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Probing the Extracellular Access Channel of the Na,K-ATPase

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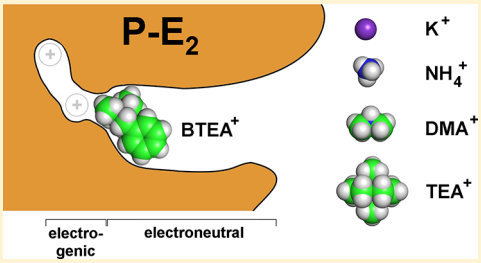
Supporting Information

ABSTRACT: When the Na,K-ATPase pumps at each turnover two K^+ ions into the cytoplasm, this translocation consists of several reaction steps. First, the ions diffuse consecutively from the extracellular phase through an access pathway to the binding sites where they are coordinated. In the next step, the enzyme is dephosphorylated and the ions are occluded inside the membrane domain. The subsequent transition to the E_1 conformation produces a deocclusion of the binding sites to the cytoplasmic side of the membrane and allows in the last steps ion dissociation and diffusion to the aqueous phase. The interaction and competition of K^+ with various quaternary organic ammonium ions have been used to gain insight into the molecular mechanism of the ion binding process from the extracellular side in the P-E_2 conformation of the enzyme. Using the electrochromic styryl dye RH421, evidence has been obtained that the access pathway consists of a wide and water-filled funnel-like part that is accessible also for bulky cations such as the benzyltriethylammonium ion, and a narrow part that permits passage only of small cations such as K^+ and NH_4^+ in a distinct electrogenic way. Benzyltriethylammonium ions inhibit K^+ binding in a competitive manner that can be explained by a stopper-like function at the interface between the wide and narrow parts of the access pathway. In contrast to other quaternary organic ammonium ions, benzyltriethylammonium ions show a specific binding to the ion pump in a position inside the access pathway where it blocks effectively the access to the binding sites.



The Na,K-ATPase is an essential ion transporter in almost all animal cells, and its structure−function relation has been a focus of scientific research for more than 50 years. This ion pump maintains the electrochemical potential gradients for Na^+ and K^+ ions across the cell membrane, which is a prerequisite for a whole series of cell functions. This so-called sodium pump is a P-type ATPase[1,2] that extracts three Na^+ ions from the cytoplasm and takes up two K^+ ions upon expenditure of the free energy released by the hydrolysis of one ATP molecule. On the basis of this stoichiometry and the consecutive ion transport, a reaction scheme was introduced that is well-known as a Post−Albers cycle[3,4] and became the prototype of the reaction scheme for all P-type ATPases.

To translocate the ions from one side of the membrane to the other, a series of reaction steps has to be performed, which are according to the Post−Albers scheme (for both ion species): ion diffusion from the aqueous bulk phase to the ion-binding site in the membrane domain, coordination in the binding site, occlusion of the ion, a transition of the conformation of the protein to allow deocclusion of the ions to the opposite side of the membrane, their release from the binding site, and diffusion to the aqueous phase.[5] Detailed studies of the single reaction steps for both the Na^+- and K^+-translocating half-cycles showed that charge movements can be detected only for the ion diffusion to or from the binding sites. Those reaction steps are termed electrogenic. Electrogenicity implicates two specific properties.[6] (1) An electrogenic transporter is a current generator that contributes to the membrane potential, and (2) its transport capacity (or transport rate) depends on the electric membrane potential and, therefore, may be modulated by an externally applied voltage.

Specific electrogenic properties of the Na,K-ATPase have been reported in great detail for Na transport,[7−11] and clarifying evidence about the mechanism has been presented, including that from recently published structural details.[12−14] It has been shown that the ion-binding sites reside almost in the middle of the membrane dielectric. This ion-binding moiety is connected to both aqueous phases by an access channel on each side. Only one of both channels is, however, opened at the same time to allow access to the binding sites. Each channel is wide enough that small cations may diffuse through it, but at least in parts so narrow that part of the transmembrane voltage drops across the length of the channel. On the cytoplasmic side, the channel stays constantly narrow, and ∼25% of the membrane potential drops between the outside and the binding sites.[8] In the P-E_2 conformation of the ion pump, when the binding sites are opened to the extracellular side, the first sodium ion released has to diffuse through a narrow release channel and traverses ∼70% of the membrane potential.[7,10] Upon its release, a conformation relaxation of the membrane domain of the Na,K-ATPase widens the access channel and allows the entrance of water molecules, and this modification causes a reduced electrogenicity of the ion movement so that both remaining Na^+ ions are affected only by ∼20% of the membrane potential each.[7,11,15]

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In the second half-cycle, during which two K^+ ions are transported into the cell, the determination of the electrogenic reaction steps is more complex. Because of the high affinity of the binding sites for K^+, they are virtually continuously occupied under physiological conditions. In the E_1 conformation and in the absence of K^+ and Na^+, they are (at least partly) occupied by protons even at physiological pH values.[16] In the P-E_2 conformation, the electrogenic K^+ binding was revealed only after reducing the K^+ concentration to values below 1 mM.[17,18] Assuming that upon the release of both Na^+ ions with the lower electrogenicity no significant change in the protein conformation occurs in the P-E_2 state, one would expect that binding of both K^+ ions is as electrogenic as the release of Na^+. This claim has been well-established experimentally, including a confinement of the electrogenicity of this process to the K^+ translocation through the access channel to the binding sites.[7,17−22] Upon occupation of both ion sites by a K^+, occlusion of the ions and enzyme dephosphorylation is triggered. Under physiological conditions, the next step is low-affinity ATP binding, which promotes the transition to the E_1 conformation, and deocclusion of the binding sites so that both K^+ ions can be released and exchanged against Na^+ on the cytoplasmic side. Extensive evidence that the P-E_2K_2 → ... → K_2E_1 reaction sequence is electroneutral has been collected,[23−25] and electrogenicity was assigned exclusively to K^+ transfer from the extracellular aqueous phase.[17,18,20,26] While in early studies the access channel was proposed as a narrow pore,[26,27] more detailed studies revealed a more sophisticated view. On one hand, cysteine mutations of amino acids that are supposed to line the access channel and thiol-specific reagents were used to probe the channel and demonstrate their accessibility.[15,28] In addition, different organic ammonium cations were applied on the extracellular side, and their competition with K^+ has been shown.[29−32] TEA^+ inhibited the Na,K-ATPase in a voltage-independent manner.[33−35] In contrast, other quaternary amines such as $BTEA^+$ showed a voltage-dependent inhibition, and this voltage sensitivity could be used to test their access to the binding sites as a function of their size.[35−38] The latter approach was used preferentially with cardiac myocytes, and the voltage dependence of the Na,K-ATPase-specific currents through the membrane was studied in the presence of those organic amines, which provide evidence of the extracellular access channel, and in addition introduced a new species of voltage-dependent inhibitors of the sodium pump.[36,37]

In this work, various organic amines were used to study their interaction with the Na,K-ATPase in purified membrane fragments isolated from rabbit kidney. The electrochromic fluorescence dye RH421 has been used to detect electrogenic ion movements in the access channel of the ion pump. Although the membrane potential as a driving force cannot be applied to solubilized membrane fragments, equilibrium titration experiments provide by all means an interesting source of information about ion permeation in the access channel on the extracellular side and on steric confinements that the ions face. The results allow a refinement of our conceptual understanding.

MATERIALS AND METHODS

Materials. The fluorescent styryl dye RH 421 was obtained from Molecular Probes (Eugene, OR) and added from a 200 μM ethanolic stock solution to produce a final concentration of 200 nM. ATP (disodium salt, special quality) was purchased from Roche Life Science, and dioleoylphosphatidylcholine was from Avanti Polar Lipids (Alabaster, AL). All other reagents were purchased from Merck or Sigma-Aldrich at the highest quality available.

