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Ion Selectivity of the Cytoplasmic Binding Sites of the Na,K-ATPase: II. Competition of Various Cations

A. Schneeberger, H.-J. Apell
Department of Biology, University of Konstanz, Fach M635, D-78457 Konstanz, Germany

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Abstract. In the E_1 state of the Na,K-ATPase all cations present in the cytoplasm compete for the ion binding sites. The mutual effects of mono-, di- and trivalent cations were investigated by experiments with the electrochromic fluorescent dye RH421. Three sites with significantly different properties could be identified. The most unspecific binding site is able to bind all cations, independent of their valence and size. The large organic cation Br_2-Titu^{3+} is bound with the highest affinity (<μM), among the tested divalent cations Ca^{2+} binds the strongest, and Na^+ binds with about the same equilibrium dissociation constant as Mg^{2+} (~0.8 mM). For alkali ions it exhibits binding affinities following the order of $Rb^+ \simeq K^+ > Na^+ > Cs^+ > Li^+$. The second type of binding site is specific for monovalent cations, its binding affinity is higher than that of the first type, for Na^+ ions the equilibrium dissociation constant is < 0.01 mM. Since binding to that site is not electrogenic it has to be close to the cytoplasmic surface. The third site is specific for Na^+, no other ions were found to bind, the binding is electrogenic and the equilibrium dissociation constant is 0.2 mM.

Key words: Sodium pump — Cytoplasmic ion binding — Electrochromic fluorescent dye — Ion transport — Monovalent ions — Magnesium effects

Introduction

The Na,K-ATPase is an integral plasma membrane protein of (virtually) all animal cells that employs the free energy derived from the hydrolysis of ATP to actively transport Na^+ and K^+ ions against their electrochemical potential gradients in a Ping-Pong type mechanism [20, 22]. During the recent years the transport cycle [1, 3, 27] has been broken down into a series of defined reaction steps and their kinetic properties were investigated and characterized [5, 10, 11, 15, 17, 40]. In the case of the sodium transport branch the reaction sequence,

$$3\ Na^+_{cyt} + E_1 \rightarrow Na_3E_1 \rightarrow (Na_3)E_1\text{-}P \rightarrow P\text{-}E_2(Na_3) \rightarrow P\text{-}E_2(Na_2) + Na^+ \rightarrow P\text{-}E_2 + Na^+_{ext},$$

is able to explain all experimental data available. Na^+ occlusion and enzyme phosphorylation as well as the conformational transition E_1 to E_2 and ion release to the extracellular side of the membrane were extensively studied [2, 6, 15–17, 40]. However, the properties of the cytoplasmic ion-binding sites, sodium binding and the competition of various cations at these sites as well as the nature and structure of these sites are only poorly understood so far. Recently, the electrogenicity of cytoplasmic Na^+ binding (in contrast to all other ions) was proven [12, 26, 32] and it was shown that the electrogenic binding of the third Na^+ ion to its (uncharged) site triggers a conformational rearrangement at the ATP-binding site which makes the enzyme competent to be phosphorylated, the next step in the transport cycle [30]. In forward direction three Na^+ ions are necessary for the phosphorylation of the enzyme, and no other ions may replace Na^+. In the second half of the physiological transport cycle 2 K^+ ions are transferred into the cytoplasm, however, it has been shown that a number of congeners, almost all monovalent cations, may replace K^+. In addition, these ions allow by binding at the cytoplasmic side the so-called backdoor phosphorylation by inorganic phosphate [8, 28]

$$E_1 + 2\ X^+_{cyt} \rightarrow X_2E_1 \rightarrow E_2(X_2) \rightarrow P\text{-}E_2X_2.$$

Correspondence to: H.-J. Apell

X^+ may be K^+, Li^+, Rb^+, Cs^+, NH_4^+, Tl^+ or H^+ [4, 14, 23]. This observation indicates that these ions are able to bind to the cytoplasmic ion binding sites, and therefore compete with Na^+ for the sites when they are present simultaneously. But it is also known that the presence of divalent or trivalent cations affects Na^+ binding in terms of modified apparent affinities of Na^+ binding [15, 18, 33]. A previously developed fluorescence method, using the electrochromic styryl dye RH421 [7], could be successfully applied to detect the electrogenic binding of the third Na^+ ion, the only reaction step which was found to produce a reliable response with respect to cytoplasmic ion binding or release [30]. Therefore, an experimental concept has been developed to utilize this single detectable reaction step as a monitor to investigate interaction between and competition of various ions at the cytoplasmic sites.

The aim of this presentation was to elucidate the interaction or competition mechanisms of cations of variable valence with respect to their binding to the ion sites of the Na,K-ATPase and to identify constraints on possible structural characteristics of the ion binding sites.

Materials and Methods

Sodium dodecylsulfate (SDS) was obtained from Pierce Chemical. Phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase, NADH and ATP (disodium salt, special quality) were from Boehringer, Mannheim. The fluorescent dyes RH421 and FITC were from Molecular Probes, Eugene, OR. Dye purity was checked by thin-layer chromatography. All other reagents were the highest grade commercially available.

Na,K-ATPase was prepared from the outer medulla of rabbit kidneys in the form of open membrane fragments using procedure C of Jørgensen [19]. Protein concentration was assessed by the Lowry method, using bovine serum albumin as a standard. Specific ATPase activity was determined by the pyruvate kinase/lactate dehydrogenase assay [34]. The specific activity was in the range of 1900 to 2300 μM P_i per mg protein and h at 37°C. Trypsin-digested protein, the so-called 19 kDa membranes, was prepared according to Capasso et al. [9, 35].

Fluorescence measurements were carried out in a Perkin-Elmer LS 50B fluorescence spectrophotometer as described in the recently published part I of this paper [30]. The excitation wavelength was set to 580 nm and the emission wavelength to 650 nm (slit width 15 nm and 20 nm, respectively). Equilibrium titration experiments were performed in standard buffer containing 25 mM histidine, 0 or 0.5 mM EDTA, pH 7.2. (In some experiments a high ionic strength of the buffer was chosen by addition of 300 mM choline chloride to check for fluorophore artifacts. No significant differences were found.) 200 nM RH421 and 9–10 μg/ml of membrane fragments containing Na,K-ATPase were added to the thermostated cuvette and equilibrated until a stable fluorescence signal, F_0, was obtained. Titrations were carried out by adding small aliquots of the indicated salt solutions from various concentrated stocks until no further changes of fluorescence could be observed. To allow a comparison between different titration experiments relative fluorescence changes, $\Delta F/F_0 = (F - F_0)/F_0$, were calculated (in %) with respect to F_0. Specific fluorescence levels could be assigned to defined states in the pump cycle of the Na,K-ATPase [15]. All experiments were performed at 16 ± 0.5°C.

