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Binding of the third Na^+ ion to the cytoplasmic side of the Na,K-ATPase is electrogenic

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Abstract A new experimental setup was constructed to allow parallel measurements of total internal reflection fluorescence and of capacitance changes in Na,K-ATPase-containing membranes. Effects correlated with cytoplasmic sodium binding to Na,K-ATPase were investigated. Ion binding-induced fluorescence changes of the electrochromic dye RH421 in membrane fragments adsorbed on a transparent capacitative electrode corresponded perfectly to capacitance increases detected by a lock-in technique. From these electric measurements it was possible to estimate a dielectric coefficient of about 0.25 for the electrogenic binding of the third Na^+ ion. Binding of K^+ to cytoplasmic sites was electroneutral.

Key words: Sodium pump; Ion movement; Electrogenic binding; Fluorescent dye; Capacitative coupling

1. Introduction

The Na,K-ATPase utilizes the free enthalpy of ATP hydrolysis to transport Na^+ and K^+ ions across cytoplasmic membranes of animal cells against their respective electrochemical potential gradients. This process has been investigated over at least two decades and resolved in a number of details [1–3]. It has been shown that translocation of three Na^+ ions can be explained by a series of reaction steps which are ion binding on the cytoplasmic side in state E_1 of the pump, ion occlusion when the enzyme is phosphorylated, conformational transition $E_1 \rightarrow E_2$, and release of the ions to the extracellular aqueous phase. To understand the molecular mechanisms of transport it is of great interest to identify and to analyze how much charge is moved through the protein in each of the reaction steps [1]. Various techniques are applied to investigate the electrogenicity of Na^+ transport: voltage jump experiments with giant patch or whole cell patch of myocardial cells [4,5], charge pulse experiments on membrane fragments adsorbed to planar lipid bilayers [6,7] or, very recently, adsorbed to solid supported bilayers [8], and a fluorescence technique applying an electrochromic styryl dye, RH421, which detects charge movements within the membrane dielectric [9,10]. The latter method provides indirect access to the so-called electrogenicity as discussed elsewhere [1,6,10].

As a results of all these investigations it has been found that in the reaction sequence of Na^+ transport

$$3\ Na^+ + E_1 \rightarrow Na_3E_1 \rightarrow (Na_3)E_1\text{-}P \rightarrow P\text{-}E_2(Na_3) \rightarrow$$

$$P\text{-}E_2(Na_2) + Na^+ \rightarrow P\text{-}E_2 + 3\ Na^+$$

the second step, Na^+ occlusion together with enzyme phosphorylation, is not electrogenic, and that the subsequent conformational change (third step) is of minor electrogenicity. The major contribution to the transmembrane current is the release of the first Na^+ to the extracellular side, $P\text{-}E_2(Na_3) \rightarrow P\text{-}E_2(Na_2) + Na^+$ which has a dielectric coefficient of about 0.65 [6,11]. The release of the other two ions generates a significant but smaller share of the electric current (0.3–0.4) [6]. The electrogenicity of the first step, binding of three Na^+ ions to the ion pump on the cytoplasmic side, has been proven so far by the RH421 method [12], deduced indirectly [6,13], and has been shown very recently in a qualitative manner with membrane fragments adsorbed on solid supported membranes [8].

The aim of this presentation is to introduce a new combined method of fluorescence and electrical measurements in which membrane fragments immobilized on a transparent capacitative electrode may be measured with respect to ion movements occurring in various partial reactions of the Na,K-ATPase pump cycle. This method has been used to demonstrate that RH421 detects charge movement within the protein when the third Na^+ ion binds to its site in the pump and then to determine the dielectric coefficient of this process by direct electric measurements.

2. Materials and methods

Choline chloride (3× crystallized) was obtained from Sigma, Deisenhofen, bis(2-hydroxyethyl)amino-tris(hydroxy-methyl)methane (Bis-Tris) from Fluka, Neu-Ulm, and mercaptopropionic acid (MPA) from Aldrich. RH421 was from Molecular Probes, Eugene, OR, all other reagents were from Merck, Darmstadt, in the purest grade available.

