FEBS Letters 532 (2002) 198–202 FEBS 26781

Do H^+ ions obscure electrogenic Na^+ and K^+ binding in the E_1 state of the Na,K-ATPase?

Hans-Jürgen Apell*, Anna Diller

University of Konstanz, Biology, Universitätsstrasse 10, Konstanz 78457 Germany

Received 25 September 2002; revised 26 October 2002; accepted 28 October 2002

First published online 11 November 2002

Edited by Stuart Ferguson

Abstract In contrast to other P-type ATPases, the Na,K-ATPase binding and release of ions on the cytoplasmic side, to the state called E_1, is not electrogenic with the exception of the third Na^+. Since the high-resolution structure of the closely related SR Ca-ATPase in state E_1 revealed the ion-binding sites deep inside the transmembrane part of the protein, the missing electrogenicity in state E_1 can be explained by an obscuring counter-movement of H^+ ions. Evidence for such a mechanism is presented by analysis of pH effects on Na^+ and K^+ binding and by electrogenic H^+ movements in the E_1 conformation of the Na,K-ATPase.

Key words: Active ion transport; Sodium pump; Binding sites; Electrogenicity; Competition

1. Introduction

The Na,K-ATPase is an ion-transport protein of the plasma membrane in animal cells. It maintains the electrochemical potential gradients of Na^+ and K^+ ions across the cytoplasmic membrane [1,2]. Ion transport is facilitated by coupling energy-providing ATP hydrolysis with the ping-pong mechanism of ion translocation [3]. This mechanism was first introduced for the Na,K-ATPase [4,5], and was later extended as the basic principle for all P-type ATPases. In Fig. 1A it is exemplified for the Na,K-ATPase. To analyze the transport at a molecular level the so-called electrogenicity of the reaction steps was investigated, i.e. the charge movements within the interior of the protein in association with the pump-cycle steps. In Fig. 1B hypothetical energy profiles for Na^+ ions in different states are visualized (adapted from [6]). When Na^+ ions are moved across the membrane, in principle all reaction steps involved (Fig. 1) could produce electric charge movements in the transmembrane domain: (1) ion binding, $E_1{\cdot}ATP \rightarrow Na_3E_1{\cdot}ATP$, (2) ion occlusion, $Na_3E_1{\cdot}ATP \rightarrow (Na_3)E_1\text{-}P$, (3) conformational transition, $(Na_3)E_1\text{-}P \rightarrow P\text{-}E_2Na_3$, and (4) ion release, $P\text{-}E_2Na_3 \rightarrow P\text{-}E_2$. To quantify the corresponding charge movements, dielectric distances were introduced, α', α'', β', β'', which express the fraction of the membrane potential that has to be traversed by ions between two neighboring pump states. Consequently, the sum of the coefficients is $\alpha'+\alpha''+\beta'+\beta''=1$ [6].

In numerous publications it has been shown that on the extracellular side of the Na,K-ATPase all ion-binding and release steps are electrogenic and that the occlusion and conformation transitions are electroneutral or of minor electrogenicity ($(Na_3)E_1\text{-}P \rightarrow P\text{-}E_2Na_3$) [7,8]. On the cytoplasmic side, only binding of the third Na^+ ion exhibited a detectable electrogenic contribution [7,9]. This was explained by the assumption that two negatively charged ion-binding sites in E_1 are located in a wide, water-filled vestibule close to the cytoplasmic surface to account for the electroneutrality of the reaction sequence $K_2E_1{\cdot}ATP \rightarrow E_1{\cdot}ATP \rightarrow Na_2E_1{\cdot}ATP$ [9].

When the 3D structure of the SR Ca-ATPase in its Ca_2E_1 conformation was published with a resolution of 2.6 Å it became obvious that both Ca^{2+} ions were bound to well-coordinating sites deep inside the membrane dielectric with a narrow access structure [10]. This explained well the findings that in the SR Ca-ATPase binding and release of Ca^{2+} and H^+ ions at both sides of the membrane are electrogenic and competitive [11,12]. In addition, very recently it was shown that H^+ and K^+ binding to the gastric H,K-ATPase is also electrogenic (A. Diller and H.-J. Apell, unpublished data).