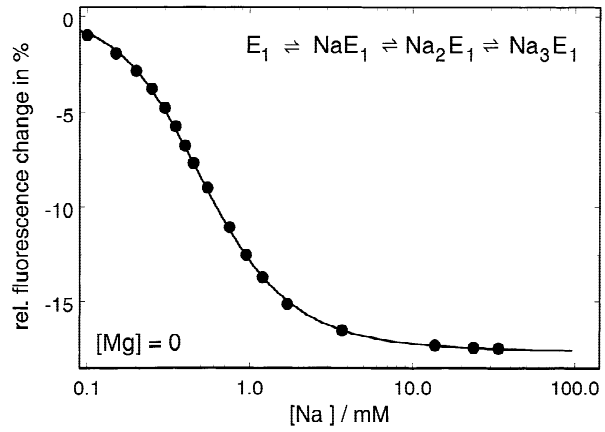

Fig. 1. Electrogenic binding of Na^+ in state E_1 of the Na,K-ATPase detected by RH421. The standard buffer (*see* Materials and Methods) contained 300 mM choline chloride and no Mg^{2+} ions. The line through the data points is a fit according to the mathematical representation of the sequential reaction scheme inserted with the equilibrium dissociation constants $K_1^{Na} = 0.005$ mM, $K_2^{Na} = 0.85$ mM, $K_3^{Na} = 0.25$ mM (*see* text).

Abbreviations

The abbreviations used are: RH421: N-(4-sulfobutyl)-4-[4-(p-dipentylaminophenyl)butadienyl]-pyridinium, inner salt; Br_2-$Titu^{3+}$: 1,3-dibromo-2,4,6-tris (methylisothiouronium) benzene; FITC: fluorescein 5-isothiocyanate (Isomer I).

Results

Binding of cations to the cytoplasmic sites of the Na,K-ATPase was investigated by the RH421 method. Since only Na^+ ions bind electrogenically in the E_1 conformation of the ion pump [12, 30], all ions other than Na^+ should have only a marginal effect on the fluorescence intensity ($\Delta F_{max}/F_0 < 3\%$). In the following it will be shown that this is true for all other monovalent cations, however, some di- and trivalent cations are able to affect the fluorescence intensity of the styryl dye. Competitive interactions at the cytoplasmic sites between Na^+ and any other cation species may be used to study not only their mutual effect but also to determine binding affinities on the basis of reaction schemes presented in the following. The concentration-dependent RH421 fluorescence change as the principally detected process is shown in Fig. 1, where in the absence of Mg^{2+} ions the cooperative Na^+ binding to the E_1 state of the Na,K-ATPase is shown. By numerous titration experiments the dependence of the RH421 fluorescence on Na^+ concentration was determined and analyzed according to the reaction sequence $E_1 \rightleftharpoons NaE_1 \rightleftharpoons Na_2E_1 \rightleftharpoons Na_3E_1$. Since the maximum fluorescence decrease, $\Delta F/F_0$ ($[Na^+] \rightarrow \infty$) can be measured independently, a fit of the numerical representation of the indicated reaction scheme to the

data depends only on the three equilibrium dissociation constants. The determined constants were $K_1^{Na} < 0.01$ mM, $K_2^{Na} = 0.86 \pm 0.13$ mM, $K_3^{Na} = 0.3 \pm 0.07$ mM. It is remarkable that the third binding site has a higher binding affinity than the second one (*see* Discussion).

Antagonistic Effects of Monovalent Ions with Respect to Na^+

K^+ ions are the physiological counter ions to Na^+ in the transport cycle. Alkali metal ions, such as Li^+, Rb^+, Cs^+ and others "congeners" of K^+, are also transported by the Na,K-ATPase. Consequently, they are able to bind to the Na,K-ATPase in its E_1 conformation. When two of these ions are bound they induce in the absence of ATP a conformational transition into an occluded state of E_2. This reaction step was widely used to determine the occlusion capacity of the ion pump by the respective radioactive isotopes of the alkali ions. The binding process itself could not be detected by this method.

To investigate the antagonistic effect between Na^+ and other monovalent cations, two different, complementary approaches may be used: In the absence of other cations but a fixed concentration of a competing ion (K^+ or one of its congeners Li^+, Rb^+, and Cs^+) binding of Na^+ is studied by a stepwise increase of the Na^+ concentration until the saturation of the Na-specific fluorescence decrease is reached. Alternatively, first Na^+ is added up to concentrations at which a saturating fluorescence decrease is obtained, and then by increasing the concentration of K^+ or one of the congeners the fluorescence decrease is reversed. The concentration dependence of the relative fluorescence intensity could be fitted by the Hill function

$$\frac{\Delta F(c)}{F_0} = \Delta F_{max} \cdot \frac{1}{1 + \left(\frac{K_M}{c}\right)^{n_H}} \qquad (1)$$

Experiments of the second type are presented in the following (Fig. 2). To a cuvette with 1 ml of standard buffer 200 nM RH421 and 9 μg/ml membrane fragments were added and the fluorescence intensity was traced until a steady-state level was reached. The presence of 0.5 mM EDTA ensure that no traces of Mg^{2+} or other divalent cations were present. Addition of 3 mM NaCl provided a Na^+ concentration high enough to almost saturate the ion binding sites of the Na,K-ATPase (>5 × $K_{1/2}^{Na}$) and to produce predominantly the state Na_3E_1. Successive additions of CsCl in aliquots between 1 and 50 mM produced a titration of the fluorescence as shown in Fig. 2*A*. The relative fluorescence changes, $\Delta F/F_0$, with respect to the level before addition of Na^+, were plotted against the corresponding concentration of Cs^+ as shown in Fig. 2*B*. The same experiments were also performed with RbCl, KCl, and LiCl. Figure 2*B* shows that the binding affinities follow the order of $Rb^+ \simeq K^+ > Cs^+ > Li^+$. The average half-saturating concentrations determined from sets of 3 experiments for each ion species were $K_{1/2}(Rb^+) = 0.8 \pm 0.05$ mM, $K_{1/2}(K^+) = 0.9 \pm 0.08$ mM, $K_{1/2}(Cs^+) = 12.0 \pm 1.37$ mM, and $K_{1/2}(Li^+) = 32.0 \pm 2.9$ mM.