Membrane preparations with a high concentration of active Na,K-ATPase (about 5000 pumps per μm^2) were prepared from outer medulla of rabbit kidneys using procedure C of Jørgensen [14]. For these preparations a specific ATPase activity was measured in the range between 2000 and 2600 μmol P_i per hour and mg protein at 37°C.

The equipment used for the fluorescence and electrical measurements was developed from a fluorescence setup applying the total internal reflection fluorescence (TIRF) technique to perform RH421 experiments with membrane fragments immobilized on a quartz trapezoid which forms the bottom of a cuvette for aqueous buffers [15]. The TIRF setup was modified by preparation of an transparent capacitative electrode on the quartz surface as shown in Fig. 1. The evanescent wave penetrated the aqueous phase with a considerable intensity in a range of about 100 nm so that fluorescence was excited

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only in dye molecules dissolved in the lipid phase of membrane fragments which were adsorbed to the transparent bottom of the cuvette. This could be verified by complete exchange of the aqueous phase after completion of the adsorption of fluorescence dye containing membrane fragments. For persistent adsorption the presence of divalent ions was prerequisite. Typically 7 mM $MgCl_2$ was added to initialize the adsorption process, which took more than 1 h to reach saturation as detected by the increasing fluorescence intensity. In the absence of divalent ions no significant adsorption of membrane fragments could be observed. The standard buffer used in the experiments presented here was 30 mM Bis-Tris, 0.5 mM EDTA, 6.8 mM $MgCl_2$, 110 mM choline chloride, pH 6.7, and various concentrations of NaCl between 0 and 30 mM or KCl between 0 and 12 mM. Experiments were performed at 21°C throughout. Adsorption occurred on a unmodified quartz surface as well as on the surface of the capacitative electrode consisting of a 'transparent' gold layer and an insulating monolayer of MPA. Scanning force microscopic images showed that under both conditions typically 40% of the surface were covered by membranes [16]. The conducting gold layer was produced by coating the quartz surface first with a 1 nm thick layer of chromium and then with a 10 nm layer of gold in a high-vacuum thermal evaporator (Uvivex 450, Leybold-Heraeus, Cologne). The mercapto group of MPA binds covalently to the gold surface when MPA is added to the aqueous solution of the assembled cuvette. The resulting dielectric layer of MPA had a typical resistance of $< 10^4$ Ω and a capacitance of 5 μF (the area of the capacitative electrode formed by the bottom of the cuvette was 0.79 cm^2). After the formation of the dielectric layer the excess MPA was eliminated by exchanging the buffer in the cuvette. The additional layer of gold and MPA affected the TIRF measurements in so far as the fluorescence intensity at the entrance window of the photomultiplier was strongly reduced when compared to the unmodified quartz trapezoid [16]. Therefore no significant fluorescence changes could be measured in the presence of a gold electrode. The TIRF data presented in Fig. 4 were taken from an uncoated quartz surface. An alternative capacitative electrode consisting of indium-tin oxide (ITO) with an insulating layer of TiO_2 had very good optical properties, however, due to the surface roughness of the available ITO layer the capacitative coupling was less effective [16].

Electrical properties of the Na,K-ATPase could be measured by the principle of capacitative coupling of the membrane fragments to a 'capacitative electrode' as previously discussed [6–8]. The crucial electric parameter evaluated in this presentation was the (apparent) capacitance of the ion-pump containing membranes as presented in detail below. According to electrochemical impedance spectroscopy [17] the capacitance and its changes were measured with an lock-in amplifier SR830 DSP (Stanford Research Systems, Sunnyvale, CA), which was used to apply a sine-wave voltage with an amplitude of 2–4 mV and a frequency in the range of 0.1–10 000 Hz. The resulting current was amplified by a factor of 10^4 with a current amplifier Model 1212 (ITHACO, Ithaca, NY). The amplitude and phase angle of the complex system impedance were transferred digitally to a computer, displayed, stored and processed with the software Testpoint (Version 3.1, Capital Equipment Corp., Burlington, MA). Details are published in the Ph.D. thesis of W. Domaszewicz [16].

Mathematical analyses of the replacement circuit (Fig. 2B) were performed with the Mathematica program system (V. 3) on a Pentium computer to derive analytical expressions for the real and imaginary part of the complex impedance and for the phase angle. Numerical simulation of the experiments were executed with programs written in FORTRAN 95 (Lahey Computer Systems, Inc., Incline Village, NV).

