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Toward an Understanding of Ion Transport through the Na,K-ATPase

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ABSTRACT: In the Na,K-ATPase the charge-translocating reaction steps were found to be binding of the third Na^+ ion to the cytoplasmic side and the release of all three Na^+ ions to the extracellular side as well as binding of the two K^+ ions on the extracellular side. The conformation transition $E_1 \rightarrow E_2$ was only of minor electrogenicity; all other reaction steps produced no significant charge movements. In the SR Ca-ATPase and the gastric H,K-ATPase, all ion-binding and -release steps were identified to move charge through the membrane. The high-resolution structure of the SR Ca-ATPase in state E_1 revealed the position of the ion-binding sites in the transmembrane part of the protein. If the same arrangement is assumed for the Na pump, the missing expected charge movements in state E_1 may to be assumed to be apparent effects. With the proposal that binding of 2 Na^+ or 2 K^+ is compensated correspondingly by H^+ ions, agreement between structural and functional aspects is obtained. Investigations of the pH-dependence of ion-binding steps indicate competition between the ions and electrogenic H^+ binding in support of this concept.

KEYWORDS: binding sites; ion transport; pH effects; electrogenicity; styryl dyes; fluorescence; competitive inhibition of ion binding

INTRODUCTION

The function of the Na,K-ATPase in the membrane of cells maintains the electrochemical potential gradient of Na^+ and K^+ ions.[1–3] Ion transport is facilitated by coupling the energy-providing enzymatic process with a ping-pong mechanism of ion translocation.[4] This process is described by the so-called post-Albers cycle[5,6] (FIG. 1A). If, for example, the Na^+ translocating pathway of the cycle is examined, we see that four reaction steps could contribute to the charge translocation: (1) ion binding; (2) ion occlusion; (3) conformation transition; and (4) ion release to the opposite side (FIG. 1B). To quantify the "dielectric" distance over which the ion is moved, dielectric coefficients were introduced that describe the fraction of the membrane dielectric over which the charge is shifted perpendicular to the plane of the membrane.[3] If the dielectric coefficient is non-zero, the accompanying reaction is termed "electrogenic." In FIGURE 1B the dielectric coefficient for cytoplasmic binding of a Na^+ ion would be α' (and $\alpha' + \beta' + \beta'' + \alpha'' = 1$ for the transfer across the whole membrane). In the last decade numerous studies were performed with differ-

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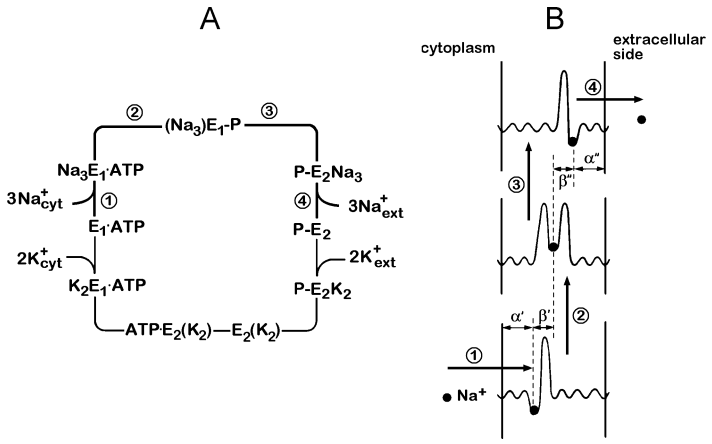

FIGURE 1. (A) Post-Albers cycle of the Na,K-ATPase. The Na^+-translocating pathway is characterized by four partial reactions: (1) Na^+ binding; (2) ion occlusion and enzyme phosphorylation; (3) conformation transition $E_1 \rightarrow E_2$; and (4) Na^+ deocclusion and release. **(B)** Schematic representation of the energy profile of the Na^+ pathway for the various states of Na^+ translocation. The Greek letters indicate the corresponding dielectric coefficients (see text).

ent electrophysiological and fluorescence spectroscopical techniques to determine the dielectric coefficients of all electrogenic partial reactions.[7–10]

On the basis of these results, a structure–function concept was constructed in which on the extracellular side, the first Na^+ ion is released through a narrow access channel, the step with the highest dielectric coefficient ($\alpha'' \approx 0.7$);[7,8] then a conformational relaxation occurs[11] before the other two Na^+ ions reach the aqueous phase with lower dielectric coefficients (0.1–0.2), probably caused by reduction of the dielectric coefficient of the transmembrane part of the pump protein, for example, by intrusion of water molecules.[7] On the cytoplasmic side only a single reaction step was detected to be electrogenic, binding of the third Na^+ ion.[12,13]

When similar studies of the electrogenicity were performed with the SR Ca-ATPase[14,15] and the gastric H,K-ATPase (unpublished data), it was found that in these P-type ATPases all ion-binding and -release reactions were electrogenic. This difference from the Na,K-ATPase is significant, since all three ATPases are believed to have closely related structures.[16] An important input into the considerations of structure–function relations was produced by the 3-D structure of the E_1 conformation of the SR Ca-ATPase at atomic resolution,[17] which revealed that the two Ca^{2+} ions were bound to extremely well coordinating ion sites in the middle of the transmembrane domains.