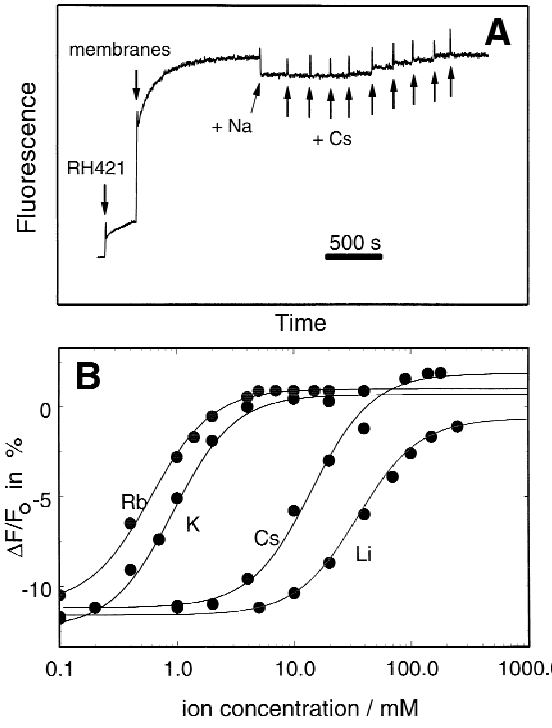

Fig. 2. Antagonistic effect of alkali ion binding in state E_1 of the Na,K-ATPase detected by RH421 fluorescence changes. (*A*) Original fluorescence trace of a Cs^+ titration experiment starting from state Na_3E_1 in which the ion binding sites were virtually saturated with Na^+. This state is visible by the fluorescence decrease of about 12% upon addition of 3 mM NaCl ("+ Na"). The final Cs^+ concentration was 190 mM. (*B*) Representation of the analyses of titration experiments similar to that shown in panel A performed with the alkali ions, Rb^+, K^+, Cs^+, and Li^+, in form of a semilogarithmic Hill-plot. A shift of the half-saturating ion concentration to higher values indicates an apparently reduced binding affinity. The data were fitted by Eq. (1) with a Hill coefficient, n_H, of 1.62 for all ions and with half-saturating ion concentrations of $K_{1/2}(Rb^+) = 0.7 \pm 0.07$ mM, $K_{1/2}(K^+) = 1.0 \pm 0.16$ mM, $K_{1/2}(Cs^+) = 11.7 \pm 1.37$ mM, and $K_{1/2}(Li^+) = 34.8 \pm 4.5$ mM.

Binding of Divalent Cations and Their Antagonistic Effects with Respect to Na^+

When experiments with divalent cations were performed, similar to that with Na^+ shown in Fig. 1, a complex behavior of the RH421 fluorescence was found upon addition of $MgCl_2$, $CaCl_2$, $SrCl_2$, and $BaCl_2$ up to 100 mM. Control experiments proved that part of the effect was caused by an unspecific interaction of the divalent

cations with the membrane fragments. As such controls membrane fragments were incubated at 58°C for one hr, after this period no enzymatic activity and no electrogenic Na^+ binding could be detected. With this inactivated enzyme and also with ouabain-inhibited ion pumps the titration experiments with divalent cations were repeated and a small fluorescence increase (<3%) was found at low concentrations. At higher concentrations the unspecific effect was an almost linear fluorescence decrease of about 8% when the concentration of the divalent ions was increased up to 100 mM. A similar concentration-dependent fluorescence behavior was found also when vesicle preparations, which consisted of phospholipids without protein, were used instead of membranes containing inactivated Na,K-ATPase. From titration experiments with native and denatured membrane preparations the unspecific fluorescence changes were determined under otherwise identical conditions and subtracted from the fluorescence changes of the untreated membranes at the same ion concentrations. The difference was plotted as Na,K-ATPase-specific signals (Fig. 3*A*). While Sr^{2+} and Ba^{2+} cause a fluorescence decrease in the order of 15% at a concentration of 100 mM, a biphasic behavior was found in the case of Mg^{2+} and Ca^{2+}. It seemed that in the case of the Ca^{2+} titration both effects, increase and decrease, almost canceled out each other. A significant initial increase was found only for Mg^{2+} ions. The Ca^{2+}-induced effect, $\Delta F_{max}/F_0$, was $\leq$ 3% and cannot be regarded as significant. The decaying part of the fluorescence intensity could be fitted with a simple binding isotherm with a K_M of 15 mM for all four ion species. The highly specific effect observed with Mg^{2+} can be fitted with a sum of two binding isotherms with $K_{M,1} = 0.5$ mM and $K_{M,2} = 15$ mM.

The effect of the presence of 10 mM divalent cations in the buffer on Na^+ binding is shown in Fig. 3*B*. A Na^+-titration experiment in the absence of divalent ions is shown for comparison (dashed line). Figure 3*B* shows that the presence of 10 mM Mg^{2+} or Ca^{2+} induced a significant reduction of the apparent Na^+-binding affinity from $K_M = 0.6$ to 9.8 mM (Mg^{2+}) and 47 mM (Ca^{2+}), while the Hill coefficient, $n_H = 1.7$, obtained from the fits to the data was increased when compared to $n_H = 1.46$ in the absence of divalent cations. The presence of Sr^{2+} and Ba^{2+} affected not only the half-saturating Na^+ concentration which increased to 2.44 mM (Ba^{2+}) and 12 mM (Sr^{2+}), but reduced the Hill coefficient to 0.7 for both ions. The decrease of the Hill coefficient from 1.7 to a value below 1 indicates a significantly altered cooperativity in the Na^+ binding process.

The observed antagonistic effect of Mg^{2+} and Na^+ ions is of potential physiological importance, and was therefore investigated in detail. The concentration of the other divalent cations in the cytoplasm may be neglected under physiological conditions. In Fig. 4*A* it is demonstrated that in state E_1 the high (apparent) Na^+ affinity of the ion-binding sites with a K^{Na} of about 0.67 ± 0.02 mM in the absence of Mg^{2+} can be apparently reduced to K^{Na} = 8.7 ± 0.4 mM by addition of 10 mM $MgCl_2$ during the course of the titration experiment. In the data shown the Mg^{2+} concentration was raised in the presence of 3 mM Na^+. The same experiments were performed with varying Na^+ concentrations at which Mg^{2+} was added (*not shown*). The resulting "low-affinity" Na^+ binding curves coincided for all experiments (corresponding to the upper solid line in Fig. 4*A*). The same transition from high to apparent low affinity could also be observed when Ca^{2+}, Sr^{2+}, or Ba^{2+} was added instead of Mg^{2+}; (*data not shown.*) In Fig. 4*B* the RH421 fluorescence increase