Na,K-ATPase was prepared from the outer medulla of rabbit kidneys using procedure C of Jørgensen.[39] The specific ATPase activity was measured by the pyruvate kinase/lactate dehydrogenase assay,[40] and the protein concentration was determined by the Lowry method,[41] using bovine serum albumin as a standard. The specific activity of the used preparations was in the range from 1800 to 2000 μmol of P_i mg^{-1} h^{-1} at 37 °C. Protein-free lipid vesicles used in control experiments were produced from dioleoylphosphatidylcholine (PC 18:1) by dialysis as described previously.[42]

Fluorescence measurements were taken with a homemade setup as described previously.[43] The thermostated cell holder was equipped with a magnetic stirrer. RH421 was excited by a HeNe laser working at 594 nm. The emitted light was collected by a photomultiplier (model R2066, Hamamatsu Photonics). An interference filter with transmission at 663 ± 18 nm selected the emitted light of the styryl dye before it entered the photomultiplier. Equilibrium titration experiments were performed in standard buffer containing 30 mM imidazole, 1 mM EDTA, and 5 mM $MgCl_2$ (pH 7.2). Unless otherwise noted, 200 nM RH421 and 8−10 μg/mL membrane fragments containing Na,K-ATPase were added to the cuvette and equilibrated until a steady fluorescence signal, F_0, was obtained. Titrations were conducted by adding small aliquots of the indicated substrates from various concentrated stock solutions until no further changes in the fluorescence signal 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 with respect to F_0. Specific fluorescence levels could be assigned to defined states in the pump cycle of the Na,K-ATPase.[19] All experiments were performed at 20 ± 0.5 °C. The steady-state fluorescence levels obtained upon addition of substrates allow the discrimination between differently charged states of the enzyme.

RESULTS

K^+ Titrations in the P-E_2 Conformation under Turnover Conditions. After 200 nM RH421 and 9 μg/mL Na,K-ATPase in membrane fragments had been equilibrated in standard buffer in a fluorescence cuvette and a steady fluorescence signal obtained, consecutively 50 mM NaCl and 500 μM Na_2ATP were added. The resulting and constant fluorescence signal, F_0, corresponds to the P-E_2 state of the Na,K-ATPase because the dephosphorylation step and reaction back to E_1 are extremely slow in the absence of K^+. Under this condition, aliquots of KCl were added and its electrogenic binding to the pump induced a decrease in the fluorescence level proportional to the occupation of the binding sites.[20,25] The K^+ concentration dependence of the fluorescence can be fit by the Hill function

$$F_{norm}([K^+]) = F_0 + \Delta F_{max}/[1 + ([K^+]/K_{1/2})^{-n}] \quad (1)$$

the binding constant, $K_{1/2}$, the maximal fluorescence change, ΔF_{max}, and the Hill coefficient, n, were obtained from the fit. The results from the fits of the titration experiments in the absence of $BTEA^+$ were as follows: $K_{1/2}$ = 286 ± 10 μM, and n = 1.4 ± 0.1. The half-saturating K^+ concentration of this protein preparation and the Hill coefficient were in agreement with

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previously published data.[20] Addition of K^+ triggers the reaction sequence that embraces the whole pump cycle

$$P\text{-}E_2 \leftrightarrow P\text{-}E_2K \leftrightarrow P\text{-}E_2K_2 \leftrightarrow E_2(K_2) \leftrightarrow E_2(K_2)ATP \leftrightarrow K_2E_1ATP \leftrightarrow \ldots \leftrightarrow P\text{-}E_2$$

in which the conformational transition to E_1 is the rate-limiting step, and thus, the fluorescence of the preceding state, $E_2(K_2)ATP$, controls primarily the detected fluorescence level in these titration experiments.[20]

The titration experiments were repeated in the presence of $BTEA^+$ with concentrations between 100 μM and 30 mM. After the steady-state fluorescence in the presence of NaCl and ATP was obtained, the respective amount of $BTEA^+$ was added. Then the K^+ titration was performed as in the absence of $BTEA^+$. At each $BTEA^+$ concentration, three titrations were performed and the results were averaged. In Figure 1, the

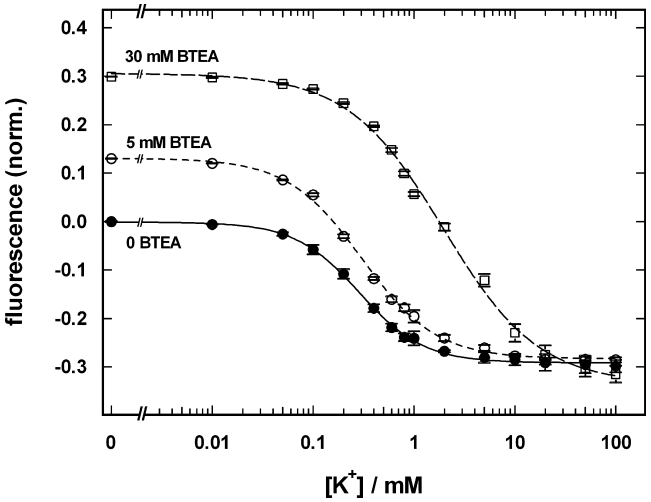

Figure 1. K^+ titrations of the Na,K-ATPase in its ATP-induced P-E_2 conformation in the presence of various $BTEA^+$ concentrations. The indicated amounts of the inhibitor were added prior the addition of KCl. The data are the average of three independent experiments. The lines represent fits obtained by the Hill function (eq 1).

analysis of single-titration experiments at 0, 5, and 30 mM $BTEA^+$ is shown. The fluorescence was normalized to the level in P-E_2 before the addition of $BTEA^+$. Three characteristic effects of $BTEA^+$ on the titrations are obvious. Additions of higher concentrations of $BTEA^+$ produced increased fluorescence levels of the P-E_2 state, F_0, up to ~0.3 at 30 mM $BTEA^+$, and the maximal fluorescence change, ΔF_{max}, increased from ~0.3 (0 mM $BTEA^+$) to ~0.6 (30 mM $BTEA^+$). Conversely, the Hill coefficient, n_{Hill}, decreased from 1.36 ± 0.05 (0 mM $BTEA^+$) to 0.95 ± 0.05 (30 mM $BTEA^+$). The half-saturating K^+ concentration, $K_{1/2}$, was shifted to larger values. In Figure 2, the corresponding $K_{1/2}$ values are compiled from the experiments with the different $BTEA^+$ concentrations. The linear dependence of $K_{1/2}$ on the $BTEA^+$ concentration is a substantial indication of a competitive inhibition between both cations, K^+ and $BTEA^+$, which is represented by eq 2

$$K_{1/2} = K_m\left(1 + \frac{[BTEA^+]}{K_I}\right) \quad (2)$$

where K_m is the half-saturating K^+ concentration in the absence of the inhibitor and K_i the half-saturating binding concentration

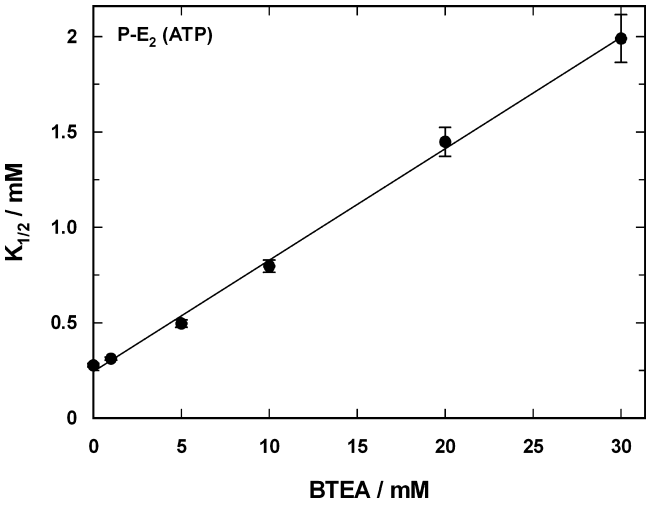

Figure 2. Competitive inhibition between K^+ and $BTEA^+$ in the P-E_2 conformation induced by ATP. K^+ titration experiments were performed in the presence of various $BTEA^+$ concentrations. The respective half-saturating K^+ concentrations, $K_{1/2}$, are plotted vs $BTEA^+$ concentration. The linear concentration dependence indicates a competitive binding kinetics according to eq 2 with a K_m of 0.24 ± 0.02 mM and a K_I of 4.2 ± 0.4 mM.

of the inhibitor $BTEA^+$. The line shown in Figure 2 is a fit of eq 2 to the data. K_m was found to be 243 ± 21 μM, and K_I of $BTEA^+$ was 4.2 ± 0.4 mM.