When we studied "backdoor phosphorylation" of the Na,K-ATPase in the absence of K^+ ions,[18] we found that the population of the state of E_2 that could be phosphorylated by P_i needed to have occluded two H^+ ions, so that the reaction sequence has to be:

$$E_1 + 2H^+_{cyt} \rightarrow H_2E_1 \rightarrow E_2(H_2) \rightarrow P\text{-}E_2(H_2) \rightarrow P\text{-}E_2 + 2H^+_{ext}$$

Because of this finding it was important to investigate the interaction of H^+ ions with the ion-binding sites to scrutinize whether instantaneous H^+ binding to empty sites of the Na,K-ATPase in state E_1 conceals otherwise electrogenic Na^+ and K^+ movements.

COMPETITION OF H^+ WITH Na^+ AND K^+ IONS AT THE CYTOPLASMIC SITES

Na^+ binding in state E_1 can be measured by equilibrium titration experiments with high accuracy using the fluorescent styryl dye RH421.[13] This dye, and others of this family, are hydrophobic substances with one polar end, so they insert into lipid membranes in an aligned manner.[19] Due to the electrochromic mechanism of their chromophore they detect changes of local electric fields in the membrane dielectric and, therefore, report charge movements in membrane preparations which are packed with ion pumps, such as Na,K-ATPase[13,18] and SR Ca-ATPase.[14,15] From Na^+-titration experiments with the Na,K-ATPase in its E_1 conformation the half-saturating concentration, $K_{1/2}$, was determined by fitting a Hill function to the Na^+-dependent fluorescence changes. These experiments were performed in buffers with a pH set between 6 and 8.5. In FIGURE 2 the $K_{1/2}$ values obtained from such experiments are shown as function of pH. They show clearly that binding of Na^+ is affected by H^+ concentration. $K_{1/2}$ increases between pH 8.5 and 6 by a factor of 5. $K_{1/2}$ corresponds approximately to the K_M value of the second of three Na^+ ions bound.[13] The fluorescence change observed in these experiments was generated by the binding of the third Na^+ ion to the "Na^+-specific site,"[12,13] and it was found that the maximum fluores-

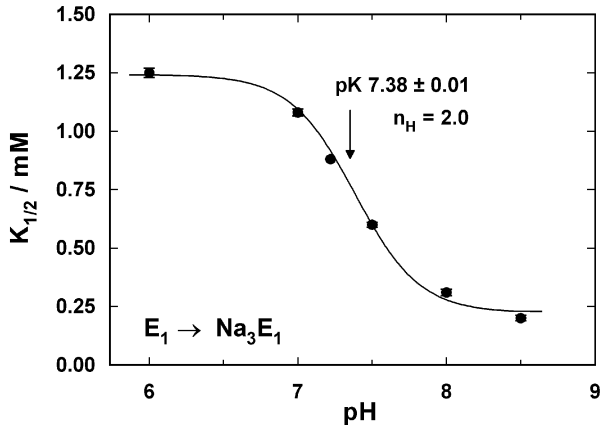

FIGURE 2. Effect of buffer pH on cytoplasmic Na^+ binding as detected by RH421 fluorescence changes. Buffers contained 25 mM histidine and 0.5 mM EDTA, and pH was adjusted by Tris or HCl. The half-saturating Na^+ concentration, $K_{1/2}$, increased with the H^+ concentration, which can be explained by competition between Na^+ and H^+ at the same site(s). The drawn line through the data represents a fit with a Hill function with a half-saturating concentration of 41.7 nM (or pK 7.38).

