw, L. C. Beadle; *J. exp. Biol.,* **1949**, *26*, 15.
42. O. H. Lowry, A. L. Rosebrough, A. L. FARR, R. J. Randall; *J. Biol. Chem.,* **1951**, *193*, 265.
43. M. A. Markwell, S. M. Haas, L. L. Bieber, N. E. Tolbert; *Anal. Biochem.,* **1978**, *87*, 206.
44. A. K. Schwartz, M. Nagano, M. Nakao, G. E. Lindenmayer, J. C. Allen; *Meth. Pharmacol.,* **1971**, *1*, 361.
45. R. L. Post, A. K. Sen, A. S. Rosenthal; *J. Biol. Chem.,* **1965**, *240*, 1437.
46. R. L. Post, P. C. Jolly; *Biochim. Biophys. Acta,* **1957**, *25*, 118.
47. De Weer P.; *J. Gen. Physiol,* **1970**, *56*, 583.
48. De Weer P., D. Geduldig; *Science,* **1973**, *179*, 1326.
49. T. L. Steck, R. S. Weinstein, J. H. Straus, D. F. Wallach; *Science,* **1970**, *168*, 255.
50. R. Blostein, L. Chu; *J. Biol. Chem.,* **1977**, *252*, 3035.
51. I. M. Glynn, J. F. Hoffman; *J. Physiol,* **1971**, *218*, 239.
52. I. M. Glynn, S. J. D. Karlish; *J. Physiol.,* **1976**, *256*, 465.
53. J. H. Kaplan, R. J. Hollis; *Nature,* **1980**, *288*, 587.
54. D. C. Gadsby, P. F. Cranefield; *Proc. Natl. Acad. Sci. U. S. A,* **1979**, *76*, 1783.
55. D. C. Gadsby, J. Kimura, A. Noma; *Nature,* **1985**, *315*, 63.
56. D. W. Hilgemann; *Science,* **1994**, *263*, 1429.
57. M. Holmgren, J. Wagg, F. Bezanilla, R. F. Rakowski, P. De Weer, D. C. Gadsby; *Nature,* **2000**, *403*, 898.
58. R. F. Rakowski, C. L. Paxson; *J. Membr. Biol.,* **1988**, *106*, 173.
59. P. Artigas, D. C. Gadsby; *Proc. Natl. Acad. Sci. U. S. A,* **2006**, *103*, 12613.
60. K. L. Dürr, N. N. Tavraz, D. Zimmermann, E. Bamberg, T. Friedrich; *Biochemistry,* **2008**, *47*, 4288.
61. N. N. Tavraz, T. Friedrich, K. L. Dürr, J. B. Koenderink, E. Bamberg, T. Freilinger, M. Dichgans; *J. Biol. Chem.,* **2008**, *283*, 31097.