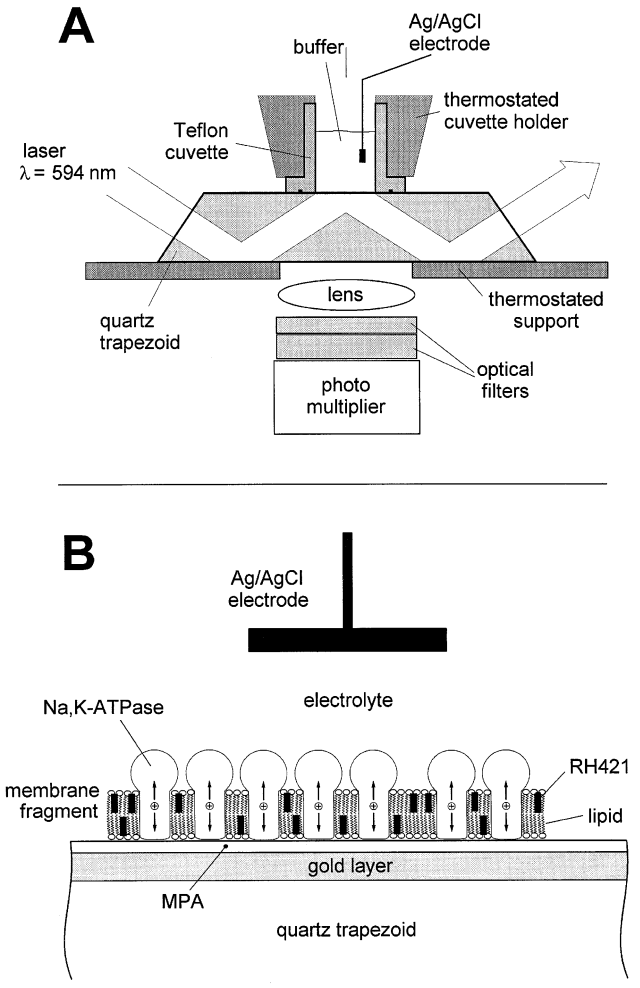

Fig. 1. Schematic drawing of the combined fluorescence and electrical setup. A: Principle of the TIRF method. The cuvette consists of a cylindrical tube of (black) Teflon and the quartz trapezoid which forms the bottom of the cuvette and guides the laser beam (He-Ne laser, $\lambda = 594$ nm) by total reflection. The whole setup can be thermostated by Peltier elements. The totally reflected light produces an evanescent wave illuminating a layer of about 100 nm of the aqueous phase in contact with the bottom of the cuvette. Membrane fragments, in which the electrochromic dye RH421 is dissolved, are adsorbed to the surface (B). The buffer in the cuvette can be exchanged and stirred during the experiments. An Ag/AgCl electrode is immersed into the electrolyte. The setup is mounted in a housing made of aluminum to shield the equipment against light and electromagnetic noise. A lens underneath the quartz trapezoid focuses the emitted fluorescence onto the entrance window of a photomultiplier. Appropriate optical filters select a window of 660 ± 15 nm for the RH421-specific emission. Contact to the gold layer of the capacitative electrode is made outside the cuvette by pressing a wire against it (not shown). B: Schematic representations of the capacitative coupling of a membrane fragment as it occurs at the bottom of the TIRF cuvette.

3. Results and discussion

3.1. Fluorescence measurements

In a first set of experiments it was shown that membrane fragments which adsorbed to the quartz or electrode surface from standard buffer (containing 640 nM RH421) produce the same characteristic fluorescence changes upon substrate-induced partial reactions in the TIRF measurements as have been observed in non-TIRF fluorescence experiments with membrane fragments floating freely in buffer [9,10,12]. In principle the same fluorescence behavior was found, although the amplitudes of the fluorescence changes showed some (minor) deviations. The fluorescence changes were calculated with respect to the fluorescence intensity, F_0, before addition of a substrate. Three partial reactions could be investigated with TIRF (not shown): (a) addition of saturating Na^+ (≥ 30 mM) in state E_1 of the Na,K-ATPase triggers the reaction $3\ Na^+ + E_1 \rightarrow Na_3E_1$, and in the TIRF experiments led to an average relative RH421-fluorescence decrease, $\Delta F/F_0 = (F - F_0)/F_0$, of −0.15 (compared to −0.2 from experi-