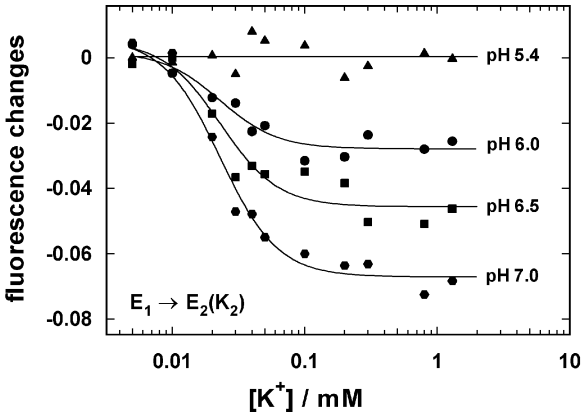

FIGURE 3. Cytoplasmic K^+ binding to the Na,K-ATPase detected by fluorescence changes of RH421 at various pH in a buffer containing 25 mM histidine, and 0.5 mM EDTA, when pH was adjusted by HCl. Data were fitted with a Hill function (*solid lines*). The Hill coefficient of 2.0 ± 0.1 was constant over a pH range of 5.4–7.

cence change was not significantly affected by the pH applied (not shown). This indicates that no competition between Na^+ and H^+ occurred at the third binding site. Therefore, it may be proposed that H^+ is able to bind to side groups of amino acids which are part of (or close to) the two binding sites which are not Na^+ specific. If so, this should be reflected also in pH effects on K^+ binding, which shows barely significant electrogenic effects when investigated at physiological pH.[12,20] Results of pH-dependent cytoplasmic K^+ binding are shown in FIGURE 3. Although the fluorescence changes were much smaller than in the case of Na^+ binding, it is clear that electrogenic K^+ binding could be seen when the buffer pH was increased from 5 to 7. The Hill fits drawn through the data had a Hill coefficient, n_H, of 2, which indicated interactions with more than one H^+ in this process. At pH 5.4 no apparent or net charge movement could be detected. Experiments with buffer pH higher than 7 could be obtained only by addition of Tris, but this addition reduced the fluorescence changes for so far unknown reasons and these data were therefore not included in the analysis.

CYTOPLASMIC AND EXTRACELLULAR H^+ BINDING

pH-titration experiments were performed in the absence of other monovalent cations in E_1, and in P-E_2 conformations of the Na,K-ATPase. The latter were obtained in the presence of Mg^{2+} ions either by addition of 500 μM P_i or by addition of 10 mM NaCl + 100 μM ATP. The pH-induced fluorescence changes are shown in FIGURE 4. Although the fluorescence changes were small, when in the E_1 conformation, H^+ ions were added in the pH range 7.2–5.5, these changes ($\Delta F = -13\%$) were nevertheless significant. In control experiments with completely blocked enzyme $\Delta F < 5\%$ was found for the same pH jump. When the reaction sequence $E_1 \rightarrow H_2E_1 \rightarrow E_2(H_2)$ was studied during backdoor phosphorylation,[18] a pK value of 8.6 was esti-

mated from the pH-dependent population of state $E_2(H_2)$, so that below pH 7.2 more than 95% of the ion sites in E_1 would have bound protons. Therefore it must be expected that further addition of H^+ would produce only minor fluorescence changes.

When the enzyme was phosphorylated, the fluorescence increased by between 30% (pH 5.5) and 40% (pH 7.2), indicating the release of at least one H^+ ion from inside the transmembrane region of the protein. This may be explained as a consequence of a dramatic decrease of the pK of one (or more) amino acid side groups to which the protons bind in the E_1 conformation when no Na^+ or K^+ ions are present. Reducing the pH in state P-E_2 by addition of aliquots of HCl resulted in a decrease of the RH421 fluorescence, which represents H^+ binding to a site within the protein dielectric (FIG. 4). The obvious differences in the fluorescence levels in the two protein conformations may be explained by the above-mentioned large shift of the pK values of the side chains of acidic amino acids to which H^+ are able to bind in, or close to, the ion-binding sites. Thus, whereas in E_1 the pK (about 8.6) is so high that in the physiological pH range the "sites" are mostly occupied by H^+ ions in the absence of other cations, the pK in P-E_2 is proposed to drop so significantly that the same sites are then largely unprotonated. A rough estimate from the titration experiment in FIGURE 4 suggests a pK < 5.5 for P-E_2.