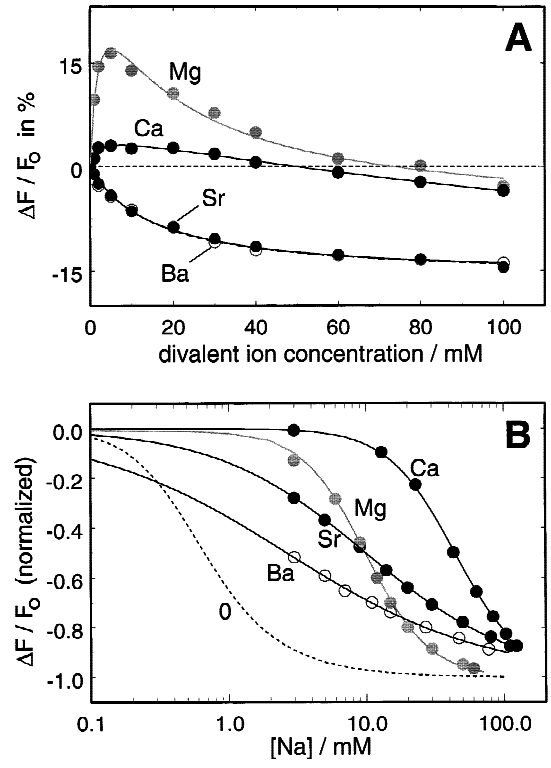

Fig. 3. Binding of divalent cations to the Na,K-ATPase detected by changes of the RH421 fluorescence intensity. (*A*) Effect of binding of Mg^{2+}, Ca^{2+}, Sr^{2+}, and Ba^{2+} in state E_1 on RH421 fluorescence in the absence of other cations. Common to all four ion species was a low affinity effect with a half-saturating concentration of ~15 mM that was represented by a relative decrease in the order of 15% at 100 mM. In the case of Ca^{2+} this decrease was mostly compensated by an almost corresponding increase with higher affinity, in the case of Mg^{2+} a dominating increase with a half-saturating Mg^{2+} concentration of ~0.5 mM could be detected. (*B*) The antagonistic effect of the divalent cations on Na^+ binding is demonstrated by Na^+ titration experiments in the absence (0) and presence of 10 mM of the indicated divalent ions. All divalent cations led to a shift of the fluorescence decrease which corresponds to a decrease of the apparent binding affinity for Na^+. In addition Sr^{2+} and Ba^{2+} altered the binding kinetics for Na^+ significantly (Hill coefficient $n_H < 1$) as can be seen from the less steep concentration dependence.

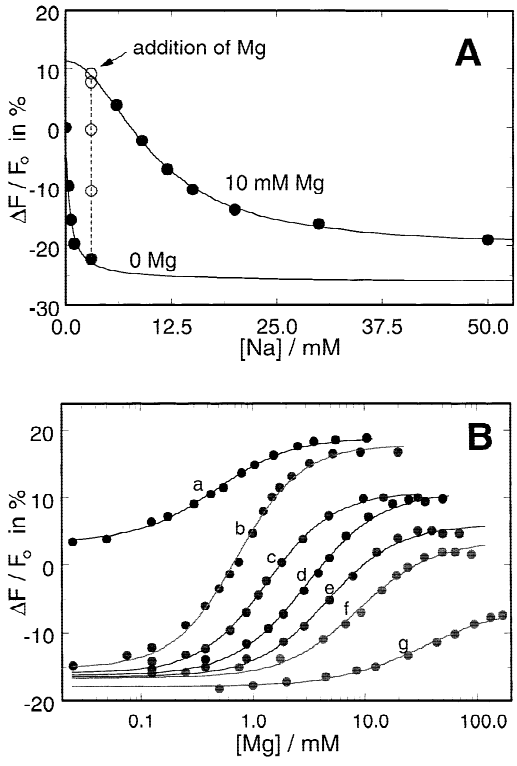

Fig. 4. Na^+/Mg^{2+} antagonism at the ion binding sites in state E_1 as detected by RH421. The fluorescence changes were calculated with respect to the fluorescence intensity prior the addition of any cations. (*A*) Transition from high-affinity Na^+ binding to low-affinity binding by subsequent addition of 10 mM Mg^{2+}. The half-saturating Na^+ concentration increases from 0.6 mM to about 9.2 mM. (*B*) Correspondingly it could be shown that the Mg^{2+} ions induced reversal of the Na^+ effect on the RH421 fluorescence. Increasing concentrations of Na^+, 0 (a), 2.5 mM (b), 5 mM (c), 7.5 mM (d), 10 (e), 15 mM (f), 50 mM (g) required higher Mg^{2+} concentrations to produce the fluorescence increase that reflects the removal of the electrogenically bound Na^+ ion from its (specific) binding site. All sets of data may be fitted by a Hill function (Eq. 1) with the same Hill coefficient of $n_H = 1.33$.

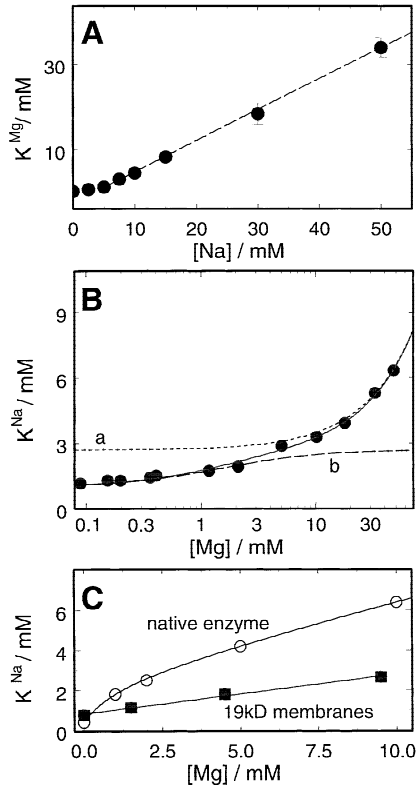

Fig. 5. Comparison of the mutual displacement of Na^+ and Mg^{2+} from their binding site(s) in the E_1 state of the Na,K-ATPase. (*A*) Half-saturating Mg^{2+} concentration, K^{Mg}, as a function of the Na^+ concentration present in the buffer. The values of K^{Mg} above 5 mM Na^+ can be fitted by a regression line (dashed line) as expected from competitive binding. (*B*) Half-saturating Na^+ concentrations, K^{Na}, as a function of the Mg^{2+} concentration in the buffer. The complex dependence on the Mg^{2+} concentration can be described by two independent processes, a competitive binding with low affinity (line a) and a noncompetitive (and saturating) binding with high affinity (line b). The total effect of both mechanisms fitted the data satisfactorily (solid line). (*C*) Comparison of the half-saturating Na^+ concentration, K^{Na}, obtained from native enzyme and 19 kD membranes. In contrast to the native enzyme, the trypsinized ion pumps show no saturating component of Mg^{2+} binding, the straight line is an indication of a competitive process between Na^+ and Mg^{2+}. Both series of experiments were performed with protein from the same preparation.

upon Mg^{2+} addition in the presence of various Na^+ concentrations between 0 and 50 mM is plotted as function of the Mg^{2+} concentration. Trace e in Fig. 4*B* corresponds to the course of the Mg^{2+} additions in Fig. 4*A* as indicated there by the dashed line. Trace a shows in an expanded presentation the rising phase of fluorescence signal for the Mg^{2+} trace in Fig. 3*A*. All Mg^{2+}-titration experiments could be fitted reasonably with a Hill coefficient of 1.33. When the Hill coefficient was varied by the fitting procedure optimal fits were found with similar half-saturating Mg^{2+} concentrations, K^{Mg}, however, the Hill coefficients varied between 1 and 1.5. The shift of K^{Mg} to higher values with increasing Na^+ concentration indicates that Mg^{2+} binds to a site which is affected by Na^+ in a competitive manner. The dependence of K^{Mg} on the Na^+ concentration is shown in Fig. 5*A*. The data presented are the average of three measurements, at most Na^+ concentrations the standard error of the mean is smaller than the size of the data point. Below a Na^+ concentration of 5 mM the value of K^{Mg} was not significantly affected, above 5 mM the half-saturating Mg^{2+} concentration increased linearly with the Na^+ concentration. The latter behavior may be described by the mechanism of competitive binding of both cations,

$$K(\mathrm{Mg}) \equiv K^{\mathrm{Mg}}([\mathrm{Na}]) = K^{\mathrm{Mg}}(0\ \mathrm{Na}) \times \left(1 + \frac{[\mathrm{Na}]}{K^{\mathrm{Na}}}\right) \qquad (2)$$

With the value of $K^{Mg}(0\ Na) = 0.5$ mM and the slope of

the regression line in Fig. 5*A* an apparent K^{Na} was determined to be 0.4 mM which is consistently close to the experimentally obtained (apparent) value of 0.67 mM (Fig. 1).