ments in suspensions), (b) addition of 200 μM ATP allows the reaction $Na_3E_1+ATP \rightarrow (Na_3)E_1\text{-}P+ADP$, the concomitant fluorescence increase, $\Delta F/F_0$, was typically +0.7 (compared to +0.75 in suspensions), (c) addition of (saturating) 26 mM K^+ led to a dephosphorylation of the protein, $P\text{-}E_2+2\ K^+ \rightarrow E_2(K_2)+P_i$, with a fluorescence decrease, $\Delta F/F_0$, of −0.38 (compared to −0.4 in suspensions). These findings reveal that adsorption of the Na,K-ATPase-containing membrane fragments to a solid substrate did not significantly affect the detection mechanism of the electrochromic styryl dye RH421.

Binding of Na^+ ions to the cytoplasmic sites has been extensively investigated with the RH421 method [12] and the dependence of the apparent binding affinity of Na^+ ions on the Mg^{2+} concentration has been shown. In the presence of 7 mM Mg^{2+} the concentration dependence in solutions could be fitted by a Hill function with a half-saturating Na^+ concentration, $K_{1/2}$, of 5 mM and a Hill coefficient, n_H, of 2. In the present study Na^+ binding to Na,K-ATPase in adsorbed membranes in state E_1 also resulted in a concentration dependence which could be fitted with a Hill function, the only difference being an apparent increase of the $K_{1/2}$ to 7.7 mM (see Fig. 4). This difference may be explained by a Guy-Chapman effect due to negative charges at the surface of quartz or MPA, which increase the local Mg^{2+} concentration and in consequence produce an slightly higher apparent $K_{1/2}$ value [12] when compared to experiments in solutions.

3.2. Electrical measurements

The analysis of the electrical measurements were based on the model which is schematically presented in Fig. 2A. The flat membrane fragments are considered to adsorb preferentially with their extracellular side to the MPA layer with low leakage conductance and fulfill the condition of capacitative coupling as observed with fragments adsorbed to lipid bilayers [8,18]. The apparent capacitance of the membrane fragments is controlled by the thickness of the membrane dielectric which has a certain size, d_1, in the absence of ions which are able to enter into binding sites. If the assumption is correct that the Na^+-specific binding site of the Na,K-ATPase is situated within the membrane dielectric (i.e., ion binding is electrogenic) as has been reasoned from the interpretation of the RH421 experiments, then the apparent capacitance of the fragments is affected when Na^+ ions are present. It is known that access of ions to the sites is fast, perhaps diffusion controlled [4,10] and that the pump density in the membranes is high, which results in an average distance between two pumps of the order of 15 nm. Therefore, the concentration-dependent occurrence of ion movement in and out of the binding site, induced by a sinusoidal voltage across the membrane, produces an apparent reduction of the thickness of the membrane dielectric. This leads to a smaller effective thickness of the membrane, $d_2 < d_1$, in the presence of a significant Na^+ concentration (Fig. 2A). The electronic replacement circuit of this layout can be represented in a first order approximation by the resistors and capacitators as shown in Fig. 2B. R_E takes into account the electrode resistance and buffer conductance. Since only part of the MPA layer is covered by fragments, covered and uncovered parts of the capacitative electrode have to be treated in parallel. R_M and C_M are resistance and capacitance of the membrane fragments, R_I and C_I are resistance and capacitance of the insulating MPA layer under