The demonstration of the position of Ca^{2+} ions in the E_1 conformation of the SR Ca-ATPase inside the membrane-spanning parts of the protein [10,13] and the generally agreed structural similarity of Na,K-ATPase and SR Ca-ATPase [13,14] argues that ion binding to the Na,K-ATPase ought to be electrogenic, not only in the $P\text{-}E_2$ form as it was proven [15,16], but also in E_1. However, K^+ binding in E_1 was found to be electroneutral [17–20], and only the binding of the third Na^+ ion appeared to be electrogenic [17,21]. This discrepancy may have two possible explanations: (1) the position of the ion-binding sites in the Na,K-ATPase is significantly different from that in the SR Ca-ATPase and H,K-ATPase, or (2) the electrogenicity of the binding and release steps in E_1 is obscured by simultaneous counter-movement of H^+ ions.

In the following we present evidence that the latter proposal satisfactorily explains the apparent 'irregular' electrogenicity of the Na,K-ATPase on basis of its assumed structural characteristics.

2. Materials and methods

ATP (disodium salt, special quality) was from Boehringer, Mannheim, Germany. RH421 was from Molecular Probes, Eugene, OR, USA. All other reagents were the highest grade commercially available.

*Corresponding author. Fax: (49)-7531-883 183.
E-mail address: h-j.apell@uni-konstanz.de (H.-J. Apell).

0014-5793/02/$22.00
PII: S0014-5793(02)03675-X

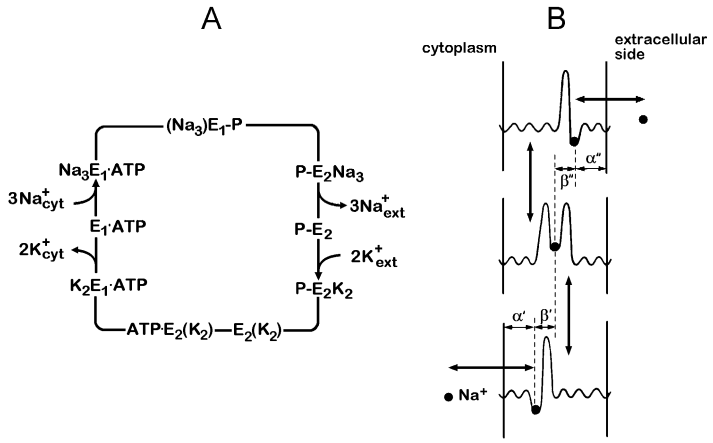

Fig. 1. A: Post–Albers scheme of the physiological pumping cycle of the Na,K-ATPase. E_1 and E_2 are conformations of the enzyme with binding sites facing the cytoplasmic and extracellular medium, respectively. $(Na_3)E_1$-P, ATP·$E_2(K_2)$ and $E_2(K_2)$ refer to occluded states in which ions are unable to exchange with either aqueous phases. B: Hypothetical energy profiles for Na^+ ions along their transport pathway through the transmembrane domain in states Na_3E_1, $(Na_3)E_1$-P, P-E_2Na_3. α', α'', β' and β'' indicate the fractional dielectric coefficients of the Na^+-translocating pathway.

Membrane preparations with a high concentration of Na,K-ATPase (about 5000 pumps per μm^2) were prepared from rabbit kidneys using procedure C of Jørgensen [22]. Their specific ATPase activity was ~2200 μmol P_i per hour and mg protein at 37°C.

Fluorescence measurements were carried out in a Perkin-Elmer LS 50B fluorescence spectrophotometer as described before [9]. The excitation wavelength was set to 580 nm and the emission wavelength to 650 nm. Experiments were performed in standard buffer containing 25 mM histidine, 0.5 mM EDTA. pH was adjusted by the addition of HCl. 200 nM RH421 and 9–10 μg/ml Na,K-ATPase in membrane fragments were added to the thermostated cuvette. After equilibration, a stable fluorescence signal, F_0, was obtained. Titrations were carried out by adding aliquots of the indicated solutions. To allow comparisons between different experiments relative fluorescence changes, $\Delta F/F_0 = (F-F_0)/F_0$, were calculated. According to the mechanism of the styryl dyes fluorescence changes report electrogenic processes in the Na,K-ATPase, fluorescence decreases report an increasing positive charge within the membrane domain of the protein [23]. All experiments were performed at 18 ± 0.5°C.