When present in the buffer (and therefore, also in or close to the ion binding sites) the effect of Mg^{2+} on the half-saturating Na^+ concentration, K^{Na}, is more complex (Fig. 5*B*). The simplest mechanism which fitted K^{Na} as function of the Mg^{2+} concentration was obtained by the assumption of two processes, a noncompetitive inhibition by Mg^{2+} with an affinity of ~1.5 mM (trace b), and a competitive inhibition which controlled the half-saturating Na^+ concentration above 5 mM Mg^{2+} (trace a). With an equation corresponding to Eq. (2) an apparent K^{Mg} of ~2 mM was determined (with $K^{Na}(0\ Mg) = 0.5$ mM). The competitive process is proposed to occur at/or in proximity to the ion-binding sites. To get additional information on the mechanism of the noncompetitive process complementary experiments were performed in which Na^+-binding was studied with 19 kDa membranes in the absence and presence of various Mg^{2+} concentrations (Fig. 5*C*). In the case of the truncated Na,K-ATPase in which most of its cytoplasmic part was removed, and the enzymatic machinery of the protein was missing, only the competitive process between Na^+ and Mg^{2+} was observed. It is known from recent experiments [35] that in the 19 kDa preparation the binding sites are accessible from the cytoplasmic side, however, the Na^+ affinity is slightly reduced ($K^{Na} = 0.83$ mM in the absence of Mg^{2+}). Therefore, it may be expected that also the (apparent) half-saturating Mg^{2+} concentration obtained in these competition experiments is altered. From the data shown in Fig. 5*C* for the 19 kDa membranes a K^{Mg} of ~4.3 mM was calculated. The disappearance of the noncompetitive process indicates that it has to be related to the removed parts of the protein. An obvious site could be the nucleotide binding site, since it is well known that ATP interacts with the ion pump as a $Mg \cdot ATP$ complex, and that Mg^{2+} is bound during backdoor phosphorylation by inorganic phosphate [29].

To obtain additional information on the nature of the Mg^{2+}-binding moiety, Mg^{2+} titration experiments were performed in the absence of Na^+ at various pH values of the buffer. The buffer used in these experiments consisted of 25 mM histidine and 20 mM TRIS, the pH was adjusted by addition of HCl. The Mg^{2+}-induced fluorescence increase (as shown in Fig. 4*B*) was measured in the pH range between 5.8 and 8. The titration curves at each pH were fitted by the Hill function (Eq. 1) and half-saturating Mg^{2+} concentrations, K^{Mg}, were obtained as plotted in Fig. 6. The Hill coefficient of the Mg^{2+} binding curves were ~1 throughout. The pH dependence of K^{Mg} could be reproduced by the Henderson-Hasselbalch equation,

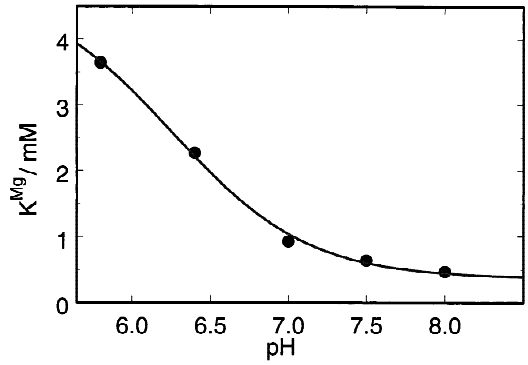

Fig. 6. pH dependence of Mg^{2+} binding to its binding moiety on the cytoplasmic side of the Na,K-ATPase. From Mg^{2+} titrations at specific pH values of the buffer the half-saturating concentrations, K^{Mg}, were obtained by fits with Michaelis-Menten functions, $\Delta F([Mg])/F_0 = \Delta F(\infty) \cdot [Mg]/(K^{Mg} + [Mg])$, and plotted as a function of the buffer pH. The line is a fit to the data points according to the Henderson-Hasselbalch equation (Eq. 3). The pK of the curve was determined to be 6.25.

$$pH = pK + \log \frac{\alpha}{1 - \alpha}, \tag{3}$$

where α is the dissociation ratio of an acidic group which is involved in Mg^{2+} binding and which can be titrated in the pH range covered by the experiments. The fit to the data shown is in agreement with the assumption that Mg^{2+} binding is pH dependent and is affected by a negatively charged side chain of an amino acid, which recombines with a proton at low pH. From Fig. 6 the apparent pK of this group was determined to be 6.25.

Binding of Trivalent Br_2-Titu^{3+} and Its Antagonistic Effects with Respect to Na^+

As has been demonstrated a couple of years ago, the large trivalent organic cation Br_2-Titu^{3+} binds to the Na,K-ATPase in the E_1 conformation and affects Na^+ binding [18]. To investigate the underlying mechanism in detail Na^+ titration experiments were performed in the presence of μmolar concentrations of Br_2-Titu^{3+}, from which half-saturating concentrations of Na^+ binding were determined as detected by RH421 experiments. It was found that the Na^+ binding affinity was decreased with increasing Br_2-Titu^{3+} (Fig. 7). The half-saturating Na^+ concentrations, K^{Na}, could be modeled satisfactorily according to the reaction mechanism shown in Fig. 7*B*, but not with a scheme including a state with mixed binding of Na^+ and Br_2-Titu^{3+}. With the constants of Na^+ binding as given in Fig. 1 the equilibrium dissociation constant for Br_2-Titu^{3+} was calculated to be 6.4 nM. The curved shape of the fitting curve, unexpected for competitive binding, is caused by the fact that three Na^+ ions bind in contrast to the single competitor ion. For very