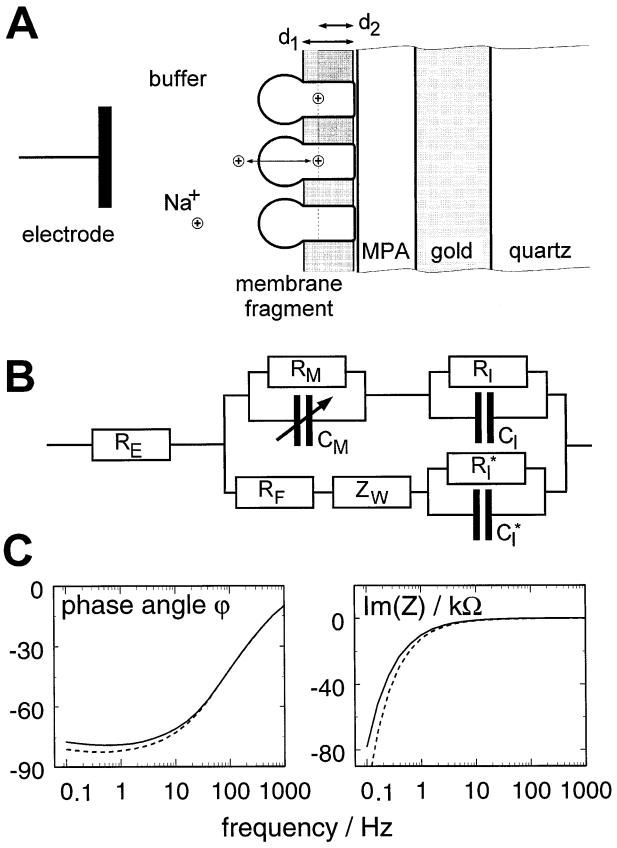

Fig. 2. Principle of the electric detection system applied to Na,K-ATPase-containing membrane fragments adsorbed to the capacitative electrode placed on the TIRF quartz surface. A: Effect of Na^+ ions which bind electrogenically to their specific site within the protein dielectric. Since no ATP is present the Na^+ ions will not be occluded in their binding sites but move in and out in a diffusion-controlled manner. Due to the high density of ion pumps (about 5000 per μm^2) an apparent reduction of the thickness of the membrane dielectric will occur at saturating Na^+ concentrations. This will be reflected by a change in the membrane capacitance which is proportional to $1/d$. B: Replacement circuit of the capacitative electrode which is partly covered by membrane fragments. For detailed explanation see text. C: Lock-in experiment. Dependence of the phase angle φ of the measured complex impedance as a function of the frequency of the applied sine voltage in the presence (solid line) and in the absence (dashed line) of membrane fragments. These curves can be fitted by the calculated response of the replacement circuit of B (not shown).

the fragments. The second pathway takes into account the MPA layer free of adsorbed membrane fragments which consists in general of a complex Warburg impedance, Z_W (which accounts for possible electrochemical reactions at the MPA/buffer interface), and R_I*, C_I* which represent the electric properties of the uncovered MPA layer. In the case of the MPA insulator Z_W could be ignored ($Z_W = 0$). The ratio of C_I*/C_I and R_I/R_I* depends on the amount of membrane fragments adsorbed to the electrode, and is 1.5 in the case of a 40% coverage. Due to the electrogenic Na^+ binding C_M is modulated by the Na^+ concentration since $C_M \propto 1/d$ (Fig. 2A). In the experiments presented here the changes of all other parameters but C_M were small when the Na^+ concentration was increased, mainly because the total ionic concentration was already high due to the presence of 110 mM (inert) choline chloride. The capacitance of the coupled capacitators was determined experimentally by the lock-in