62. S. M. Goldin, S. W. Tong; *J. Biol. Chem.,* **1974**, *249*, 5907.
63. S. Hilden, H. M. Rhee, L. E. Hokin; *J. Biol. Chem.,* **1974**, *249*, 7432.
64. B. M. Anner, L. K. Lane, A. Schwartz, B. J. R. Pitts; *Biochim. Biophys. Acta,* **1977**, *467*, 340.
65. E. Skriver, A. B. Maunsbach, B. M. Anner, P. L. Jørgensen; *Cell Biol. Int. Rep.,* **1980**, *4*, 585.
66. B. Forbush, III; *Anal. Biochem.,* **1984**, *140*, 495.
67. B. Forbush, III; *J. Biol. Chem.,* **1987**, *262*, 11104.
68. B. Forbush, III; *J. Biol. Chem.,* **1987**, *262*, 11116.
69. H.-J. Apell, M. M. Marcus, B. M. Anner, H. Oetliker, P. Läuger; *J. Membr. Biol.,* **1985**, *85*, 49.
70. H.-J. Apell, B. Bersch; *Biochim. Biophys. Acta,* **1987**, *903*, 480.
71. H.-J. Apell, B. Bersch; *Prog. Clin. Biol. Res.,* **1988**, *268A*, 469.
72. J. H. Kaplan, B. Forbush, III, J. F. Hoffman; *Biochem.,* **1978**, *17*, 1929.
73. J. A. McCray, L. Herbette, T. Kihara, D. R. Trentham; *Proc. Natl. Acad. Sci. U. S. A,* **1980**, *77*, 7237.
74. K. Fendler, E. Grell, M. Haubs, E. Bamberg; *EMBO J.,* **1985**, *4*, 3079.
75. H.-J. Apell, R. Borlinghaus, P. Läuger; *J. Membr. Biol.,* **1987**, *97*, 179.
76. R. Borlinghaus, H.-J. Apell; *Biochim. Biophys. Acta,* **1988**, *939*, 197.
77. H. Thirlwell, J. E. T. Corrie, G. P. Reid, D. R. Trentham, M. A. Ferenczi; *Biophys. J.,* **1994**, *67*, 2436.
78. V. S. Sokolov, H.-J. Apell, J. E. Corrie, D. R. Trentham; *Biophys. J.,* **1998**, *74*, 2285.
79. V. S. Sokolov, S. M. Stukolov, N. M. Gevondyan, H.-J. Apell; *Ann. N. Y. Acad. Sci.,* **1997**, *834*, 364.
80. V. Yu. Tashkin, A. N. Gavrilchik, A. I. Ilovaisky, H.-J. Apell, V. S. Sokolov; *Biochemistry (Moscow) Supplement Series A,* **2015**, *9*, 92.
81. W. Domaszewicz, H.-J. Apell; *FEBS Lett.,* **1999**, *458*, 241.
82. J. Pintschovius, K. Fendler; *Biophys. J.,* **1999**, *76*, 814.
83. J. Pintschovius, K. Fendler, E. Bamberg; *Biophys. J.,* **1999**, *76*, 827.
84. F. Tadini-Buoninsegni, P. Nassi, C. Nediani, A. Dolfi, R. Guidelli; *Biochim. Biophys. Acta,* **2003**, *1611*, 70.
85. S. J. Karlish, D. W. Yates; *Biochim. Biophys. Acta,* **1978**, *527*, 115.
86. S. J. Karlish, D. W. Yates, I. M. Glynn; *Nature,* **1976**, *263*, 251.
87. S. J. Karlish, D. W. Yates, I. M. Glynn; *Biochim. Biophys. Acta,* **1978**, *525*, 252.