3. Results

3.1. pH effects on cytoplasmic Na^+ binding

Membrane fragments were equilibrated in standard buffer with 36 mM Tris at various pH values between 8.0 and 5.5 (adjusted with HCl) before aliquots of NaCl were added in the absence of other cations. In Fig. 2A fluorescence changes of three Na^+-titration experiments are shown. The fluorescence change was fitted by the Hill function, $\Delta F([Na^+]) = \Delta F_{max}/(1+([Na^+]/K_{1/2})^{-nH})$, with a (pH-independent) Hill coefficient, n_H, of 1.4 ± 0.1 (S.E.M., $n = 4$) and a pH-dependent half-saturating Na^+ concentration, $K_{1/2}$, (Fig. 2B). The $K_{1/2}$ values revealed that Na^+ binding was competitively affected by the H^+ concentration with a pK of 7.38. The maximal fluorescence change, $\Delta F_{max} = 0.175 \pm 0.003$ (S.E.M., $n = 6$), at saturating Na^+ concentration was almost pH independent (Fig. 2C).

3.2. pH effects on cytoplasmic K^+ binding

K^+-binding experiments were performed in the absence of other cations in a pH range between pH 5.4 and 7.0 (Fig. 3). Since the fluorescence changes were small ($\Delta F_{max} < 8\%$), the experiments were repeated up to five times and averaged to obtain reliable data. At the lowest pH of 5.4 no significant K^+-dependent fluorescence changes were observed. To obtain a buffer pH higher than 7 we tried addition of Tris base to avoid possible cation interactions with the binding sites. However, the presence of Tris produced a 50% reduction of the K^+-induced fluorescence changes when compared to experiments without Tris. Therefore, these data are not included. (The Na^+-titration experiments were not significantly affected by Tris.) The origin of this effect could not be identified so far. The lines drawn in Fig. 3 are fits with the Hill function with a pH-independent half-saturating concentration, $K_{1/2}$, of 22.9 ± 0.1 μM. From this constancy it can be concluded that there was no detectable competitive binding between K^+ and H^+ at the binding sites. K^+ binding is electroneutral at low pH, while at higher pH an electrogenic component of K^+ binding could be detected.

3.3. H^+ binding to the Na,K-ATPase

In the absence of other cations the H^+ concentration dependence of fluorescence intensity in E_1 was studied by pH-titration experiments. Experiments were started in standard buffer with 10 mM $MgCl_2$, pH 7.2. Fluorescence emission was monitored while HCl was added to the cuvette in the fluorescence spectrophotometer, and in parallel, to an identical cuvette with a pH electrode inserted to record pH changes. At the end the pH difference in the two cuvettes was less than 0.1 pH units. The result of three averaged experiments with Na,K-ATPase in E_1 conformation is shown in Fig. 4 (■). Lowering pH to 5.5 produced a 10% fluorescence decrease. In control experiments with ouabain-inhibited Na,K-ATPase no significant RH421 fluorescence response was found upon pH decrease to 5.5. In another experiment 10 mM NaCl and 100 μM ATP were added to the buffer at pH 7.2 so that the enzyme proceeded preferentially to state P-E_2 [24]. This transition led to a fluorescence increase of about 45% (Fig. 4). When a pH titration was then performed the fluorescence decrease reflected H^+ binding to the extracellular sites. The averaged result of three experiments is also shown in Fig. 4 (●). As control, backdoor phosphorylation was performed in the absence of Na^+ and K^+ ions by addition of inorganic phosphate [25]. The results of experiments at pH 7.0 and 6.5 are also included (○). The lines in Fig. 4 are two calculated binding isotherms, $F(pH) = F_\infty - \Delta F_{max}/(1+10^{pH-pK})$,

with the same fluorescence intensity at low pH ($F_\infty - \Delta F_{max} = -0.1$) and high pH ($F_\infty = 0.44$) but with different p$K$, 7.9 ($E_1$) and 5.8 (P-$E_2$).