CONSEQUENCES FOR THE STRUCTURE–FUNCTION RELATIONSHIP

On the basis of these results, together with the generally accepted constraint that all P-type ATPases have closely related structures,[16] a concept for the position and characteristics of the ion-binding sites may be developed that is able to describe the

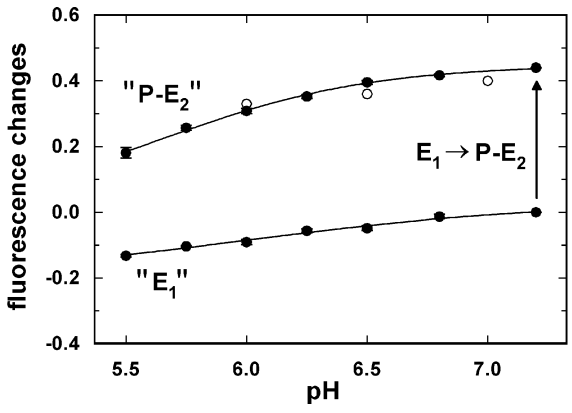

FIGURE 4. pH titration of the ion sites in the two principal conformations, E_1 and P-E_2, in the absence of other monovalent cations. The *vertical arrow* indicates the RH421 fluorescence change induced by phosphorylation of the Na,K-ATPase with P_i in the absence of other monovalent cations, or by addition of 10 mM NaCl + 100 μM ATP. Initial buffer composition was 25 mM histidine, 0.5 mM EDTA, and 10 mM $MgCl_2$, at pH 7.2.

electrogenicity as well as the detected interaction, or competition, of the cations binding to these sites. To maintain the homology of ion sites in the P-type ATPases, it is assumed that in the E_1 conformation two sites are in the middle of the membrane dielectric, where they were recently identified for the SR Ca-ATPase.[17] In the case of the Na,K-ATPase these sites are able to bind various monovalent cations[21] including H^+, as shown above. Binding of a third (Na^+) ion, which is a special feature of the Na,K-ATPase, occurs only after two Na^+ ions have already bound.[21] This third site is positioned about 25% of the dielectric thickness into the membrane from the cytoplasmic side[12] and is virtually exclusively selective for Na^+. Such a topographical arrangement, however, appears to contradict the observation that the cytoplasmic binding or release of the two K^+ or of the first two Na^+ ions is not electrogenic.[12,20] To resolve this problem, the reported H^+ binding to the cytoplasmic sites may be brought into play.

When under physiological conditions the Na,K-ATPase reaches state K_2E_1 in the pump's cycle, the subsequent release of K^+ ions may be accompanied (and supported) by an uptake of one H^+ per K^+ ion that binds to an acidic side group of an amino acid at the binding site: $K_2E_1 + 2H^+ \rightarrow H_2E_1 + 2K^+$. Such a reaction would be apparently electroneutral. Only at high pH approaching the pK of the H^+ binding groups, would a significant electrogenic contribution of K^+ binding and release become detectable (cf. FIG. 4).

A corresponding reaction is to be expected for binding of the first two Na^+ ions to H_2E_1. Moreover, any small effect of incompletely matched exchange of Na^+ for H^+ on the RH421 fluorescence at physiological pH would be concealed because binding of the third Na^+ is electrogenic and generates a large and pH-independent contribution. Nevertheless, the competition between Na^+ and H^+ ions for the same sites is evident in the pH dependence of the half-saturating Na^+ concentration for binding of the first two Na^+ ions (FIG. 2). The fact that, despite the almost complete occupancy of the sites by H^+ under physiological conditions, Na^+ binding is so fast that it could not be resolved so far may be explained by the fact that H^+ ions are extremely small and can exchange between a carboxylate and a water molecule without steric hindrance for (or by) cation that is shedding off its hydration shell to enter the binding site. In addition, a multiply coordinated (alkali) cation and a "free" H_3O^+ ion are an energetically much more favorable combination than a free cation and a protonated carboxylate.

CONCLUSIONS

The placement of two ion-binding sites of the Na,K-ATPase inside of the membrane dielectric, as suggested by the structure of the SR Ca-ATPase and the apparent electroneutrality of cytoplasmic K^+ release and binding of the first two Na^+ ions under physiological conditions, can be explained by a transient binding of two H^+ ions to carboxylate groups in or close to the ion binding sites. This leads to a modification of the post-Albers cycle (cf. FIG. 1A) for the E_1 conformation in the following way:

$$\cdots \rightarrow K_2E_1ATP \rightarrow H_2E_1ATP \rightarrow Na_2E_1ATP \rightarrow Na_3E_1ATP \rightarrow \cdots$$

After binding of two Na^+ ions in exchange for two H^+ ions the highly specific third site becomes available and the third Na^+ ion binds electrogenically. This non-

single file mechanism allows the last ion bound to be released first after the conformation transition to the P-E_2 states. In P-E_2 the ion sites are modified such that they may have shifted slightly within the dielectric, but certainly such that the affinity for Na^+ and H^+ ions has decreased by orders of magnitude, whereas that for K^+ remains almost constant.[10] Due to the low affinity of the binding sites for H^+ in P-E_2 under physiological conditions the sites remain unoccupied, and consequently release of Na^+ and binding of K^+ are found to be electrogenic.