K^+ Titrations in the P-E_2 Conformation upon Backdoor Phosphorylation. Under turnover conditions, electrogenic K^+ binding is immediately followed by electroneutral ion occlusion and enzyme dephosphorylation. Because previous work on $BTEA^+$ inhibition of the Na,K-ATPase has shown that $BTEA^+$ inhibits the pump current without being occluded,[36] it would be interesting to study the effect of $BTEA^+$ on K^+ binding in the P-E_2 conformation when the states of the Na,K-ATPase are confined to the P-E_2 conformation without further progress through the pump cycle. Such a condition can be maintained by so-called backdoor phosphorylation.[44−47] It has been shown that in the absence of both Na^+ and K^+, and in the presence of 500 μM inorganic phosphate, P_i, more than 96% of the α1 isoform of the Na,K-ATPase used in these experiments prevails in the P-E_2 conformation with empty binding sites.[47] When K^+ titration experiments are performed under this condition, the reaction sequence is confined to

$$P\text{-}E_2 \leftrightarrow P\text{-}E_2K \leftrightarrow P\text{-}E_2K_2 \leftrightarrow P\text{-}E_2(K_2)$$

The questions of whether the third step, P-$E_2K_2 \leftrightarrow$ P-$E_2(K_2)$, occurs and whether it also is triggered by dephosphorylation, P-$E_2K_2 \leftrightarrow E_2(K_2) + P_i$, have not been finally clarified. Opposite to a number of publications in which such a coupling is presumed,[4,48,49] Forbush suggests that the K^+-occluded intermediate may be phosphorylated.[50,51] It has been shown, however, that in the absence of Na^+ and in the presence of Mg^{2+}, inorganic phosphate, P_i, and ATP a K^+−K^+ exchange is performed; i.e., the ion pump shuttles back and forth through the P-E_2 + $2K^+_{ext} \leftrightarrow$ P-$E_2K_2 \leftrightarrow E_2(K_2) \leftrightarrow E_2(K_2)ATP \leftrightarrow K_2E_1ATP \leftrightarrow E_1ATP + 2K^+_{cyto}$ reaction sequence.[52−54] Furthermore, it was found that addition of P_i to the ion pump in the $E_2(Rb_2)$ state leads to deocclusion of the Rb^+ ions, which are congeners of K^+, and their exchange against K^+ ions from the extracellular bulk phase.[55] This indicates on average that in the steady state of this partial reaction at least a

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considerable fraction of the ion pumps has to be in the deoccluded states. When the Na,K-ATPase is backdoor phosphorylated in the absence of Na^+ and K^+ ions, the binding sites are initially occupied by protons that are immediately released to the extracellular side upon phosphorylation.[47] In summary, this behavior indicates a deocclusion of the binding sites. Therefore, we may conclude that in the presence of saturating P_i concentrations the ion pumps are confined at a major fraction to the $P\text{-}E_2 \leftrightarrow P\text{-}E_2K \leftrightarrow P\text{-}E_2K_2$ reaction sequence when K^+ ions are added, and ion occlusion may go hand in hand with dephosphorylation as proposed by various authors.[4,48,49]

This reaction sequence, which comprises electrogenic K^+ binding, can also be investigated by the RH421 method, and the occupation of the binding sites can be studied. In Figure 3 three control experiments are shown. First, 9 μg/mL Na,K-ATPase in membrane fragments and 200 nM RH421 were equilibrated in standard buffer at pH 7.2. After a steady fluorescence signal was obtained, 20 mM KCl was added, and a fluorescence decrease of ~6% was observed (Figure 3A). This effect is explained by the fact that in the absence of Na^+ and K^+ the ion-binding sites in the E_1 conformation are occupied partly and in a pH-dependent manner by H^+.[16] At pH 7.2, on average ~83% of the binding sites are taken. Addition of a saturating concentration of K^+ (20 mM) leads to an electroneutral replacement of the H^+ by K^+ and an electrogenic binding of K^+ to the remaining empty sites. When subsequently the enzyme is phosphorylated by 500 μM P_i, the binding sites are deoccluded in the $P\text{-}E_2$ conformation to the extracellular side but the fluorescence does not change because the binding sites remain occupied because of the high binding affinity of K^+ in $P\text{-}E_2$ (Figure 1). If the experiment is repeated in pH 5.5 buffer (Figure 3B), neither K^+ nor P_i modifies the fluorescence signal because almost all binding sites are (and remain) occupied from the beginning and throughout the experiment. If the Na,K-ATPase is backdoor phosphorylated in the absence of K^+ (here at pH 7.2), the protons act as congeners of K^+ and mediate the transition to the deoccluded $P\text{-}E_2$ state. Because the binding affinity for H^+ is significantly lower in $P\text{-}E_2$ than in E_1,[47] the protons are released and a corresponding fluorescence increase is observed (Figure 3C). Addition of 20 mM K^+ leads to saturating binding of K^+ to the $P\text{-}E_2$ state, which produces a decrease in fluorescence to the same level observed at the end of the experiment in Figure 3A. Addition of 0.5 mM MgATP promotes the subsequent reaction steps, $P\text{-}E_2K_2 \leftrightarrow E_2(K_2) \leftrightarrow E_2(K_2)ATP \leftrightarrow K_2E_1ATP$. This partial reaction includes also a conformational rearrangement, a significant shift of the steady state to the E_1 conformation of the Na,K-ATPase, and, therefore, a drain of the deoccluded states in $P\text{-}E_2$. This process did, however, not produce any fluorescence changes (Figure 3C). The complete absence of a fluorescence change indicates that the induced reaction steps, including the occlusion and dephosphorylation step, ATP binding, and the conformational transition, do not contribute to the signal detected by RH421. These controls show that under the condition of a backdoor phosphorylation the RH421 technique can be used to study K^+ binding in the $P\text{-}E_2$ state ($P\text{-}E_2 \rightarrow P\text{-}E_2K_2$) and how this partial reaction is affected by $BTEA^+$.

To perform the K^+ titration under backdoor phosphorylation, 9 μg/mL membrane fragments were equilibrated in standard buffer with 200 nM RH421. When 500 μM Tris-P_i was added, the transition from the H_xE_1 state (because no Na^+ and K^+ were present, $x \approx 1.6$ at pH 7.2) to the $P\text{-}E_2$ state occurred, which was accompanied by a fluorescence increase of ~0.4. Subsequently, appropriate aliquots of KCl solutions were added, and the fluorescence decrease was recorded, plotted against the K^+ concentration, and fit with the Hill function (similarly as shown in Figure 1). Such experiments were performed in the absence and presence of various concentrations of $BTEA^+$ up to 30 mM. At each concentration, three experiments were performed, and the resulting averaged $K_{1/2}$ values of K^+ binding are shown in Figure 4 as a function of $BTEA^+$ concentration. Again, the linear $BTEA^+$ concentration dependence indicates competitive inhibition of K^+ by $BTEA^+$. For comparison, the data of Figure 2 are included. In addition, K^+ titrations were also performed in the E_1 conformation of the Na,K-ATPase, where the effect of $BTEA^+$ on K^+ binding was also linear but significantly less effective. The kinetic

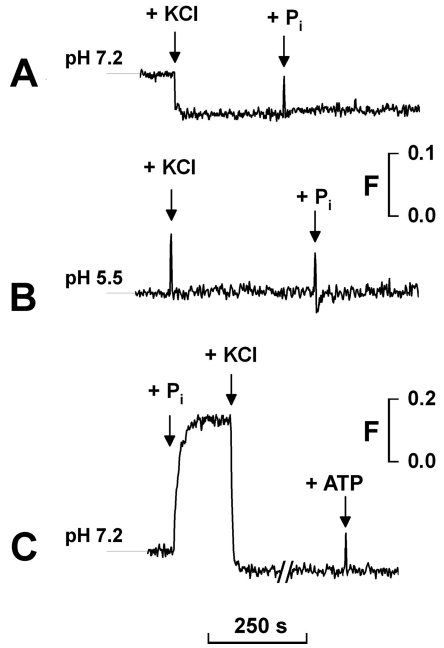

Figure 3. Response of the RH421 fluorescence to different reaction sequences under the condition of backdoor phosphorylation of the Na,K-ATPase. (A) In the absence of Na^+, addition of 20 mM KCl induced K^+ binding, conformational transition, and ion occlusion[$E_1 + 2K^+ \rightarrow K_2E_1 \rightarrow E_2(K_2)$]. At pH 7.2, a small fraction of the K^+ binds electrogenically and modifies the RH421 fluorescence while the major fraction of K^+ binding is an electroneutral exchange of H^+ against K^+. Addition of 500 μM P_i leads to enzyme phosphorylation and deocclusion of the binding sites [$E_2(K_2) + P_i \rightarrow P\text{-}E_2K_2$]. Because of the high binding affinity of K^+, the binding sites remain occupied, as reflected by the unchanged RH421 fluorescence. (B) Repetition of the experiment at pH 5.5. Because at this pH all binding sites are occupied initially by H^+, additions of 20 mM KCl and 500 μM P_i do not induce any electrogenic reaction step. Therefore, the fluorescence signal remains constant. (C) When the Na,K-ATPase is phosphorylated by P_i in the absence of K^+, H^+ acts as congener of K^+ and the $H_2E_1 \rightarrow E_2(H_2) \rightarrow P\text{-}E_2H_2 \rightarrow P\text{-}E_2$ reaction sequence is initiated. Because in the $P\text{-}E_2$ conformation the binding affinity of H^+ is much lower than in E_1, (most of) the protons are released electrogenically to the aqueous bulk phase and correspondingly the RH421 fluorescence increases. Addition of saturating 20 mM KCl leads to saturation of the ion-binding sites ($P\text{-}E_2 + 2K^+ \rightarrow P\text{-}E_2K_2$), and the intensity of the fluorescence signal decreases to the same level as at the end of trace A. Further addition of 0.5 mM MgATP allows $K^+ - K^+$ exchange across the membrane and also includes population of the state $E_2(K_2)ATP$, and promotes a transition to the K_2E_1ATP state. It does not change the fluorescence signal.