88. S. J. Karlish, L. A. Beauge, I. M. Glynn; *Nature,* **1979**, *282*, 333.
89. S. J. D. Karlish; *J. Bioenerg. Biomembr.,* **1980**, *12*, 111.
90. C. Hegyvary, P. L. Jørgensen; *J. Biol. Chem.,* **1981**, *256*, 6296.
91. C. T. Carilli, R. A. Farley, D. M. Perlman, L. C. Cantley; *J. Biol. Chem.,* **1982**, *257*, 5601.
92. R. A. Farley, C. M. Tran, C. T. Carilli, D. Hawke, J. E. Shively; *J. Biol. Chem.,* **1984**, *259*, 9532.
93. J. G. Kapakos, M. Steinberg; *Biochim. Biophys. Acta,* **1982**, *693*, 493.
94. P. A. Tyson, M. Steinberg, E. T. Wallick, T. L. Kirley; *J. Biol. Chem.,* **1989**, *264*, 726.
95. M. Steinberg, S. J. Karlish; *J. Biol. Chem.,* **1989**, *264*, 2726.
96. W. Stürmer, H.-J. Apell, I. Wuddel, P. Läuger; *J. Membr. Biol.,* **1989**, *110*, 67.
97. J. C. Skou, M. Esmann; *Biochim. Biophys. Acta,* **1981**, *647*, 232.
98. J. C. Skou, M. Esmann; *Biochim. Biophys. Acta,* **1983**, *727*, 101.
99. J. C. Skou; *Biochim. Biophys. Acta,* **1982**, *688*, 369.
100. L. M. Loew, S. Scully, L. Simpson, A. S. Waggoner; *Nature,* **1979**, *281*, 497.
101. A. Grinvald, L. Anglister, J. A. Freeman, R. Hildesheim, A. Manker; *Nature,* **1984**, *308*, 848.
102. I. Klodos, B. Forbush, III; *J. Gen. Physiol.,* **1988**, *92*, 46A (abstr.).
103. R. Bühler, W. Stürmer, H.-J. Apell, P. Läuger; *J. Membr. Biol.,* **1991**, *121*, 141.
104. W. Stürmer, R. Bühler, H.-J. Apell, P. Läuger; *J. Membr. Biol.,* **1991**, *121*, 163.
105. R. J. Clarke, P. Schrimpf, M. Schoneich; *Biochim. Biophys. Acta,* **1992**, *1112*, 142.
106. P. R. Pratap, J. D. Robinson; *Biochim. Biophys. Acta,* **1993**, *1151*, 89.
107. N. U. Fedosova, F. Cornelius, I. Klodos; *Biochemistry,* **1995**, *34*, 16806.
108. G. Bartolommei, N. Devaux, F. Tadini-Buoninsegni, M. R. Moncelli, H.-J. Apell; *Biophys. J.,* **2008**, *95*, 1813.
109. G. A. Figtree, C. C. Liu, S. Bibert, E. J. Hamilton, A. Garcia, C. N. White, K. K. Chia, F. Cornelius, K. Geering, H. H. Rasmussen; *Circ. Res.,* **2009**, *105*, 185.
110. S. E. Faraj, M. Centeno, R. C. Rossi, M. R. Montes; *Biochim. Biophys. Acta,* **2018**, *1861*, 355.
111. M. Habeck, E. Cirri, A. Katz, S. J. Karlish, H.-J. Apell; *Biochemistry,* **2009**, *48*, 9147.
112. J. C. Skou; *Biochim. Biophys. Acta,* **1960**, *42*, 6.
113. R. L. Post, A. S. Rosenthal; *J. Gen. Physiol.,* **1962**, *45*, 614A (abstr.).
114. J. S. Charnock, R. L. Post; *Nature,* **1963**, *199*, 910.
115. L. E. Hokin, P. S. Sastry, P. R. Galsworthy, A. Yoda; *Proc. Natl. Acad Sci U. S. A,* **1965**, *54*, 177.
116. K. Nagano, T. Kanazawa, N. Mizuno, Y. Tashima, T. Nakao, M. Nakao; *Biochem. Biophys. Res. Commun.,* **1965**, *19*, 759.