4. Discussion

On the basis of these results a mechanism is proposed which overcomes the discrepancy of structural [13] and functional [21] constraints for cytoplasmic ion binding and release reactions of the Na,K-ATPase. The required but missing electrogenicity of K^+ and Na^+ binding (or release) has to be discussed in the light of the presented pH effects.

Previous indications that H^+ ions bind to state E_1 came from investigations of backdoor phosphorylation [25]. It was found that in the absence of other monovalent cations the kinetics of the phosphorylation reaction with inorganic phosphate, P_i, were controlled by the concentration of the state $E_2(H_2)$. The transport-reaction sequence for backdoor phosphorylation was found to be $E_1 + 2H^+_{cyt} \rightarrow H_2E_1 \rightarrow E_2(H_2) \rightarrow P\text{-}E_2(H_2) \rightarrow P\text{-}E_2 + 2H^+_{ext}$.

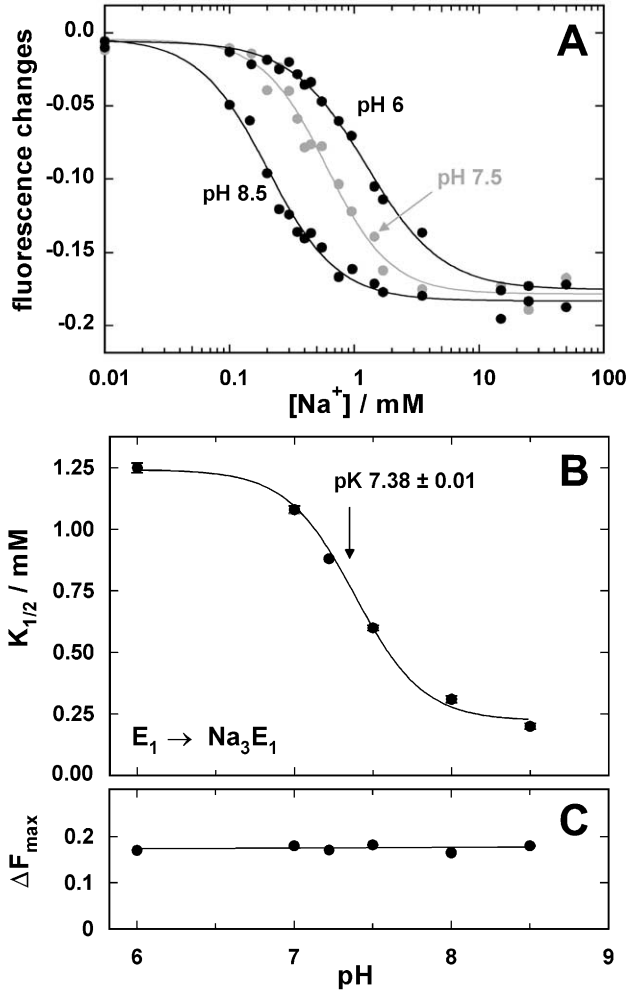

Fig. 2. pH effects on cytoplasmic Na^+ binding. A: The fluorescence decrease reflects the increasing occupancy of the third site by electrogenic Na^+ binding [21]. The concentration dependence could be fitted by the Hill function (see text). B: pH dependence of the half-saturating Na^+ concentration, $K_{1/2}$, from experiments as shown in A, fitted by a Hill function with a pK of 7.38 ($n_H = 2$). C: The maximum fluorescence decrease, ΔF_{max}, of 50 mM cytoplasmic Na^+ binding was pH independent.