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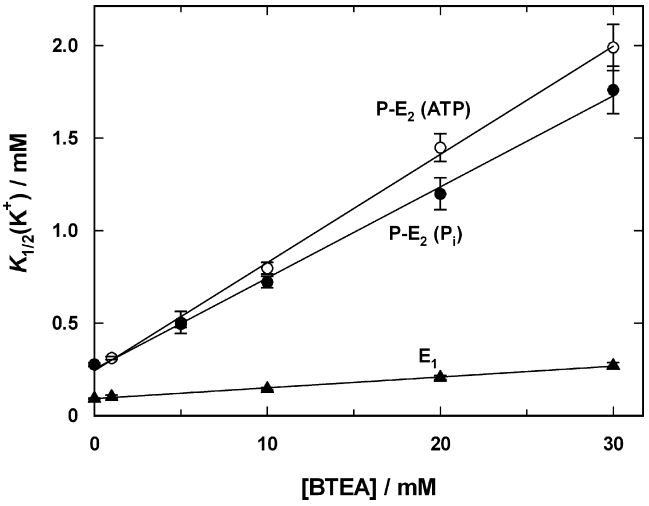

Figure 4. Inhibition of K^+ binding by $BTEA^+$ under different experimental conditions. K^+ titration experiments were performed under backdoor phosphorylation [P-E_2 (P_i)] and, for comparison, under turnover conditions [P-E_2 (ATP)], and in the E_1 conformation, i.e., when the ion-binding sites can be accessed from the cytoplasmic side. The lines are fits of eq 2 to the data. The respective half-saturating K^+ concentrations, K_m, in the absence of $BTEA^+$ and the binding constant of the inhibitor $BTEA^+$, K_I, are listed in Table 1.

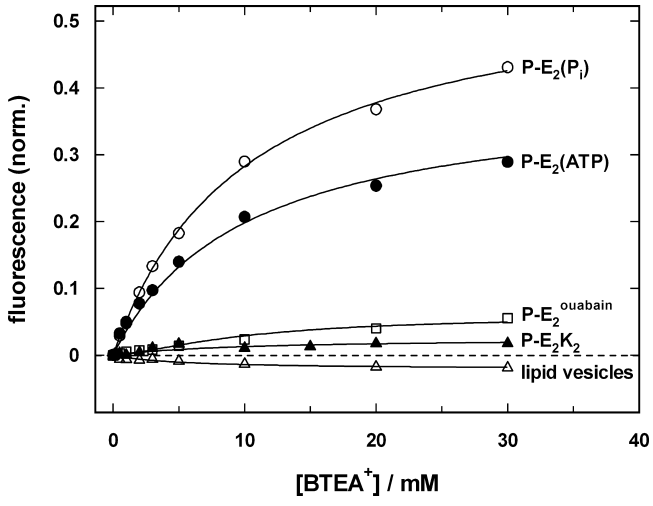

Figure 5. Effects of $BTEA^+$ on the RH421 fluorescence under different experimental conditions. $BTEA^+$ titrations were performed in the presence of RH421, membrane fragments, 50 mM NaCl, and 500 μM ATP ["P-E_2(ATP)"], or 500 μM P_i only ["P-E_2(P_i)"]. In both cases, the ion pumps were preferentially in the P-E_2 conformation. The lines drawn through both data sets are fits of the Hill function (eq 1) with no significant difference in the half-saturating concentration, $K_{1/2}$, of ~10.4 mM. For comparison are shown the effects of the corresponding $BTEA^+$ impact on pure lipid vesicles stained with 200 nM RH421 ("lipid vesicles") as well as on Na,K-ATPase in its P-E_2 conformation with saturating 20 mM KCl present ("P-E_2K_2"), and the phosphorylated state inhibited with 12.5 μM ouabain ("P-$E_2^{ouabain}$"). The effect on the fluorescence under the latter three conditions demonstrates that artifactual fluorescence changes are insignificant and the major part of the fluorescence changes in the P-E_2 state is specific to the functional ion pump. The lines through these three data sets are drawn to guide the eyes.

parameters, K_m and K_I, derived from all three series of experiments are listed in Table 1. While the half-saturating K^+ concentration, K_m, obtained from the titration experiments was the same in the enzyme phosphorylated by ATP and P_i, the inhibitory potency of $BTEA^+$ was ~20% higher when the pumps were phosphorylated by ATP. In the latter case, there was also 50 mM Na^+ present and the pumps worked under turnover conditions. In the E_1 conformation, the inhibitory effect of $BTEA^+$ was a factor of 4–5 lower than in the P-E_2 conformation. The respective titration experiments in E_1 were also performed in standard buffer containing 9 μg/mL membrane fragments and 200 nM RH421.

Table 1. Competition between K^+ and $BTEA^+$ at the Ion-Binding Sites of the Na,K-ATPase[a]

	E_1	P-E_2 (P_i)	P-E_2 (ATP)
K_m (mM)	0.09 ± 0.01	0.25 ± 0.02	0.24 ± 0.02
K_i (mM)	15.9 ± 0.8	5.1 ± 0.5	4.2 ± 0.4

[a]Under three experimental conditions, the half-binding K^+ concentration, K_m, and the inhibitor binding constant, K_i, were determined according to eq 2.

Possible $BTEA^+$–RH421 Interactions. When $BTEA^+$ was added to the Na,K-ATPase in the P-E_2 conformation in the absence of K^+, an increase in RH421 fluorescence was observed (Figure 1). To study this effect, $BTEA^+$ titrations were performed in the P-E_2 conformation, in two standard buffer solutions containing either 50 mM NaCl, 0 mM KCl, and 500 μM ATP ["P-E_2(ATP)"] or 0 mM NaCl, 0 mM KCl, and 500 μM P_i ["P-E_2(P_i)"]. The results are shown in Figure 5. At high $BTEA^+$ concentrations (30 mM), a fluorescence increase of up to 40% was found. The $BTEA^+$ concentration dependence was fit by the Michaelis–Menten function (eq 1, n fixed at 1.0), and the half-saturating concentrations were the same in both experiments {10.3 ± 2.1 mM [P-E_2(ATP)] and 10.4 ± 1.5 mM [P-E_2(P_i)]}. The only significant difference was observed in the maximal fluorescence increases, ΔF_{max}, of 0.40 ± 0.03 and 0.58 ± 0.03, respectively. According to the detection mechanism of RH421, the fluorescence increase seems to indicate a reduction of positive charge inside the membrane domain. Under both conditions, the ion-binding sites were, however, thought to be unoccupied (0 Na^+, pH 7.2) or occupied at a rather low level (50 mM Na^+, pH 7.2). Therefore, the question of whether the $BTEA^+$-induced fluorescence change is an artifact caused by unspecific $BTEA^+$–RH421 interactions had to be examined. Thus, a number of control experiments were performed. The control experiments are included also in Figure 5. First, the effect of $BTEA^+$ on RH421 was investigated by testing RH421-stained lipid vesicles formed from PC18:1 ("lipid vesicles"). In these vesicles, no Na,K-ATPase was present so that only potential artifacts could be detected. The observed fluorescence decrease of <2% at 30 mM $BTEA^+$ may be considered as insignificant as has been reported recently.[38] Therefore, a direct effect of $BTEA^+$ on RH421 can be excluded. When Na,K-ATPase was present in the P-E_2 state obtained by the addition of 500 μM P_i and after addition of 20 mM K^+ ("P-E_2K_2"), all ion-binding sites are permanently occupied by K^+, and in this case, only a minor increase in RH421 fluorescence (<2%) was found at 30 mM $BTEA^+$. Similarly, the Na,K-ATPase was phosphorylated in the presence of 50 mM Na^+ by 0.5 mM ATP and then inhibited by the addition of 12.5 μM ouabain (which has a K_D of <0.5 μM). Under this condition, almost all pumps may be assumed to be inhibited. At $BTEA^+$ concentrations above 5 mM, a small fluorescence increase ("P-$E_2^{ouabain}$") could be observed (<6% at 30 mM $BTEA^+$). This effect may be

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assigned to the reversibility of ouabain binding, so that at sufficiently high $BTEA^+$ concentrations, an equilibrium state may be obtained in which $BTEA^+$ replaces ouabain in a small fraction of ion pumps. Bound ouabain is located in the access channel to the ion-binding sites.[56] The experiments with lipid vesicles in the presence of saturating K^+ clearly reveal that $BTEA^+$ interacts neither with the RH421 dye in the lipid phase of the membrane nor in an unspecific manner with the Na,K-ATPase. From these observations, it may be deduced that in the $P\text{-}E_2$ conformation with virtually unoccupied ion-binding sites, $BTEA^+$ is able to produce a condition that resembles a withdrawal of further positive charge from the membrane dielectric of the pump's membrane domain.