117. J. C. Skou; *Physiol Rev.,* **1965**, *45*, 596.
118. A. Schwartz, G. E. Lindenmayer, J. C. Allen; *Curr. Top. Membranes Transp.,* **1972**, *3*, 1.
119. R. Gibbs, P. M. Roddy, E. Titus; *J. Biol. Chem.,* **2018**, *240*, 2181.
120. R. Blostein; *Biochem. Biophys. Res. Commun.,* **1966**, *24*, 598.
121. S. Fahn, G. J. Koval, R. W. Albers; *J. Biol. Chem.,* **1966**, *241*, 1882.
122. T. Hexum, F. E. Samson, Jr., R. H. Himes; *Biochim. Biophys. Acta,* **1970**, *212*, 322.
123. F. Bastide, G. Meissner, S. Fleischer, R. L. Post; *J. Biol. Chem.,* **1973**, *248*, 8385.
124. I. Nishigaki, F. T. Chen, L. E. Hokin; *J. Biol. Chem.,* **1974**, *249*, 4911.
125. G. E. Shull, A. Schwartz, J. B. Lingrel; *Nature,* **1985**, *316*, 691.
126. S. Lutsenko, J. H. Kaplan; *Biochem.,* **1995**, *34*, 15607.
127. H. Matsui, H. Homareda; *J. Biochem. (Tokyo),* **1982**, *92*, 193.
128. M. Esmann, J. C. Skou; *Biochem. Biophys. Res. Comm.,* **1985**, *127*, 857.
129. I. Klodos, J. G. Norby, I. W. Plesner; *Biochim. Biophys. Acta,* **1981**, *643*, 463.
130. J. G. Nørby, I. Klodos, N. O. Christiansen; *J. Gen. Physiol,* **1983**, *82*, 725.
131. A. Yoda, S. Yoda; *Mol. Pharmacol.,* **1982**, *22*, 693.
132. J. G. Nørby, I. Klodos; *Progr. Clin. Biol. Res.,* **1988**, *268A*, 249.
133. J. D. Cavieres; *Curr. Top. Membr. Trans.,* **1983**, *19*, 677.
134. R. W. Albers, G. J. Koval, Siegel; *Mol. Pharmacol.,* **1968**, *4*, 324.
135. G. E. Lindenmayer, A. H. Laughter, A. Schwartz; *Arch. Biochem. Biophys.,* **1968**, *127*, 187.
136. G. J. Siegel, G. J. Koval, R. W. Albers; *J. Biol. Chem.,* **1969**, *244*, 3264.
137. A. K. Sen, T. Tobin, R. L. Post; *J. Biol. Chem.,* **1969**, *244*, 6596.
138. H. Matsui, A. Schwartz; *Biochim. Biophys. Acta,* **1966**, *128*, 380.
139. I. A. Svinukhova, A. A. Boldyrev; *FEBS Letters,* **1987**, *214*, 335.
140. J. D. Robinson; *Biochim. Biophys. Acta,* **1974**, *341*, 232.

141. J. J. Lacapère, N. Bennett, Y. Dupont, F. Guillain; *J. Biol. Chem.,* **1990**, *265*, 348.
142. A. M. Romani; *Arch. Biochem. Biophys.,* **2011**, *512*, 1.
143. M. D. Forgac; *J. Biol. Chem.,* **1980**, *255*, 1547.
144. Y. Fukushima, R. L. Post; *J. Biol. Chem.,* **1978**, *253*, 6853.
145. C. H. Pedemonte, L. Beauge; *Biochim. Biophys. Acta,* **1983**, *748*, 245.
146. P. A. Pedersen, J. R. Jørgensen, P. L. Jørgensen; *J. Biol. Chem.,* **2000**, *275*, 37588.
147. M. Palasis, T. A. Kuntzweiler, J. M. Argüello, J. B. Lingrel; *J. Biol. Chem.,* **1996**, *271*, 14176.
148. J. B. Lingrel, J. M. Argüello, J. van Huysse, T. A. Kuntzweiler; *Ann. N. Y. Acad. Sci.,* **1997**, *834*, 194.
149. E. Or, E. D. Goldshleger, D. M. Tal, S. J. Karlish; *Biochemistry,* **1996**, *35*, 6853.
150. H. Ogawa, T. Shinoda, F. Cornelius, C. Toyoshima; *Proc. Natl. Acad. Sci. U. S. A,* **2009**, *106*, 13742.
151. L. Yatime, M. Laursen, J. P. Morth, M. Esmann, P. Nissen, N. U. Fedosova; *J. Struct. Biol.,* **2011**, *174*, 296.
152. M. Laursen, L. Yatime, P. Nissen, N. U. Fedosova; *Proc. Natl. Acad. Sci. U. S. A,* **2013**, *110*, 10958.
153. M. Y. Abeywardena, E. J. McMurchie, G. R. Russell, J. S. Charnock; *Biochem. Pharmacol.,* **1984**, *33*, 3649.
154. J. M. Hamlyn, R. Ringel, J. Schaeffer, P. D. Levinson, B. P. Hamilton, A. A. Kowarski, M. P. Blaustein; *Nature,* **1982**, *300*, 650.
155. J. M. Hamlyn, M. P. Blaustein, S. Bova, D. W. DuCharme, D. W. Harris, F. Mandel, W. R. Mathews, J. H. Ludens; *Proc. Natl. Acad. Sci. U. S. A,* **1991**, *88*, 6259.
156. A. Kawamura, J. Guo, Y. Itagaki, C. Bell, Y. Wang, G. T. Haupert, Jr., S. Magil, R. T. Gallagher, N. Berova, K. Nakanishi; *Proc. Natl. Acad. Sci. U. S. A,* **1999**, *96*, 6654.
157. W. Schoner; *Exp. Clin. Endocrinol. Diabetes,* **2000**, *108*, 449.
158. J. M. Hamlyn, M. P. Blaustein; *Hypertension,* **2016**, *68*, 526.
159. J. G. Kaplan; *Annu. Rev. Physiol,* **1978**, *40*, 19.
160. Z. Xie, A. Askari; *Eur. J. Biochem.,* **2002**, *269*, 2434.
161. A. Zulian, C. I. Linde, M. V. Pulina, S. G. Baryshnikov, I. Papparella, J. M. Hamlyn, V. A. Golovina; *Am. J. Physiol Cell Physiol,* **2013**, *304*, C324-C333.