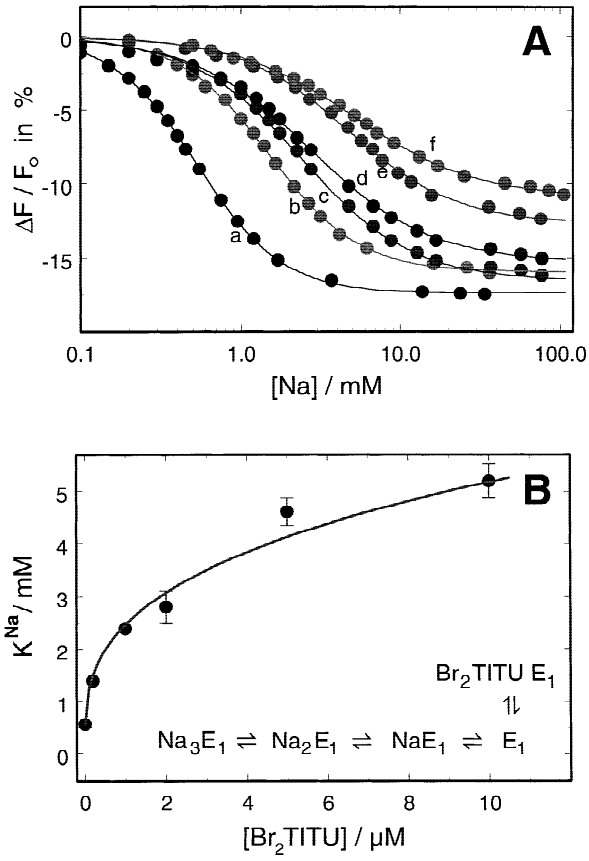

Fig. 7. Effect of the trivalent organic cation Br_2-$Titu^{3+}$ on the Na^+ binding to the Na,K-ATPase. (*A*) Titration of the cytoplasmic binding sites with Na^+ in the absence (a) and presence of various concentrations of the competitor Br_2-$Titu^{3+}$ (b: 0.2 μM, c: 1 μM, d: 2 μM, e: 5μM, f: 10 μM). To determine the half-saturating Na^+ concentrations, K^{Na}, the experiments were fitted with Hill functions (Eq. 1). The maximum fluorescence changes were $\Delta F_{max} = 15.8 \pm 1.8$, the Hill coefficients $n_H = 1.34 \pm 0.2$. (*B*) The values of K^{Na} were plotted against the concentration of Br_2-$Titu^{3+}$ and fitted by the analytically determined function of K^{Na} from the reaction scheme shown as inset. The only free parameter was the equilibrium dissociation constant of Br_2-$Titu^{3+}$ since those of Na^+ binding were set as obtained independently (Fig. 1).

high concentrations of the competitor the calculated value of K^{Na} increase linearly with [Br_2-$Titu^{3+}$], as it is expected from competitive binding (*not shown*).

Discussion

The data presented in this paper are all related to the interaction of cations with the cytoplasmic ion-binding sites of the Na,K-ATPase. They allow, together with other recent findings, an extensive characterization of the properties of these sites and the formulation of constraints on structure-function relations for the sodium pump.

Binding of Na^+ in the Absence of Other Cations

In the sequence of ion binding and release steps of monovalent cations in the E_1 conformation of the Na,K-ATPase only a single step can be detected directly by the methods known so far, which is binding of the third Na^+ ion, $Na_2E_1 \rightleftharpoons Na_3E_1$. This reaction can be monitored by fluorescent probes such as RH421 and FITC [30] or by direct electric measurements of changes of the membrane capacitance, and it has been shown that all three methods detect identical behavior of the protein as a function of the Na^+ concentration [12]. Since the experiments with RH421 as well as the changes of the membrane capacitance are related to electrogenic processes, it could be proven that binding of the third Na^+ ion is the only electrogenic event on the cytoplasmic side of the Na,K-ATPase. The observation that exclusively Na^+ is able to bind as a third ion indicates that this site is extremely selective for Na^+; no other ions have been found to introduce a positive charge into the protein dielectric. From recent studies is was shown that the Na^+ ion has to cross about 25% of membrane dielectric (or membrane potential) to reach its site [12, 25].

The special role of the third Na^+-binding site can be seen also from the titrations with Na^+ only. The concentration dependence of the RH421 fluorescence as shown in Fig. 1 revealed that under the assumption of sequential binding of three Na^+ ions a fit of the data always led to the result that the equilibrium dissociation constant of the third Na^+ was smaller than that of the second (or: that the affinity of the third site for Na^+ was higher than for the second). This may occur only in the case that the third site becomes available after the first two sites are occupied, otherwise the third site would be filled before the second.

This observation has implications on the mechanism of ion binding which may be proposed on the basis of either of two models published for the SR-Ca-ATPase. In the first model Tanford et al. proposed a so-called jaw-closing model [36] in which by a single-file mechanism the second Ca^{2+} binding site is formed "on top" of the first by a structural change of the entrance funnel of the protein. The second, more recent proposal dispenses with the single file mechanism and proposes two sites which are positioned in the protein abreast [24]. Menguy and collaborators suggested that the cytoplasmic loop located between transmembrane segments 6 and 7, L6/7, controls the access to the first and second Ca^{2+} binding site, and that this process could be related with the occlusion of the first Ca^{2+} by a movement of the L6/7 over the first site, thus opening the second [24]. The recent publication of the crystal structure of the SR Ca-ATPase has proven the side by side binding of Ca^{2+} ions, although the loop L6/7 has been given a different assignment as to how it affects Ca^{2+} binding [37]. Due to the

fact that the structure of the SR-Ca-ATPase and the Na,K-ATPase are closely related [38, 39] an analogous conclusion may be drawn on the basis of the recent proposal, especially since it was found that in the case of the Na,K-ATPase the L6/7 is of similar importance for the interaction of cations with the cytoplasmic ion sites [35]. The side-by-side model would imply that the third, Na^+-specific binding site becomes accessible by a movement of L6/7 only after two Na^+ ions are already bound and "pre-occluded" [31].

Competition Between Monovalent Cations

In the absence of a detectable signal specific to binding of K^+ (or its congeners Li^+, Rb^+, Cs^+) this process can be studied only in competition with Na^+, or by the subsequent conformational transition, $K_2E_2 \rightleftharpoons E_2(K_2)$, if the enzyme was labeled with FITC [21, 30]. It was found that all these ions (and in addition NH_4^+ and Tl^+, *not shown*) are able to displace (at least the electrogenically bound) Na^+ from its site(s) (Fig. 2). Obviously the mechanism is not caused by a direct interaction of ions but by a shift of the steady-state population to the left side in the following reaction scheme,

$$E_2(K_2) \rightleftharpoons K_2E_1 \rightleftharpoons KE_1 \rightleftharpoons E_1 \rightleftharpoons NaE_1 \rightleftharpoons Na_2E_1 \rightleftharpoons Na_3E_1,$$

which is possible under the reasonable assumption that forward and backward reaction steps occur with rate constants which are high compared to the time of the experimental resolution. In the absence of $Mg \cdot ATP$ and inorganic phosphate the pump is confined to the reaction sequence shown in the scheme above.

According to the varying affinities of K^+ and its congeners different concentrations were needed to reverse Na^+ binding. The half-saturating concentrations of the ions competing with Na^+ may be plotted against the radius of the respective dehydrated ions (Fig. 8). To include into Fig. 8 also some information on the binding affinity for Na^+ we estimated a (relative) effect for Na^+ from independently determined half-saturating concentrations for Na^+ and K^+ in the absence of all other cations as they were obtained from experiments with FITC-labeled Na,K-ATPase [30], in which the ratio of $K_{1/2}{}^{Na}/K_{1/2}{}^{K}$ was 4.2. From the dependence of the $K_{1/2}$ values it can be seen that (dehydrated) ions with a radius of about 1.4 Å were bound strongest to the ion site. Smaller cations, such as Na^+ and Li^+, may easily enter the site, however, their coordination with the elements of the protein was significantly less stable. The larger cation Cs^+ apparently had to deform the binding site to fit into the pocket so that the higher energy needed for this process reduced the binding affinity by more than an order of magnitude when compared with K^+ or Rb^+. The results summarized in Fig. 8 indicate that the structure ion binding sites has to be rather rigid to produce the observed tenfold changes in binding affinity when the radius of the ion differed by +0.2 Å or −0.5 Å.