Comparison to Other Organic Ammonium Ions. A further set of titration experiments was performed in the P_i-induced $P\text{-}E_2$ conformation with ammonium chloride (NH_4^+), dimethylammonium chloride (DMA^+), and tetraethylammonium chloride (TEA^+), corresponding to the respective K^+ titration experiments with $BTEA^+$. Na,K-ATPase (9 μg/mL) in membrane fragments and 200 nM RH421 were equilibrated in standard buffer at pH 7.2. After a steady fluorescence signal was obtained, 500 μM Tris phosphate was added, and a fluorescence increase of ~40% was observed (cf. Figure 3C). Subsequently, aliquots of differently concentrated solutions of NH_4^+ were added to a final concentration of 100 mM. The NH_4^+-induced fluorescence decrease was plotted against the ion concentration and fit by the Hill function (eq 1). The same procedure was repeated with DMA^+ and TEA^+. The experimental results are compiled in Figure 6, in which for comparison also the results obtained from titrations with K^+ and $BTEA^+$ are included. Electrogenic binding of NH_4^+ could be observed, like that of K^+, only with a lower binding affinity ($K_{1/2}$ = 2.3 ± 0.1 mM). The maximal fluorescence decrease (ΔF_{max} = 0.27 ± 0.02) was comparable to that of K^+ binding (0.29 ± 0.01). NH_4^+ ions are known to act as congeners of K^+.[57]

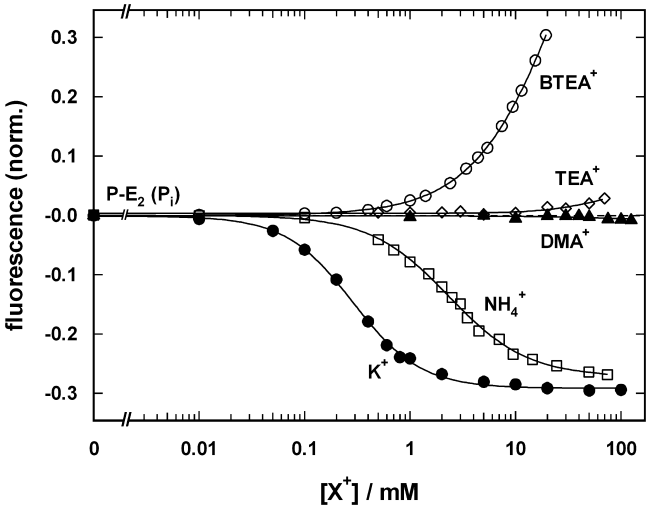

Figure 6. Effects of different inorganic and organic cations on the Na,K-ATPase in the P_i-induced $P\text{-}E_2$ conformation. K^+ and NH_4^+ are able to bind to the ion-binding sites with different half-saturating concentrations, $K_{1/2}$, of 0.29 ± 0.01 and 2.31 ± 0.04 mM, respectively. DMA^+ has no significant effects on the occupation of the ion-binding sites. TEA^+ and $BTEA^+$ are unable to bind to the ion-binding sites, the latter showing a significant fluorescence increase at concentrations above 1 mM that corresponds to an apparent removal of positive charge from the membrane domain of the ion pump (see the text).

In the case of DMA^+, no significant fluorescence change was observed up to a concentration of 125 mM. This observation indicates that no binding of DMA^+ occurs in this concentration range. When TEA^+ was added, no significant fluorescence change was observed up to a concentration of 10 mM. In the range between 10 and 70 mM, a small increase (<2.9%) was observed. The fluorescence increase (instead of a decrease) indicates that no electrogenic binding of TEA^+ to the Na,K-ATPase occurred, although this compound was able to inhibit K^+ binding in rat cardiac myocytes at a K_I of 26.6 mM.[35] The tendency to increase the fluorescence could be caused by the same effect that was observed much more distinctly with $BTEA^+$.

To study the competition between K^+ and the organic ammonium ions, K^+ titrations were performed at various concentrations of these compounds. In a first series, K^+ titrations were performed in the $P\text{-}E_2$ conformation upon backdoor phosphorylation (see above) and in the presence of different concentrations of NH_4^+ (1−5 mM). Because NH_4^+ binds electrogenically to the ion sites (cf. Figure 6), the remaining fluorescence decrease produced by K^+ binding in the presence of NH_4^+ concentrations above 5 mM was so small that the determination of the $K_{1/2}$ values for K^+ binding became too imprecise. In the concentration range between 1 and 5 mM, the value of K_m increased linearly (Figure 7A), and according to eq 2, this indicates again competitive binding of K^+ and NH_4^+. The K_I value obtained from the fit of eq 2 to the data was 2.3 ± 0.1 mM, which is in perfect agreement with the $K_{1/2}$ value obtained from the NH_4^+ titration experiments (Figure 6). This result strengthens the argument that both ions, K^+ and NH_4^+, bind to the same sites.

When the experiments were repeated in the presence of DMA^+ (1−20 mM), no significant effect of the compound on the K^+ binding kinetics was found (Figure 7B). The increase in the half-saturating K^+ concentration is too small to allow the estimation of a reasonable value of K_I. The effect of TEA^+ on K^+ binding was studied up to 60 mM TEA^+ (Figure 7C). The K_I value obtained from the fit of eq 2 to the data was 33.6 ± 2.1 mM. The TEA^+ molecule was an effective competitor of K^+ binding in contrast to the smaller DMA^+. As in the case of $BTEA^+$, TEA^+ does not bind to the ion-binding sites but is nevertheless able to affect K^+ binding in a competitive manner.

Effect of $BTEA^+$ on Na^+ Binding in the E_1 Conformation of the Na,K-ATPase. Binding of ions in the E_1 conformation is also performed through a narrow access channel,[5] and therefore, it is interesting to check the effect of $BTEA^+$ on ion binding also to the cytoplasmic side of the Na,K-ATPase. Because binding of K^+ to the E_1 conformation in the absence of ATP entails also a conformational transition to the occluded $E_2(K_2)$ state, Na^+ binding was chosen as a control experiment. In the absence of ATP, the reaction sequence is limited to the $E_1 \leftrightarrow NaE_1 \leftrightarrow Na_2E_1 \leftrightarrow Na_3E_1$ sequence. Na^+ titrations were performed in standard buffer with 9 μg/mL Na,K-ATPase in membrane fragments and 200 nM RH421 (pH 7.2). NaCl was added in appropriate aliquots to cover the concentration range between 0 and 100 mM. The experiments were performed in triplicate in the absence and presence of 10 and 20 mM $BTEA^+$. In Figure 8, one set of experiments is shown. The concentration dependence was fit by the Hill function (eq 1). The results show that also in the E_1 conformation in the absence of Na^+ addition of $BTEA^+$ to