162. Z. Xie, T. Cai; *Mol. Interv.,* **2003**, *3*, 157.
163. W. Schoner, G. Scheiner-Bobis; *Am. J. Physiol Cell Physiol,* **2007**, *293*, C509-C536.
164. Y. Yan, J. I. Shapiro; *Curr. Opin. Pharmacol.,* **2016**, *27*, 43.
165. L. C. Cantley, L. Josephson, R. Warner, M. Yanagisawa, C. Lechene, G. Guidotti; *J. Biol. Chem.,* **1977**, *252*, 7421.
166. L. C. Cantley, L. G. Cantley, L. Josephson; *J. Biol. Chem.,* **1978**, *253*, 7361.
167. I. M. Glynn; *Biochem. J.,* **1962**, *84*, 75P (abstr.).
168. J. Järnefelt; *Biochim. Biophys. Acta,* **1962**, *59*, 643.
169. S. Fahn, M. R. Hurley, G. J. Koval, R. W. Albers; *J. Biol. Chem.,* **1966**, *241*, 1890.
170. H. Homareda, T. Ishii, K. Takeyasu; *Eur. J. Pharmacol.,* **2000**, *400*, 177.
171. R. Kanai, H. Ogawa, B. Vilsen, F. Cornelius, C. Toyoshima; *Nature,* **2013**, *502*, 201.
172. H.-J. Apell; *Bioelectrochemistry.,* **2004**, *63*, 149.
173. A. Schneeberger, H.-J. Apell; *J. Membr. Biol.,* **2001**, *179*, 263.
174. R. Goldshlegger, S. J. Karlish, A. Rephaeli, W. D. Stein; *J. Physiol (Lond),* **1987**, *387*, 331.
175. A. Schneeberger, H.-J. Apell; *J. Membr. Biol.,* **1999**, *168*, 221.
176. H.-J. Apell, A. Diller; *FEBS Lett.,* **2002**, *532*, 198.
177. V. S. Sokolov, A. A. Scherbakov, A. A. Lenz, Yu. A. Chizmadzhev, H.-J. Apell; *Biochemistry (Moscow) Supplement Series A,* **2008**, *2*, 161.
178. H. J. Apell, T. Hitzler, G. Schreiber; *Biochemistry,* **2017**, *56*, 1005.
179. J. V. Møller, C. Olesen, A. M. Winther, P. Nissen; *Q. Rev. Biophys.,* **2010**, *43*, 501.
180. H.-J. Apell; *Ann. N. Y. Acad. Sci.,* **1997**, *834*, 221.
181. P. De Weer, D. C. Gadsby, R. F. Rakowski; *Prog. Clin. Biol. Res.,* **1988**, *268A*, 421.
182. M. Nakao, D. C. Gadsby; *J. Gen. Physiol,* **1989**, *94*, 539.
183. A. Sagar, R. F. Rakowski; *J. Gen. Physiol,* **1994**, *103*, 869.
184. T. Friedrich, G. Nagel; *Biophys. J.,* **1997**, *73*, 186.
185. D. C. Gadsby, F. Bezanilla, R. F. Rakowski, P. De Weer, M. Holmgren; *Nat. Commun.,* **2012**, *3*, 669.
186. R. V. Gradinaru, H.-J. Apell; *Biochemistry,* **2015**, *54*, 2508.
187. B. Forbush, III; *Prog. Clin. Biol. Res.,* **1988**, *268A*, 229.
188. R. F. Rakowski, L. A. Vasilets, J. LaTona, W. Schwarz; *J. Membr. Biol.,* **1991**, *121*, 177.
189. F. Jaisser, P. Jaunin, K. Geering, B. C. Rossier, J. D. Horisberger; *J. Gen. Physiol,* **1994**, *103*, 605.
190. R. Bühler, H.-J. Apell; *J. Membr. Biol.,* **1995**, *145*, 165.
191. R. D. Peluffo, J. R. Berlin; *J. Physiol,* **1997**, *501*, 33.
192. R. D. Peluffo, Y. Hara, J. R. Berlin; *J. Gen. Physiol,* **2004**, *123*, 249.
193. R. D. Peluffo, R. M. Gonzalez-Lebrero, S. B. Kaufman, S. Kortagere, B. Orban, R. C. Rossi, J. R. Berlin; *Biochemistry,* **2009**, *48*, 8105.