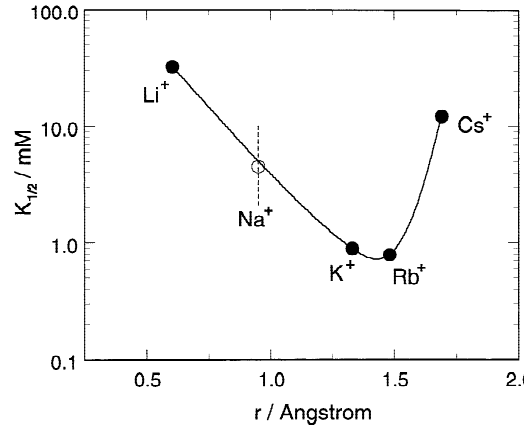

Fig. 8. Competition between monovalent cations at the cytoplasmic binding sites. The half-saturating concentrations, $K_{1/2}$, of the alkali ions were determined in the presence of 3 mM Na^+ (Fig. 2*B*) and plotted against the radius of the dehydrated ions. The solid line was drawn to guide the eye. The vertical dashed line indicates the radius of the Na^+ ion, the open circle represents an estimated value for Na^+ binding from the ratio of half-saturating concentrations for Na^+ and K^+ binding in the absence of other ions [28].

Effects of Divalent Cations

It was observed that addition of divalent cations in the absence of Na^+ and K^+ produced changes of the RH421 fluorescence intensity. Part of the observed effect was an unspecific interaction of the ions with the lipid membrane, however, even when this contribution was subtracted significant changes were detected (Fig. 3). The physiologically relevant Mg^{2+} ion produced a biphasic fluorescence response as a function of its concentration in the buffer while Ca^{2+} had no significant effect ($|\Delta F/F_0| \leq 3\%$), and Sr^{2+} and Ba^{2+} produced a significant fluorescence decrease at concentrations higher than 10 mM, which were probably correlated with an (irreversible) inactivation of the ion pump. Because of the large radii of Sr^{2+} and Ba^{2+} (1.34 Å and 1.49 Å, respectively) and their two positive charges it may be excluded that they produced the decrease of RH fluorescence by binding electrogenically to the Na^+-specific site within the protein dielectric. Therefore, we assume that the Na,K-ATPase is affected in an unspecific way by high concentrations of the divalent cations. The interaction may occur directly or on the lipid molecules of the surrounding membrane; such interactions are known to inhibit the ion pump [13].

The biphasic fluorescence change which depends on the Mg^{2+} concentration has a rising phase with a $K^{Mg} \sim$ 0.5 mM, which is comparable to the concentrations that are characteristic for Mg^{2+} binding to both, the competi-

tive and noncompetitive sites (Fig. 5). According to the electrochromic mechanism an increase of the RH421 fluorescence represents a less positive electric potential in the protein/membrane dielectric when Mg^{2+} is bound. Comparable effects were found also in the case of 19 kDa membranes. This process is not understood so far. For example it could be induced by a rearrangement of carboxylic side chains of amino acids or the dipoles of the transmembrane α helices (detailed investigations are planned). The decreasing phase of the concentration dependent fluorescence change at high concentrations is comparable to the Sr^{2+} or Ba^{2+} induced effect.

Figure 3*B* shows that the divalent cations affect Na^+ binding. Mg^{2+} and Ca^{2+} mainly shift the half-saturating Na^+ concentrations to higher values, the Hill coefficient is not strongly increased. It is obvious that Ca^{2+} is more tightly bound than Mg^{2+} since a fivefold higher Na^+ concentration is needed to get the third Na^+ ion bound in the presence of 10 mM of the divalent cation. The cytoplasmic loop L6/7 was recently proposed as a possible candidate for the site to which the divalent cations could bind [35]. This observation is in agreement with corresponding findings for the Ca-ATPase [24].

Remarkable in Fig. 3*B* is the significantly different concentration dependence in the presence of 10 mM Sr^{2+} and Ba^{2+}. The data were fitted by the Hill function (Eq. 1) with a Hill coefficient of 0.7, which is an indication for a negative cooperativity. A possible explanation could be that (in contrast to the findings in the presence of Mg^{2+} and Ca^{2+}) binding of the first Na^+ ion to its assigned site on the pump significantly destabilizes binding of the (larger) Ba^{2+} and Sr^{2+} ions.

Physiologically relevant for the function of the Na,K-ATPase is mainly Mg^{2+} since the concentration of other divalent cations is controlled strictly and kept well below 1 mM. Figures 4*A* and 5 demonstrate that at physiological concentrations of 2 mM up to 10 mM Mg^{2+} the interaction of Na^+ with its binding sites is significantly affected. From the results shown in Fig. 5 it may be derived that Na^+ and Mg^{2+} compete for the same site or, if the sites are not identical, that they have to be so close to each other that they sterically overlap and exclude simultaneous occupation by both species.

Due to the fact that Mg^{2+} binding does not produce a fluorescence decrease at concentrations below 10 mM in the RH421 experiments it may be excluded that the competition occurs at the third, "Na-specific" site. Initially each of the other two Na^+ sites which are discriminated by their equilibrium dissociation constants could be proposed to interfere with the Mg^{2+}-binding site. The search for the simplest reaction scheme which convincingly reproduces the titration experiments led to the reaction model shown in Fig. 9*A*. In this model it is assumed that Mg^{2+} binds to or affects the site with the lower Na^+ affinity and that a mixed state is possible in