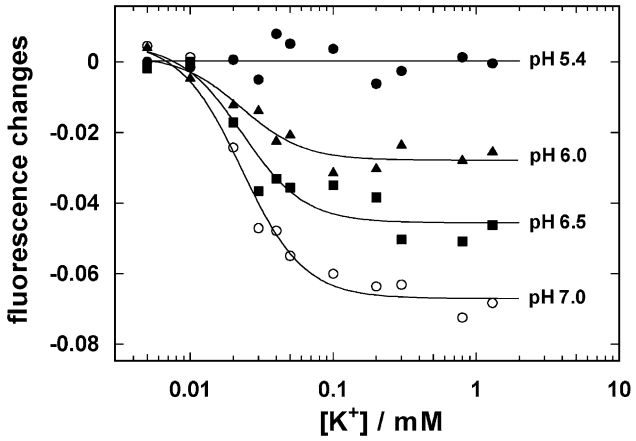

Fig. 3. pH-dependent cytoplasmic K^+ binding. K^+ was detected by RH421 fluorescence changes at the indicated buffer pH. Electrogenic contributions of K^+ binding were found at pH > 5.4. Data were fitted by a Hill function with $K_{1/2} = 22.9$ μM ($n_H = 2$).

When H^+ ions are able to bind to the sites of the Na,K-ATPase in the absence of K^+ and Na^+ it has to be expected that they compete with these ions. This was demonstrated in the experiments shown in Figs. 2 and 3. In the case of competition with Na^+ the half-saturating Na^+ concentration, $K_{1/2}$, showed a distinct pH dependence. It was shown [21] that $K_{1/2}$ represents mainly the equilibrium dissociation constant of the second Na^+ bound, $NaE_1 + Na^+ \rightleftarrows Na_2E_1$. Therefore, we can conclude that binding of at least the second Na^+ is affected by H^+ in such a way that both ions compete for (part of) the same site. From Fig. 2B an apparent pK for this H^+-binding process can be estimated to be in the order of 7.4. The reason that the fluorescence change, ΔF_{max}, is pH independent (Fig. 2C) is that RH421 monitors the electrogenic binding of the third Na^+, which was found to bind to an exclusively Na^+-specific site [21], without competition with H^+.

Replacement of K^+ and H^+, or vice versa, is demonstrated by the results in Fig. 3. It is obvious that K^+ binding is completely electroneutral at low pH, i.e. binding of K^+ is completely compensated by a displacement of one H^+ per

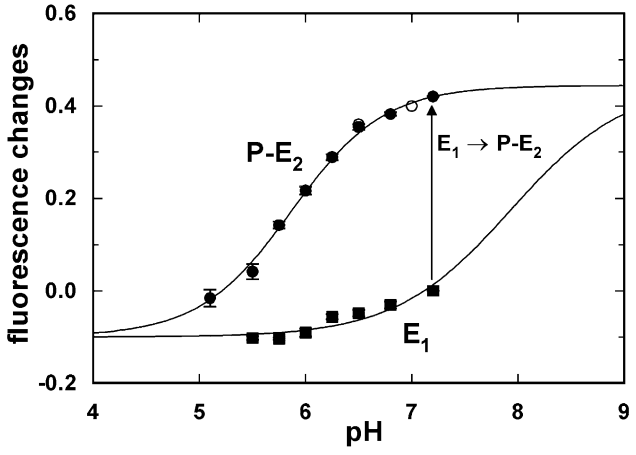

Fig. 4. H^+ binding in both principal enzyme conformations, E_1 (■) and P-E_2 (●). The ATP-induced transition into state P-E_2 was accompanied by a fluorescence increase of ~40% (arrow). When phosphorylated by 'backdoor phosphorylation' with P_i (○), a similar fluorescence increase was obtained.

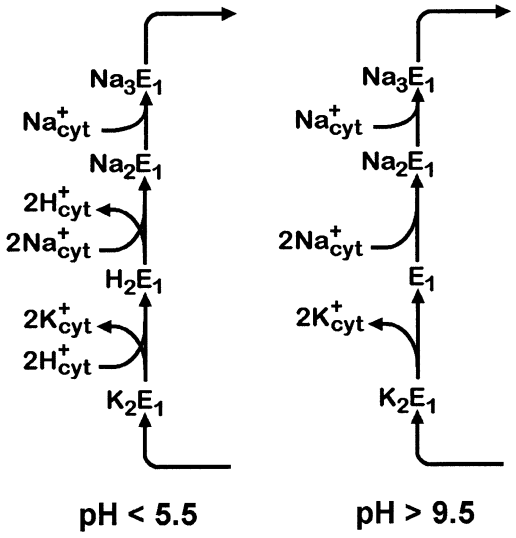

Fig. 5. Extended cytoplasmic part of the Post–Albers pump scheme which accounts for the observed pH effects in the E_1 conformation. Both pathways are alternatives at extremely low or high pH. Under physiological conditions they occur in parallel. Equally strong contribution are expected at the assumed pK of 7.9. The arrows indicate the direction in the normal Na,K-pump mode.