194. R. D. Peluffo, J. R. Berlin; *Mol. Pharmacol.,* **2012**, *82*, 1.

195. L. J. Mares, A. Garcia, H. H. Rasmussen, F. Cornelius, Y. A. Mahmmoud, J. R. Berlin, B. Lev, T. W. Allen, R. J. Clarke; *Biophys. J.,* **2014**, *107*, 1352.
196. R. Goldshlegger, S. J. Karlish, A. Rephaeli, W. D. Stein; *J. Physiol,* **1987**, *387*, 331.
197. A. Bahinski, M. Nakao, D. C. Gadsby; *Proc. Natl. Acad. Sci. U. S. A,* **1988**, *85*, 3412.
198. B. Forbush, III; *J. Biol. Chem.,* **1988**, *263*, 7961.
199. H.-J. Apell, M. Roudna, J. E. Corrie, D. R. Trentham; *Biochemistry,* **1996**, *35*, 10922.
200. S. J. D. Karlish, D. W. Yates; *Biochim. Biophys. Acta,* **1978**, *527*, 115.
201. I. M. Glynn, D. E. Richards; *J. Physiol.,* **1982**, *330*, 17.
202. R. E. Moore, P. J. Scheuer; *Science,* **1971**, *172*, 495.
203. M. Ikeda, K. Mitani, K. Ito; *Naunyn Schmiedebergs Arch. Pharmacol.,* **1988**, *337*, 591.
204. M. T. Tosteson, J. A. Halperin, Y. Kishi, D. C. Tosteson; *J. Gen. Physiol,* **1991**, *98*, 969.
205. H. Ozaki, H. Nagase, N. Urakawa; *Eur. J. Biochem.,* **1985**, *152*, 475.
206. E. Habermann; *Toxicon,* **1989**, *27*, 1171.
207. X. Wang, J.-D. Horisberger; *FEBS Letters,* **1997**, *409*, 391.
208. M. T. Tosteson, G. S. Bignami, D. R. L. Scriven, A. K. Bharadwaj, D. C. Tosteson; *Biochim. Biophys. Acta,* **1994**, *1191*, 371.
209. P. Artigas, D. C. Gadsby; *Proc. Natl. Acad. Sci. U. S. A,* **2003**, *100*, 501.
210. P. Artigas, D. C. Gadsby; *J. Gen. Physiol,* **2004**, *123*, 357.
211. N. Harmel, H.-J. Apell; *J. Gen. Physiol,* **2006**, *128*, 103.
212. N. Reyes, D. C. Gadsby; *Nature,* **2006**, *443*, 470.
213. A. Takeuchi, N. Reyes, P. Artigas, D. C. Gadsby; *Nature,* **2008**, *456*, 413.
214. T. L. Hill, *Free Energy Transduction in Biology*, Academic Press, New York, **1977**, p. 229.
215. T. L. Hill, *Free Energy Transduction and Biochemical Cycle Kinetics*, Springer, New York, **1989**, p. 119.
216. H. J. Schatzmann; *Protoplasma,* **1967**, *63*, 136.
217. A. M. Rose, R. Valdes; *Clin. Chem.,* **1994**, *40*, 1674.
218. K. J. Swoboda, E. Kanavakis, A. Xaidara, J. E. Johnson, M. F. Leppert, M. B. Schlesinger-Massart, L. J. Ptacek, K. Silver, S. Youroukos; *Ann. Neurol.,* **2004**, *55*, 884.
219. T. Friedrich, N. N. Tavraz, C. Junghans; *Front Physiol,* **2016**, *7*, 239.
220. A. N. Shrivastava, V. Redeker, N. Fritz, L. Pieri, L. G. Almeida, M. Spolidoro, T. Liebmann, L. Bousset, M. Renner, C. Lena, A. Aperia, R. Melki, A. Triller; *EMBO J.,* **2015**, *34*, 2408.