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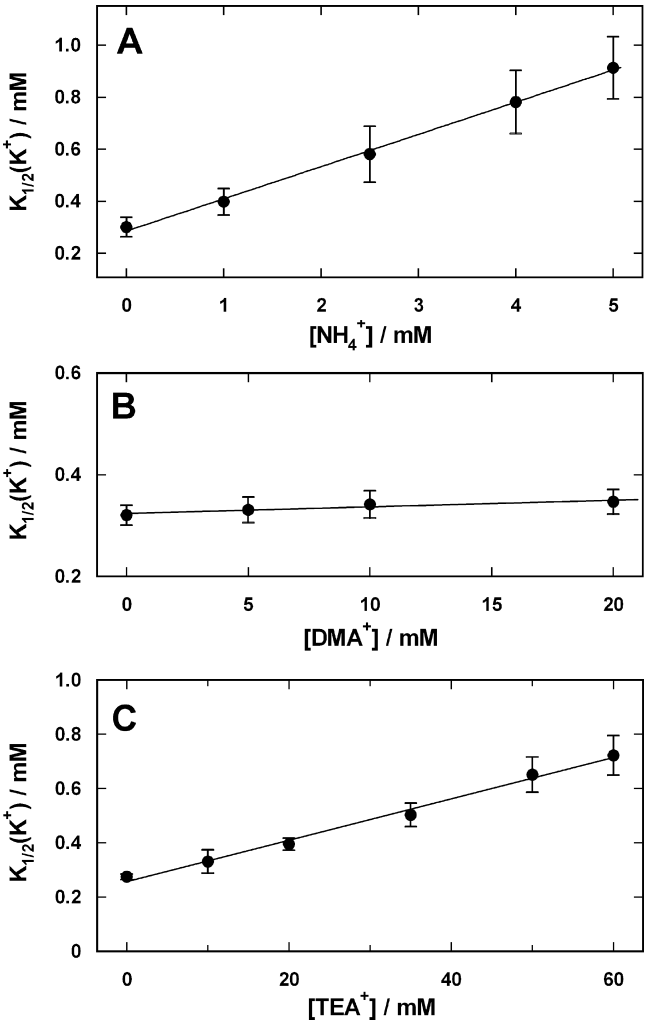

Figure 7. Competition between K^+ binding and (A) NH_4^+, (B) DMA^+, and (C) TEA^+ in the P_i-induced P-E_2 conformation of the Na,K-ATPase. The lines are fits of eq 2 to the data and indicate competitive inhibition. While a competition is obvious between NH_4^+ and K^+ with a K_I of 2.3 mM for NH_4^+, and between TEA^+ and K^+ with a K_I of 33.6 mM, in the case of DMA^+ no significant competition was observed in the concentration range of ≤20 mM.

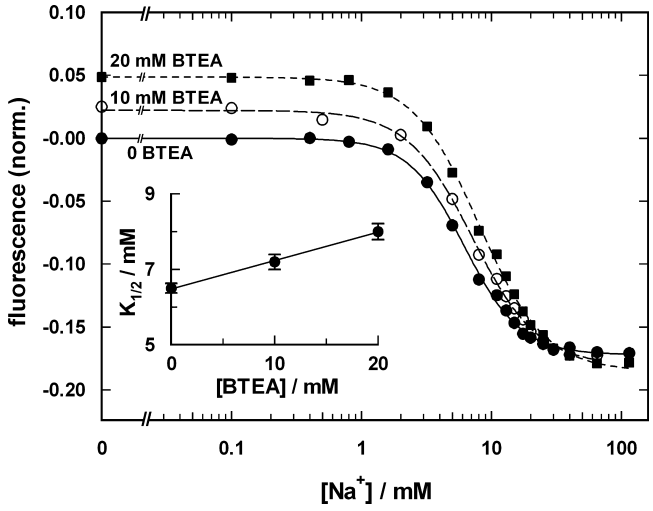

Figure 8. Competitive inhibition of Na^+ binding by $BTEA^+$ in the E_1 conformation of the Na,K-ATPase. Na^+ titrations were performed in the absence and presence of 10 and 20 mM $BTEA^+$. The concentration dependence of the RH421 fluorescence was fit with the Hill function (eq 1). The inset shows a plot of the half-saturating Na^+ concentration, $K_{1/2}$, vs $BTEA^+$ concentration. The linear dependence was fit with eq 2, which represents the kinetics of competitive binding. The inhibition constant of $BTEA^+$, K_I, was calculated to be 86.6 mM.

the ion pumps in the E_1 conformation led to a fluorescence increase, although one significantly smaller than that in the P-E_2 conformation. At 20 mM $BTEA^+$, the fluorescence is increased by <5%, while in P-$E_2(P_i)$, an increase of 24% was observed (Figure 5). As one can see in the inset in Figure 8, the $K_{1/2}$ of K^+ binding increases from 6.2 ± 0.1 mM (0 mM $BTEA^+$) to 8.0 ± 0.2 mM (20 mM $BTEA^+$). From these values, an inhibition constant, K_I, of $BTEA^+$ can be estimated to be 87 ± 3 mM. At saturating Na^+ concentrations, the fluorescence levels are almost independent of the $BTEA^+$ concentration at approximately −17%. An explanation of the minor effects of $BTEA^+$ on the cytoplasmic side of the Na,K-ATPase can be proposed in analogy to the impact of Mg^{2+} on Na^+ binding to the E_1 conformation. It was found that Mg^{2+} is able to bind to the L6−L7 loop of the α subunit close to the entrance of the access channel to the ion-binding sites and thus reduces the local Na^+ concentration by electrostatic repulsion (according to Debye−Hückel theory).[58] $BTEA^+$ may bind to the same site with the observed low affinity, and as a result of this location, it diminishes the apparent Na^+ affinity, which is similar to the effect of Mg^{2+}. This explanation is also in agreement with the detected $BTEA^+$-independent final fluorescence level.

DISCUSSION

The pump cycle of the Na,K-ATPase, the so-called Post−Albers cycle, in which enzyme and transport activity are correlated, was discovered more than 40 years ago,[3,4] but only in recent years have experimental tools and knowledge about structural details become available that allow, in combination with kinetic data, an analysis of the molecular processes that take place while the pumps run around their cycle. One focus has been ion binding and release on the extracellular side of the Na,K-ATPase. Binding and release of the three Na^+ ions have been studied in great detail because this process exhibits a strong electrogenicity. This property made it a welcome target of electrophysiological techniques, and these studies brought forth a well-accepted mechanistic explanation.[7,10,11,59,60] The subsequent binding of the two K^+ ions has been investigated by a variety of techniques as already mentioned in the introductory section. Different experimental approaches provided details that have been interpreted with different concepts that do not necessarily concur in a unanimous mechanism.

The process summarily named "binding of two K^+ ions" is in fact a sequence of several reaction steps. A K^+ ion diffuses from the external aqueous phase through a part of the membrane domain of the Na,K-ATPase, the access channel, to a site at which then the dehydrated ion can be coordinated by the protein matrix. The latter is the actual ion binding step. The same action comes to pass for the second K^+ ion to a second site. It has been found that both sites are occupied in a defined, sequential order.[50] After this binding step, a conformational rearrangement that blocks the ion-binding sites from the access channel occurs, the ions becoming occluded. This step is correlated with (or followed by) enzyme dephosphorylation. For the separation of single steps in this sequence of ion diffusion, coordination, and occlusion/dephosphorylation,

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different approaches are possible. A proposal of Forbush to use ions that are able to diffuse to the binding sites and bind but are not occluded[61] became the starting point of a series of investigations that confirmed the results from different approaches that claimed that the diffusion to the binding sites is electrogenic and demonstrated that the ion occlusion step is electroneutral.[36] The ions used to separate the access to the binding sites from the occlusion are quaternary amines.[35−37,61,62] Recently, extensive electrophysiological studies were performed exploiting whole-cell patch clamp experiments with ventricular myocytes.[18,35−37]

A completely different approach is presented here. The electrochromic styryl dye, RH421, has been used to detect charge movements in the Na,K-ATPase isolated in purified microsomal membrane fractions. This dye has been applied to study the electrogenic binding of K^+ from the extracellular side, and the observed fluorescence decrease has been assigned to the movement of the cation to the binding sites inside the membrane domain.[20] The effect of $BTEA^+$ on K^+ binding may be also detected by RH421 in terms of a shifted K_m value of K^+ binding (Figure 2), and the inhibition constant for $BTEA^+$ determined from these experiments (K_I = 4.2 ± 0.4 mM) is in good agreement with the value obtained from electrophysiological experiments (4.0 ± 0.3 mM).[35] In contrast to electrophysiological experiments that detected a potential-dependent inhibition of the Na,K-ATPase by $BTEA^+$ intrusion through the access channel toward the binding sites, the experiments with RH421 do not show a fluorescence decrease that is expected when cations are bound electrogenically inside the membrane domain, but a fluorescence increase (Figures 1 and 5). In a recent paper, it was proposed, therefore, that the missing fluorescence decrease could be the result of an alternative detection mechanism of RH421 in a way that it detects an occlusion-related conformational transition of the Na,K-ATPase that deforms the surrounding lipid phase.[38] Because $BTEA^+$ is not occluded, the RH421 fluorescence decrease then would fail to appear.