K^+. In buffer with a higher pH the binding sites are less completely occupied by H^+ so that a titration of the sites with K^+ exhibits an electrogenic contribution, as is visible in Fig. 3. The observation that the K^+-binding affinity in E_1, $K_{1/2}$, is not affected by pH indicates that the multiply coordinated K^+ cations fit perfectly into their sites [21], and bound K^+ ions together with 'free' H_3O^+ ions are energetically much more favorable than a free cation and a protonated carboxylate in the (proximity of the) binding site.

Direct H^+ binding could be studied only in a limited pH range, between 5 and 7.2, since substances such as Tris, glycylglycine or $MgOH_2$, which elevate the buffer pH, affected the small RH421 fluorescence response of K^+ as well as of H^+ binding by a so far unknown mechanism. pH-dependent fluorescence changes in E_1 and P-E_2 (Fig. 4) were checked to be specific to H^+ binding. As shown by the calculated binding isotherms the fluorescence decrease can be interpreted by binding of H^+ as congener of K^+. Under the assumption that the fluorescence intensity of empty and occupied ion-binding sites in both principal protein conformations are similar, i.e. $F_\infty(E_1) \approx F_\infty(P\text{-}E_2)$ and $\Delta F_{max}(E_1) \approx \Delta F_{max}(P\text{-}E_2)$, the difference of H^+-binding properties in both conformations is reduced to a shift of the apparent pK of the H^+-binding structures by about two units.

From this evidence we propose an ion-binding and exchange mechanism for the E_1 conformation of the Na,K-ATPase as shown in Fig. 5, in which the cytoplasmic part of the Post–Albers cycle (Fig. 1A) is extended. At high pH (>9.5) the standard pump cycle is valid. At low pH (<5.5) the binding sites are completely occupied by H^+ in the absence of other monovalent cations. Charge movements in Na^+ or K^+ binding or release are compensated by an opposite H^+ movement. With a pK 7.9 for H^+ binding to E_1, at a physiological pH of 7.2 more than 83% of the sites would have bound an H^+ ion, a mixture of both pathways in Fig. 5 will occur, the majority being electroneutral, and binding or release of two Na^+ and K^+ ions will contribute only with minor electrogenicity, in agreement with published findings.

Binding of H^+ ions to the Na,K-ATPase was reported also by Polvani and Blostein [26]. They found in experiments with inside-out vesicles from red blood cells that in the absence of cytoplasmic Na^+ and/or extracellular K^+ ions enzymatic activity of the Na,K-ATPase was nevertheless observed. This activity could be assigned to a replacement of the ion species transported in both directions by protons.

H^+ binding to the cytoplasmic sites allows also an alternative explanation of intriguing experiments with chimera of Na,K-ATPase and H,K-ATPase [27]. Mense and collaborators found that an exchange of three amino acids in the fourth transmembrane segment and of the loop between the third and fourth transmembrane segment from the Na,K-ATPase to the H,K-ATPase sequence produced a phenotype that had at pH 6 and in the absence of Na^+ already 50% of its maximum enzymatic activity with saturating Na^+ [27]. In the light of our data the underlying mechanism could be that the amino acid exchange either switched the Na^+-specific third binding site into an H^+ site, or introduced a condition that signaled to the enzymatic machinery a permanent occupancy of that third site. With such a modification the Na,K-ATPase mimics on principle features of the H,K-ATPase.