221. T. Ohnishi, M. Yanazawa, T. Sasahara, Y. Kitamura, H. Hiroaki, Y. Fukazawa, I. Kii, T. Nishiyama, A. Kakita, H. Takeda, A. Takeuchi, Y. Arai, A. Ito, H. Komura, H. Hirao, K. Satomura, M. Inoue, S. Muramatsu, K. Matsui, M. Tada, M. Sato, E. Saijo, Y. Shigemitsu, S. Sakai, Y. Umetsu, N. Goda, N. Takino, H. Takahashi, M. Hagiwara, T. Sawasaki, G. Iwasaki, Y. Nakamura, Y. Nabeshima, D. B. Teplow, M. Hoshi; *Proc. Natl. Acad. Sci. U. S. A,* **2015**, *112*, E4465-E4474.
222. F. Beuschlein, S. Boulkroun, A. Osswald, T. Wieland, H. N. Nielsen, U. D. Lichtenauer, D. Penton, V. R. Schack, L. Amar, E. Fischer, A. Walther, P. Tauber, T. Schwarzmayr, S. Diener, E. Graf, B. Allolio, B. Samson-Couterie, A. Benecke, M. Quinkler, F. Fallo, P. F. Plouin, F. Mantero, T. Meitinger, P. Mulatero, X. Jeunemaitre, R. Warth, B. Vilsen, M. C. Zennaro, T. M. Strom, M. Reincke; *Nat. Genet.,* **2013**, *45*, 440.
223. E. A. Azizan, H. Poulsen, P. Tuluc, J. Zhou, M. V. Clausen, A. Lieb, C. Maniero, S. Garg, E. G. Bochukova, W. Zhao, L. H. Shaikh, C. A. Brighton, A. E. Teo, A. P. Davenport, T. Dekkers, B. Tops, B. Kusters, J. Ceral, G. S. Yeo, S. G. Neogi, I. McFarlane, N. Rosenfeld, F. Marass, J. Hadfield, W. Margas, K. Chaggar, M. Solar, J. Deinum, A. C. Dolphin, I. S. Farooqi, J. Striessnig, P. Nissen, M. J. Brown; *Nat. Genet.,* **2013**, *45*, 1055.
224. T. A. Williams, S. Monticone, V. R. Schack, J. Stindl, J. Burrello, F. Buffolo, L. Annaratone, I. Castellano, F. Beuschlein, M. Reincke, B. Lucatello, V. Ronconi, F. Fallo, G. Bernini, M. Maccario, G. Giacchetti, F. Veglio, R. Warth, B. Vilsen, P. Mulatero; *Hypertension,* **2014**, *63*, 188.
225. C. P. Roenn, M. Li, V. R. Schack, I. C. Forster, R. Holm, M. S. Toustrup-Jensen, J. P. Andersen, S. Petrou, B. Vilsen; *J. Biol. Chem.,* **2018**, doi: 10.1074/jbc.RA118.004591.
226. P. Lassuthova, A. P. Rebelo, G. Ravenscroft, P. J. Lamont, M. R. Davis, F. Manganelli, S. M. Feely, C. Bacon, D. S. Brozkova, J. Haberlova, R. Mazanec, F. Tao, C. Saghira, L. Abreu, S. Courel, E. Powell, E. Buglo, D. M. Bis, M. F. Baxter, R. W. Ong, L. Marns, Y. C. Lee, Y. Bai, D. G. Isom, R. Barro-Soria, K. W. Chung, S. S. Scherer, H. P. Larsson, N. G. Laing, B. O. Choi, P. Seeman, M. E. Shy, L. Santoro, S. Zuchner; *Am. J. Hum. Genet.,* **2018**, *102*, 505.
227. Castaneda M.S., E. Zanoteli, R. S. Scalco, V. Scaramuzzi, C. Marques, V, R. U. Conti, A. M. S. da Silva, B. O'Callaghan, R. Phadke, E. Bugiardini, R. Sud, S. McCall, M. G. Hanna, H. Poulsen, R. Mannikko, E. Matthews; *Brain,* **2018**, *141*, 3308.
228. M. V. Clausen, F. Hilbers, H. Poulsen; *Front Physiol,* **2017**, *8*, 371.