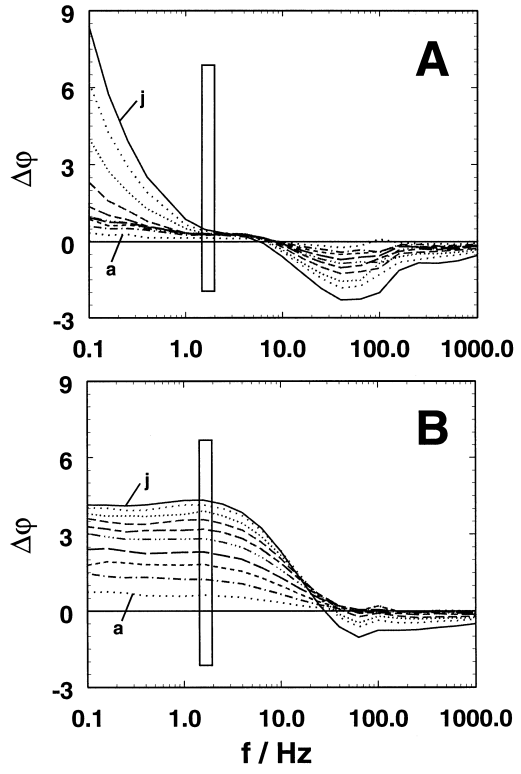

Fig. 3. Lock-in measurements of the capacitative electrode without (A) and with (B) adsorbed membrane fragments containing Na,K-ATPase. The difference of the phase angle of the complex impedance, $\Delta\varphi = \varphi([Na^+]) - \varphi(0\ Na^+)$, is plotted in both panels. $\Delta\varphi$ is a function of the Na^+ concentration and the frequency of the applied sine voltage of 4 mV amplitude. The Na^+ concentration was increased from a to j and had the values (in mM): 2.0 (a), 3.9, 5.8, 7.7, 9.7, 11.6, 15.5, 19.3, 23,1, 30.5 (j). The MPA monolayer was incubated with buffer containing 14 μg/ml membrane fragments which resulted in a surface coverage of about 40%. For further analysis the data points obtained at a frequency of 1.6 Hz were used (as indicated by the box).

method which allowed the measurement of the impedance of the system, $|Z|$ (not shown), and the phase angle, φ (Fig. 2C). The imaginary part of Z, ImZ, is $|Z| \sin\varphi$. $|Z|$ and φ were obtained as functions of the frequency of the sine voltage applied to the system. In the frequency range between 0.1 Hz and 100 Hz the data could be fitted reasonably by the predictions of the replacement circuit as shown in Fig. 2B. It was obvious that the differences of the impedance with and without membrane fragments were small, therefore, appropriately long sampling intervals of the lock-in amplifier were chosen (e.g. 20 s at 0.1 Hz) to obtain phase angles with a signal/noise ratio of >3000.

To identify the effect of increasing Na^+ concentration on the capacitative electrode, Na^+ titrations were performed in the absence of membrane fragments. For a series of 10 increasing Na^+ concentrations, as listed in the legend of Fig. 3, the impedance was measured at frequencies, f, between 0.1 Hz and 1 kHz and the capacitance-dependent signals $\varphi(f)$ and $|Z(f)|$ were recorded. For each Na^+ concentration changes with respect to the signal in the absence of Na^+, $\Delta\varphi = \varphi([Na^+]) - \varphi(0)$, were calculated and plotted (Fig. 3A). It was found that $\Delta\varphi(f)$ (and ΔIm $Z(f)$, not shown) were independent of the Na^+ concentration in the frequency window between 1.5 Hz and 6 Hz. In a second series of experiments membrane fragments (14 μg/ml) were added to the buffer and equilibrated with the capacitative electrode until a steady state of adsorbed membrane fragments was obtained. Then the Na^+ titration was repeated with the same concentration steps and the results analyzed accordingly (Fig. 3B). The frequency dependence of $\Delta\varphi$ of the complex system of capacitative electrode and membrane fragments (which covered about 40% of the electrode) differed significantly from that of the MPA layer only (Fig. 3). This is expected from simulations of the replacement circuit. In the frequency window between 0.1 Hz and 5 Hz the change of the phase angle, $\Delta\varphi$, was frequency independent and $\Delta\varphi$ for the uncovered MPA layer was near zero for all Na^+ concentrations. Therefore, we selected the results at 1.6 Hz for further analysis of the Na^+ dependence of the Na,K-ATPase, where no Na^+ concentration-dependent corrections of the contributions of the capacitative electrode free of membrane fragments were necessary.

Taking into account that the increase of the NaCl concentration in the presence of a high choline chloride concentration does not significantly alter the ohmic resistances in the investigated system (Fig. 2B), the Na^+-induced capacitative changes are reflected preferentially in changes of Im Z (as known from basic principles) and of φ (tan $\varphi = (\text{Im } Z)/(\text{Re } Z)$). Since the changes of the phase angle were small, $\Delta\varphi \ll \varphi$, we can approximate $\Delta\varphi \propto \Delta\tan\varphi$, and plot $\Delta\varphi$ as well as ΔIm Z as a function of the Na^+ concentration and compare them with the results of the RH421 fluorescence experiments (Fig. 4). All three data sets in Fig. 4 were normalized to their respective amplitude at 30 mM Na^+. They show perfect agreement in their Na^+ concentration dependence and may be fitted with a Hill function, $A = A_\infty/(1+(K_{1/2}/c_{Na})^n$. From all three types of measurements a half-saturation Na^+ concentration, $K_{1/2}$, of 7.7 mM and a Hill coefficient, n, of 2.0 were determined. This result is in agreement with previously obtained