In summary, the obvious ability of the two non-Na^+-specific binding sites to bind two H^+ ions in E_1 with an apparent pK that is higher than the cytoplasmic pH can explain the apparently electroneutral Na^+ and K^+ binding. This process conserves besides the strict structural relationship also a mechanistic agreement between Na,K-ATPase, gastric H,K-ATPase and SR Ca-ATPase.

Acknowledgements: The authors thank Milena Roudna for excellent technical assistance. This work was financially supported by the Deutsche Forschungsgemeinschaft (AP45/4) and INTAS (Project 01-0224).

References

[1] Glitsch, H.G. (2001) Physiol. Rev. 81, 1791–1826.
[2] Jørgensen, P.L. and Pedersen, P.A. (2001) Biochim. Biophys. Acta 1505, 57–74.
[3] Apell, H.-J. (1997) Ann. New York Acad. Sci. 834, 221–230.
[4] Albers, R.W. (1967) Annu. Rev. Biochem. 36, 727–756.
[5] Post, R.L., Hegyvary, C. and Kume, S. (1972) J. Biol. Chem. 247, 6530–6540.
[6] Läuger, P. and Apell, H.-J. (1988) Biochim. Biophys. Acta 945, 1–10.
[7] Apell, H.-J. and Karlish, S.J.D. (2001) J. Membr. Biol. 180, 1–9.
[8] de Weer, P., Gadsby, D.C. and Rakowski, R.F. (2000) in: The Na/K Pump and Related ATPases (Taniguchi, K. and Kaya, S., Eds.), pp. 27–34, Elsevier Science, Amsterdam.
[9] Schneeberger, A. and Apell, H.-J. (2000) in: Na/K-ATPase and Related ATPases (Taniguchi, K. and Kaya, S., Eds.), pp. 319–326, Elsevier Science, Amsterdam.
[10] Toyoshima, C., Nakasako, M., Nomura, H. and Ogawa, H. (2000) Nature 405, 647–655.
[11] Butscher, C., Roudna, M. and Apell, H.-J. (1999) J. Membr. Biol. 168, 169–181.
[12] Peinelt, C. and Apell, H.-J. (2000) Biophys. J. 78, 74a.
[13] Toyoshima, C. and Nomura, H. (2002) Nature 418, 605–611.
[14] Sweadner, K.J. and Donnet, C. (2001) Biochem. J. 356, 685–704.
[15] Holmgren, M., Wagg, J., Bezanilla, F., Rakowski, R.F., de Weer, P. and Gadsby, D.C. (2000) Nature 403, 898–901.
[16] Wuddel, I., Stürmer, W. and Apell, H.-J. (1994) in: The Sodium Pump (Bamberg, E. and Schoner, W., Eds.), pp. 577–580, Steinkopf, Darmstadt.
[17] Domaszewicz, W. and Apell, H.-J. (1999) FEBS Lett. 458, 241–246.

[18] Goldshlegger, R., Karlish, S.J., Rephaeli, A. and Stein, W.D. (1987) J. Physiol. 387, 331–355.
[19] Pintschovius, J., Fendler, K. and Bamberg, E. (1999) Biophys. J. 76, 827–836.
[20] Stürmer, W., Bühler, R., Apell, H.-J. and Läuger, P. (1991) J. Membr. Biol. 121, 163–176.
[21] Schneeberger, A. and Apell, H.-J. (2001) J. Membr. Biol. 179, 263–273.
[22] Jørgensen, P.L. (1974) Methods Enzymol. 32, 277–290.
[23] Pedersen, M., Roudna, M., Beutner, S., Birmes, M., Reifers, B., Martin, H.-D. and Apell, H.-J. (2001) J. Membr. Biol. 185, 221–236.
[24] Heyse, S., Wuddel, I., Apell, H.-J. and Stürmer, W. (1994) J. Gen. Physiol. 104, 197–240.
[25] Apell, H.-J., Roudna, M., Corrie, J.E. and Trentham, D.R. (1996) Biochemistry 35, 10922–10930.
[26] Polvani, C. and Blostein, R. (1988) J. Biol. Chem. 263, 16757–16763.
[27] Mense, M., Dunbar, L.A., Blostein, R. and Caplan, M.J. (2000) J. Biol. Chem. 275, 1749–1756.