This ambiguity, but in particular a proposal of more details of the reaction mechanism, will be discussed in the following on the basis of the presented effects of quaternary ions on K^+ binding. We decided to make use of the backdoor phosphorylation as a tool to select a modified partial reaction of the Post−Albers cycle for the RH421 experiments. Because of the presence of ATP in the cytoplasm, a confinement of the possible reaction steps to the $P\text{-}E_2$ states cannot so easily be established during *in vivo* experiments. As explained in the introductory section, the Na,K-ATPase can be transferred in the absence of Na^+ and K^+ by saturating concentrations of inorganic phosphate (and Mg^{2+}) quantitatively into the $P\text{-}E_2$ state with empty ion-binding sites.[47] In studies in which the binding sites were loaded initially with radioactive $^{86}Rb^+$ or $^{42}K^+$, a rapid exchange of the bound radioactive ion against stable $^{39}K^+$ ions was observed upon backdoor phosphorylation. This finding indicates that on average a considerable fraction of the binding sites exists in the deoccluded phosphorylated state, even in the presence of high K^+ concentrations.[51,55] This condition allows restriction of the reaction sequence initiated by addition of K^+ to the $P\text{-}E_2$ state preferentially to the electrogenic diffusion of the ions through the access channel and their coordination in and release from the binding sites ($P\text{-}E_2 \leftrightarrow P\text{-}E_2K \leftrightarrow P\text{-}E_2K_2$).

The results found under the condition of backdoor phosphorylation confirm first of all the conclusion published by Peluffo et al.[35,36] that $BTEA^+$ is not occluded and does not interfere with K^+ occlusion because the K_I values (∼5 mM) obtained for $BTEA^+$ are not significantly different under turnover and backdoor phosphorylation conditions (Table 1). Also, the half-saturating concentration ($K_{1/2}$) for $BTEA^+$ of ∼10.3 mM was the same in both types of experiments (Figure 5). The fact that K_I is only half of the half-saturating binding constant of $BTEA^+$, $K_{1/2}$, is remarkable, and it indicates that $BTEA^+$ is obviously able to affect K^+ binding by just being present, without necessarily being bound. This observation will be discussed below. A second conclusion from the experiments under the condition of backdoor phosphorylation is that the K^+-induced fluorescence decrease is in favor of the assumption that RH421 detects the electrogenic movement of ions into their binding sites.

Additional information about K^+ binding is obtained when the effects of differently sized added quaternary ions are compared. A comparison of the effects of NH_4^+, DMA^+, TEA^+, and $BTEA^+$ on K^+ binding and the RH421 fluorescence levels reveals interesting details. Because it has been shown that, especially in the case of $BTEA^+$, artifacts due to unspecific interaction between RH421 and these ions can be excluded, three kinds of interaction of these compounds with K^+ may be discriminated. First, NH_4^+ competes with K^+ directly in the ion-binding sites. NH_4^+ is a congener of K^+, and it is coordinated with a lower binding affinity in the binding sites and transported by the Na,K-ATPase. This is reflected by the fact that $K_{1/2}$ and K_I of NH_4^+ were identical (2.3 mM), and K^+ and NH_4^+ caused the same saturating amplitude of the RH421 fluorescence decrease (Figure 6). Second, TEA^+ and $BTEA^+$ showed competitive inhibition of K^+ binding (Figures 2 and 7C) but did not cause a RH421 fluorescence decrease that would indicate an electrogenic binding in the ion-binding sites as NH_4^+ does (Figure 6). In contrast, $BTEA^+$ induced a significant fluorescence increase at concentrations above 0.5 mM. In the case of TEA^+, a minor fluorescence increase was indicated above 20 mM. This observation will be discussed below. The inhibitory effect of both compounds on K^+ binding has been observed previously.[33−35] A third kind of "interaction" was found for DMA^+. According to the missing effect on the RH421 fluorescence (Figure 6), this compound does not enter into the ion-binding sites or affect K^+ binding (Figure 7B); in contrast to the other compounds, no competition at all could be proven.

Two further details have to be considered before mechanistic proposals may be discussed. An effect of $BTEA^+$ on the RH421 fluorescence failed to appear when the ion-binding sites were occupied by two K^+ ions, and it was dramatically reduced when the pump was inhibited by ouabain (Figure 5). Using the same type of Na,K-ATPase preparation, it has been shown before that in the ouabain-inhibited state the ion-binding sites are occupied by two H^+ or Na^+ ions,[63] or possibly by one Mg^{2+}.[64] On the other hand, it is known that bound ouabain blocks the access channel and prevents binding of an ion to or release of an ion from the binding sites. Therefore, inhibition of the pump by ouabain will also prevent the entrance of $BTEA^+$ into the access channel. The second detail is that the presence of two K^+ ions in the binding sites, even if not occluded in the phosphorylated state, disables the interaction of $BTEA^+$ with the pump in a way that $BTEA^+$ is not able to reach the site or position where it causes the RH421 fluorescence to increase.

In electrophysiological experiments, a reduced electrogenicity of the release of the second and third Na^+ has been found upon

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comparison to the first Na^+ leaving the pump in its P-E_2 conformation.[7,10,11,15] The electrogenicity of binding of both K^+ ions corresponds to that of the second and third Na^+ released.[19] The obvious explanation of these observations is that after the release of the first Na^+ a conformational rearrangement of the P-E_2 state of the Na,K-ATPase widens the extracellular access channel so that it becomes filled with numerous water molecules. This process leads to a strong deformation of the equipotential lines of the membrane potential in the water-filled cavity and reduces the electrogenicity of ion binding under this condition. Such a cavity must be visible in a high-resolution structure of this state of the Na,K-ATPase. Unfortunately, such a structure has not been determined. Until now, only the structure of the occluded $E_2(K_2)$ has been published with a resolution of 2.4 Å [Protein Data Bank (PDB) entry 2ZXE].[13] Another related structure is the ouabain-inhibited Na,K-ATPase, P-$E_2^{ouabain}$, with a resolution of 2.7 Å (PDB entry 4HYT).[64] A vestibule is visible, although binding of the inhibitor molecule in the access channel leads to a blockage of this pathway. To gain nevertheless further information, one may look up the structure of the sarcoplasmic reticulum Ca-ATPase (SERCA) in the open P-E_2 conformation. Because corresponding structures of the Na,K-ATPase and SERCA, as far as those are available, show a reasonable conformity, one may scrutinize the SERCA structure resembling closest the empty P-E_2 state of the Na,K-ATPase. For this purpose, the E_2-BeF_3^- complex of SERCA was chosen (PDB entry 3B9B)[65] that has a resolution of 2.65 Å. This structure may be discussed as a template of the P-E_2 state of the Na,K-ATPase and has been used for homology modeling.[15] It shows a funnel-like vestibule with a maximal width of >10 Å at the entrance (see Figure S1 of the Supporting Information), which is in contact with the aqueous phase, and a depth of ∼30 Å. At the bottom of this pit, close to the ion-binding sites, the cross section is reduced to a diameter that allows only (electrogenic) permeation of small cations. This spatial arrangement is represented as a schematic drawing in Figure 9. The volume of the cavity of the published SERCA structure can be estimated to be >2000 $Å^3$ and, therefore, is able to contain more than 60 water molecules. $BTEA^+$ molecules are able to enter the access pathway, and it is quite conceivable that a single $BTEA^+$ can act as a stopper at the narrow opening to the binding sites as indicated in Figure 9 and proposed by Peluffo et al.[35] A comparison of the molecule size of the ammonium ion and its organic derivatives tested in this study is shown in Figure S2 of the Supporting Information. In their 2009 paper, Peluffo et al. propose on the basis of molecular dynamics simulations two $BTEA^+$-binding sites, one close to the surface of the protein and the other partly overlapping with the ion-binding sites.[36]

On the basis of this structural assignment, constraints may be formulated with respect to the effect of the different quaternary organic ammonium ions on K^+ binding (and transport). Ion movements in the wide, water-filled part of the access pathway are not or only to a very small extent electrogenic and will not be detected by RH421. In contrast, NH_4^+ is small enough to slip, like K^+, through the narrow part of the pathway and generates an electrogenic current contribution while approaching the binding sites. NH_4^+ can be coordinated in the binding sites and eventually transported to the cytoplasm. Because these binding sites show a distinct selectivity for K^+ (or NH_4^+) over Na^+, the flexibility of this moiety must be rather restricted. The other three ions, DMA^+, TEA^+, and $BTEA^+$, are able to enter and move only in the wide part of the access pathway and produce different, size-dependent effects. DMA^+ is not interacting with the protein anywhere inside the access pathway. In addition, it is small enough not to affect significantly K^+ movements inside the access channel, so that no change in K^+ binding affected by DMA^+ has been detected experimentally (Figure 7B). The more bulky TEA^+ molecule displaces already a significant number of water molecules in the access pathway, and it can be expected that TEA^+ impedes in a detectable way the movement of K^+ ions that have to be assumed to be still hydrated in this section of their trajectory. This causes an apparent and TEA^+ concentration-dependent reduction of the K^+ binding affinity (Figure 7C). The size of TEA^+ excludes an entrance into the narrow access channel and explains the absence of electrogenic binding that can be detected by RH421 (Figure 6). The competitive behavior has been reported previously[33,34] and may be explained by the space-filling, hampering size of TEA^+ molecules (Figure S2 of the Supporting Information). The presence of a TEA^+ molecule close to the entrance to the narrow access channel reduces thus effectively the level of K^+ binding.