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REFERENCES

1. GLITSCH, H.G. 2001. Electrophysiology of the sodium-potassium-ATPase in cardiac cells. Physiol. Rev. **81:** 1791–1826.
2. JORGENSEN, P.L. & P.A. PEDERSEN. 2001. Structure-function relationships of Na^+, K^+, ATP, or Mg^{2+} binding and energy transduction in Na,K-ATPase. Biochim. Biophys. Acta **1505:** 57–74.
3. LÄUGER, P. 1991. Electrogenic Ion Pumps: 1–313. Sinauer. Sunderland, MA.
4. APELL, H.-J. 1997. Kinetic and energetic aspects of Na^+/K^+-transport cycle steps. Ann. N. Y. Acad. Sci. **834:** 221–230.
5. ALBERS, R.W. 1967. Biochemical aspects of active transport. Annu. Rev. Biochem. **36:** 727–756.
6. POST, R.L., C. HEGYVARY & S. KUME. 1972. Activation by adenosine triphosphate in the phosphorylation kinetics of sodium and potassium ion transport adenosine triphosphatase. J. Biol. Chem. **247:** 6530–6540.
7. WUDDEL, I. & H.-J. APELL. 1995. Electrogenicity of the sodium transport pathway in the Na,K-ATPase probed by charge-pulse experiments. Biophys. J. **69:** 909–921.
8. HOLMGREN, M. *et al.* 2000. Three distinct and sequential steps in the release of sodium ions by the Na^+/K^+-ATPase. Nature **403:** 898–901.
9. DE WEER, P., D.C. GADSBY & R.F. RAKOWSKI. 2000. The Na/K-ATPase: a current-generating enzyme. *In* The Na/K Pump and Related ATPases. K. Taniguchi & S. Kaya, Eds.: 27–34. Elsevier. Amsterdam.
10. APELL, H.J. & S.J. KARLISH. 2001. Functional properties of Na,K-ATPase, and their structural implications, as detected with biophysical techniques. J. Membr. Biol. **180:** 1–9.
11. HILGEMANN, D.W. 1994. Channel-like function of the Na,K pump probed at microsecond resolution in giant membrane patches. Science **263:** 1429–1432.
12. DOMASZEWICZ, W. & H.-J. APELL. 1999. Binding of the third Na^+ ion to the cytoplasmic side of the Na,K-ATPase is electrogenic. FEBS Lett. **458:** 241–246.
13. SCHNEEBERGER, A. & H.-J. APELL. 1999. Ion selectivity of the cytoplasmic binding sites of the Na,K-ATPase: I. Sodium binding is associated with a conformational rearrangement. J. Membr. Biol. **168:** 221–228.
14. BUTSCHER, C., M. ROUDNA & H.-J. APELL. 1999. Electrogenic partial reactions of the SR-Ca-ATPase investigated by a fluorescence method. J. Membr. Biol. **168:** 169–181.
15. PEINELT, C. & H.-J. APELL. 2002. Kinetics of the Ca^{2+}, H^+ and Mg^{2+} interaction with the ion-binding sites of the SR-Ca-ATPase. Biophys. J. **82:** 170–181.
16. SWEADNER, K.J. & C. DONNET. 2001. Structural similarities of Na,K-ATPase and SERCA, the Ca^{2+}-ATPase of the sarcoplasmic reticulum. Biochem. J. **356:** 685–704.

17. TOYOSHIMA, C. *et al.* 2000. Crystal structure of the calcium pump of sarcoplasmatic reticulum at 2.6 Å resolution. Nature **405:** 647–655.
18. APELL, H.-J. *et al*. 1996. Kinetics of the phosphorylation of Na,K-ATPase by inorganic phosphate detected by a fluorescence method. Biochemistry **35:** 10922–10930.
19. PEDERSEN, M. *et al*. 2001. Detection of charge movements in ion pumps by a family of styryl dyes. J. Membr. Biol. **185:** 221–236.
20. PINTSCHOVIUS, J., K. FENDLER & E. BAMBERG. 1999. Charge translocation by the Na^+/K^+-ATPase investigated on solid supported membranes: cytoplasmic cation binding and release. Biophys. J. **76:** 827–836.
21. SCHNEEBERGER, A. & H.-J. APELL. 2001. Ion selectivity of the cytoplasmic binding sites of the Na,K-ATPase: II. Competition of various cations. J. Membr. Biol. **179:** 263–273.