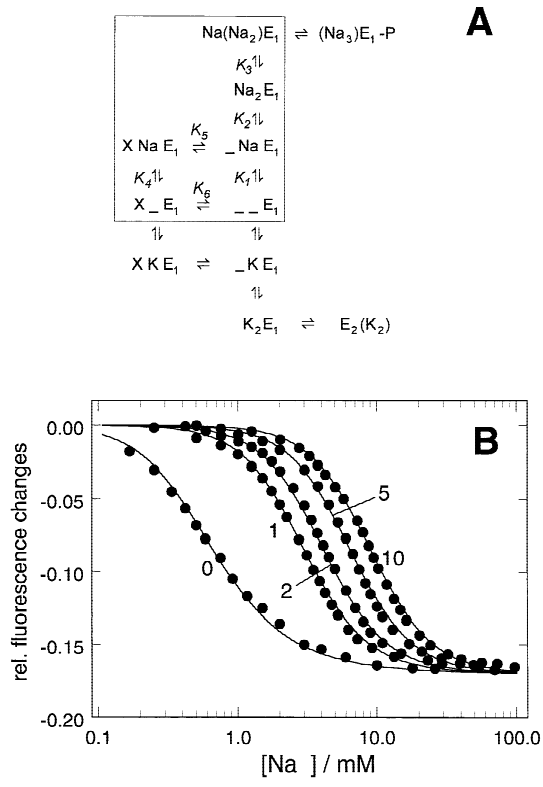

Fig. 9. Schematic representation of the competition of cations for the cytoplasmic binding sites of the Na,K-ATPase. (*A*) Reaction model for a satisfactory simulation of all experiments presented in this paper. "X" signifies a cation of any valence, e.g., Mg^{2+}, Ca^{2+}, Br_2-Titu^{3+}. "_" represents an empty and accessible site. The equilibrium dissociation constants, $K_1, \ldots, K_6$, were used when simulating the Na^+/Mg^{2+} antagonism. The box denotes the possible reaction steps when only Na^+ and Mg^{2+} were present in the buffer. (*B*) Inhibition of Na^+ binding in the presence of various Mg^{2+} concentrations. The solid lines were calculated according to the framed part of the scheme shown in *A*. For an explanation of the choice of the equilibrium dissociation constants *see* Discussion. The numbers connected to the data indicate the Mg^{2+} concentration (in mM) present in these experiments.

which Na^+ is able to bind to the high-affinity site while the other is still blocked by Mg^{2+} (or any other cation). An application of this model is shown in Fig. 9*B*. To fit the fluorescence decrease of the Mg^{2+}-dependent Na^+ titration experiments the boxed six-state system in Fig. 9*A* was used. The equilibrium dissociation constants for Na^+ binding, K_1, K_2, K_3, were taken from the experiment in Fig. 1. In addition it was assumed that Na^+ or Mg^{2+} binding to one site would be independent of the presence of another ion at the second site, i.e., $K_1 = K_4$ and $K_5 = K_6$. Under this assumption the Mg^{2+} effect was accounted for with a single constant for the reaction steps, $E_1 \rightleftharpoons MgE_1$ and $NaE_1 \rightleftharpoons MgNaE_1$, $K_5 = K_6 = 0.031$ mM. This value differs by about an order of magnitude from the experimentally determined $K^{Mg} = 0.5$ mM, a fact which has to be taken as hint that the assumption, $K_1 = K_4$ and $K_5 = K_6$, may be an oversimplification. In

addition, we found that numerous (simpler) schemes without the mixed state, $MgNaE_1$, were unable to reproduce the varying shapes of the titration curves in Fig. 9*B* (*not shown*). The pH dependence of Mg^{2+} binding as presented in Fig. 6 supports the proposal that one or more amino acids with a negatively charged side chain at physiological buffer pH are part of the binding domain for the cation.

All observations presented here, together with the recent proposals from the Ca-ATPase [24] and from 19 kDa membranes [35], support the concept that binding of Na^+ or K^+ (or its congeners) to one of the ion sites is competitively blocked by Mg^{2+} (or other divalent cations), which is assumed to bind close to the entrance port that is formed at least in part by the cytoplasmic loop L6/7 [3, 35]. According to the scheme in Fig. 9*A* applied for the simulation of the Na^+/Mg^{2+} antagonism the occupation of the "Mg^{2+} site" would have an influence preferentially on the Na^+ site with the low affinity ($K_2^{Na} = 0.85$ mM).

Effects of Br_2-Titu^{3+}

The observed interaction between Na^+ and Br_2-Titu^{3+} (Fig. 7) could be simulated also by the mechanism of competitive binding of both cations at the same site. Reproduction of the concentration dependence of the fluorescence change and half-saturating Na^+ concentrations, K^{Na}, was possible only without introduction of a mixed state, such as Br_2-Titu$^{3+} \cdot NaE_1$. Thus either Na^+ or Br_2-Titu bind to the pump. Exclusive binding of one ion species could be achieved by one of the following mechanisms: Binding of Br_2-Titu^{3+} could completely block the access of Na^+ to its sites and binding of Na^+ prevents binding of the blocking ion by steric reasons, by Coulomb repulsion or effects of protein conformation. The differences in kinetics which were found to describe the antagonistic effects of Na^+ and divalent cations as well as the large organic Br_2-Titu^{3+} has to be scrutinized with respect to its significance in future experiments.

Conclusions

The interaction of various cations with the cytoplasmic binding sites of the Na,K-ATPase as presented in this paper can be reproduced essentially on the basis of the reaction scheme shown in Fig. 9*A*. The concentration dependence of ion binding and of mutual effects between ion species is controlled exclusively by the specific equilibrium dissociation constants of the ion sites accessible. Three different sites could be discriminated. The first one is either identical or spatially very close to the binding site for di- or trivalent cations so that all cations compete at this site for binding. It is assumed that the cytoplasmic loop L6/7 with its negatively charged aspartate and glutamate residues affect access and binding of the cations. The binding affinity of this "first site" was found to be highest for Br_2-Titu^{3+} (<μM, Fig. 7). Ca^{2+} binds tighter than Mg^{2+} (Fig. 3), and Mg^{2+} has approximately the same affinity as Na^+ (0.5 mM *vs.* 0.85 mM). The part of the site which binds alkali ions has to be shaped rather rigidly to exhibit such a high selectivity as shown in Fig. 8, where K^+ and Rb^+ have the highest affinity for this site. In the binding sequence of Na^+ this site would be related to the second ion bound. The first Na^+ ion is assumed to bind to a site formed by transmembrane α-helices (which could be M4–M6 and M8 according to [37]). In the case of Na,K-ATPase this site is not accessible for di- or trivalent cations. The first two sites have to be situated close to the cytoplasmic interface of the ion pump since ion binding to these sites is not electrogenic. As a mechanistic concept it could be proposed that when two K^+ ions (or its congeners) are bound these ions become occluded, accompanied by a movement of the L6/7 loop, and due to their tight fit the pump immediately undergoes the conformational transition into state $E_2(K_2)$. When two Na^+ ions are bound to these sites they are (pre-)occluded; however, a transition into an E_2 state is either not stable or not possible, but the third (cytoplasmic) ion binding site becomes accessible in the (then preserved) E_1 state, which is exclusively selective for Na^+ and has a higher binding affinity than the second Na^+ site. Even though a Mg^{2+} ion is bound to the unspecific site a Na^+ ion can bind to its high-affinity site; thus producing the mixed state, $MgNaE_1$, which is essential for the observed binding kinetics (Fig. 9). In contrast, if the unspecific site is occupied by Br_2-Titu the mixed state, Br_2-Titu $\cdot NaE_1$, seemed to be kinetically inhibited, since the interaction of both ions could be described perfectly with the linear reaction sequence shown in Fig. 7*B*. The third binding site is placed inside the protein dielectric (about 25%), is uncharged, exclusively selective for Na^+, and binding to this site promotes a conformational relaxation that is propagated, possibly by another movement of the L6/7 loop [32, 37], to the nucleotide binding site where Asp372 becomes competent to be phosphorylated by ATP [12, 30, 31].

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