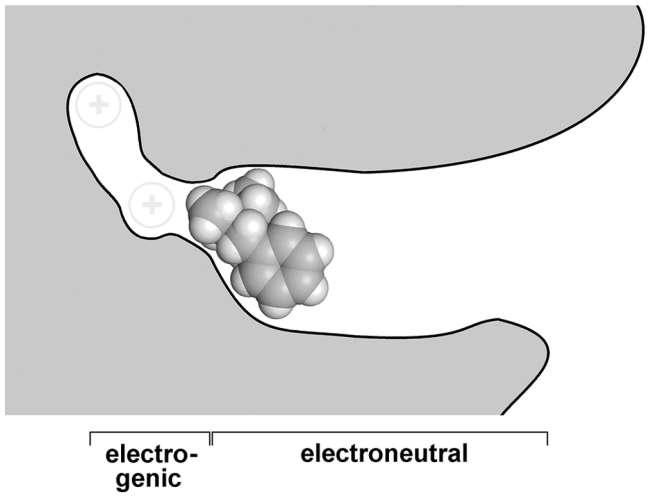

Figure 9. Schematic representation of the extracellular access pathway to the ion-binding sites in the P-E_2 conformation of the Na,K-ATPase. The basis of this cartoon is the shape of the SR Ca-ATPase in the E_2-BeF_3^- conformation (PDB entry 3B9B).[65] The position of both K^+ ions (according to the position of the coordinating amino acid side chains) is included in light gray. A $BTEA^+$ molecule in space-filling representation is added at a proposed deepest position in the access pathway (cf. also Figure S1 of the Supporting Information).

$BTEA^+$ and TEA^+ are similar molecules. The only difference is that in $BTEA^+$ one ethyl residue is replaced by a benzyl residue, and it is intriguing but so far not understood that the additional, apolar benzyl group introduces an electrogenic binding behavior.[36] Actually, it has to be expected that $BTEA^+$ behaves like TEA^+, insofar as is should not enter the electrogenic part of the access pathway and inhibits competitively K^+ binding. With a K_I of 5.1 mM, it is, however, 6 times more effective than TEA^+ (K_I = 33.6 mM). Furthermore, a RH421-detected binding kinetics of $BTEA^+$ as shown in Figure 5 could not be observed for TEA^+ in the concentration range covered by the experiments (≤70 mM). Binding of $BTEA^+$ to a location different from the K^+-binding sites is indicated by a (so far unexplained) fluorescence increase with a $K_{1/2}$ of 10 mM, which is twice the value of K_I obtained from K^+ binding studies. This observation points to two

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different interaction mechanisms. On one hand, $BTEA^+$ is able, like TEA^+, to block K^+ binding by its mere presence in the access pathway because of its charge and its size, it being even more bulky than TEA^+. On the other hand, the additional benzyl residue seems to amplify the interaction with the surface of the protein inside the access cavity and induces a binding behavior therein. This could be caused either by a spatial fit of the benzyl group into a kind of binding pocket ("lock-and-key" principle) or by an appropriate van der Waals attraction between the benzyl residue and an amino acid side chain in the access pathway. On the basis of this assumption, a proposal can be offered for the electrophysiologically observed potential-dependent inhibition of binding of K^+ to the Na,K-ATPase[37] by $BTEA^+$ but not by TEA^+: The externally applied membrane potential produces a small electric field along the access pathway that supports a diffusion of cations toward the binding sites, and both TEA^+ and $BTEA^+$ act as a stopper at the entrance to the narrow channel (similar to what has been observed for TEA^+ as an inhibitor of the voltage-dependent K^+ channel in nerve cell membranes). When located in this position, the benzyl residue of $BTEA^+$ interacts with the protein and thus stabilizes its inhibitory placement, in contrast to TEA^+. This proposal is further supported by the observation that the addition of *p*NBTEA$^+$, in which a NO_2 group is added in the "para" position of the aromatic ring, increases severalfold the apparent affinity for inhibition of the K^+-activated current while keeping the voltage dependence of the block.[32] The effect of *p*NBTEA could be due to changes in the π-electron cloud of the aromatic ring (thus, possibly increasing the number of cation$-\pi$ interactions with the protein) or a direct effect of the NO_2 group itself. TEA^+ lacks such an additional interaction with the protein, and the small electric field produced by the membrane potential in the access pathway alone cannot reinforce the stopper function. Therefore, it inhibits less effectively K^+ binding, and no significant effect of the membrane voltage on TEA^+ inhibition can be detected.

The intriguing effect of $BTEA^+$ on the RH421 fluorescence remains to be resolved. The control experiments have shown that it is no artifact caused by direct interaction between both compounds or by unspecific binding to the protein. A significant fluorescence increase in the P-E_2 conformation was observed only when the ion-binding sites were accessible and not occupied by ions (Figure 5), and this increase could be reversed by addition of saturating K^+ concentrations (Figure 1). In contrast to $BTEA^+$, TEA^+ that lacks the benzyl group is unable to produce such a significant fluorescence increase in the concentration range up to 70 mM. The $BTEA^+$-induced increase in RH421 fluorescence corresponds to a change in the local electric field (by a change in the local dielectric constant) that is produced either by less positive charge inside the membrane domain of the Na,K-ATPase or by a weaker alignment of electric dipoles formed by the protein matrix and/or the water-filled access pathway. Taking these observations into account allows the consideration of possible mechanisms.

A first concept could be that binding of $BTEA^+$ induces a significant conformational rearrangement such as a constriction of the access funnel. The resulting displacement of the water molecules is followed by a relaxation of the amino acid side chains that are no longer in contact with the polar water molecules, and this could easily produce a significant modification of the local electric field. Against such a mechanism, however, it may be argued that electrogenic binding of K^+ in the presence of $BTEA^+$ is fast upon addition (within the time resolution of the setup) and without delay. In contrast, the RH421 response on ouabain binding has a time constant on the order of several seconds.[63] An alternative mechanism could be that binding of $BTEA^+$ at the bottom of the access funnel displaces numerous water molecules, repels cations from the funnel, aligns the dipole moments of the residual water molecules differently, and modifies thus the local electric field in a way that is detected by the RH421 molecules surrounding the ion pumps in the lipid phase. Such a process would occur in the microsecond time range. Furthermore, not completely excluded may be the hypothesis that the ion-binding sites in the P-E_2 conformation at physiological pH and low Na^+ concentrations are not completely empty as has been proposed previously[47] but contain at least one H^+ that shuttles in and out until it is replaced by a K^+ bound to the sites. If true, $BTEA^+$ could bind as a stopper and block the binding sites preferentially when no H^+ is located in the sites, and the now effectively empty sites would lead to an increase in the RH421 fluorescence level. The discrimination between those (or further different) proposals has to be, however, subject to future studies.

■ ASSOCIATED CONTENT

Supporting Information

Structural arrangements of the extracellular access channel of the SR Ca-ATPase in the E_2-BeF_3^- conformation (PDB entry 3B9B) that has been used as a template for the P-E_2 conformation of the Na,K-ATPase to depict the size of this part of the ion pathway and a comparison of the sizes and chemical structures of the tested substrate molecules. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

RH421, *N*-(4-sulfobutyl)-4-{4-[4-(dipentylamino)phenyl]-butadienyl}pyridinium, inner salt; $BTEA^+$, benzyltriethylammonium ion; TEA^+, tetraethylammonium ion; DMA^+, dimethylammonium ion; *p*NBTEA$^+$, *p*-nitrobenzyltriethylammonium ion.

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