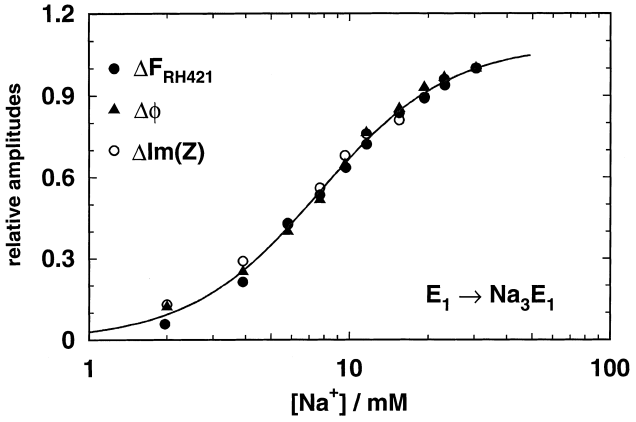

Fig. 4. Concentration dependence of cytoplasmic Na^+ binding to the Na,K-ATPase. Comparison of the results obtained by optical and electrical measurements. The experimentally determined signals were normalized to their respective amplitude at 30 mM Na^+ and plotted as changes of the RH421 fluorescence, ΔF_{RH421}, of the phase angle of the complex system impedance, $\Delta\varphi$, and of the imaginary part of the impedance, ΔIm(Z), with respect to a Na^+-free buffer. The RH421 data presented in this plot are from measurements with fragments adsorbed to an uncoated quartz surface. The line shown is the least-square fit of the Hill function with $K_{1/2} = 7.7$ mM and the Hill coefficient $n = 2$ to all three sets of data. The data at 30 mM Na^+ ($\sim 4 \times K_{1/2}$) reached about 90% of the amplitude at saturating Na^+ concentrations.

data on cytoplasmic Na^+ binding to the Na,K-ATPase in solutions of membrane fragments [10,12]. Recently published cytoplasmic cation binding to sodium pumps from pig kidney adsorbed to solid supported membranes using a flow-through method [8] showed a Na^+ binding-induced charge transfer which followed first-order Michaelis-Menten kinetics with a $K_{1/2}$ value of 15 mM in the absence of ATP, and a Hill function with $K_{1/2}$, of 2.6 mM and n of 2.0 in the presence of ATP under turnover conditions of the pumps.

Other cations which bind to the Na,K-ATPase in its E_1 state and which are able to bind to the two negatively charged ion binding sites on the cytoplasmic interface of the pump are K^+ and its congeners such as Rb^+ or Cs^+. They do this in an electrically silent fashion. It has been demonstrated that they produce no significant changes of RH421 fluorescence. The effect of K^+ binding on the capacitance of membrane fragments coupled to the capacitative electrode was investigated by experiments similar to those for Na^+ binding. Since the apparent binding affinity for K^+ in the E_1 conformation is of the order of 0.1 mM [12], concentrations between 44 μM to 10 mM were chosen. In the whole range of K^+ no concentration-dependent changes of $|\Delta\varphi| > 0.3°$ were observed. (Under the assumptions discussed in the next paragraph this would correspond to dielectric coefficients for cytoplasmic K^+ binding smaller than 0.02, which is not significant.) This is in agreement with previously published electrophysiological studies [19], with RH421 experiments in solutions [10], with membrane fragments on solid supported membranes [8], and with reconstituted vesicles [20]. By all these experiments it has been ruled out that cytoplasmic K^+ binding is electrogenic.

The dielectric coefficient of cytoplasmic Na^+ binding, α, may be introduced as $\alpha = d_1 - d_2$, where d_1 and d_2 are dielectric distances, which are not necessarily geometric distances (both quantities would coincide when the dielectric constant of membrane/protein is homogeneous in the dielectric phase of the membrane). Under the assumption that the ion pumps are homogeneously distributed within the membrane fragments and that $\Delta C_M \ll C_M$ the dielectric coefficient may by estimated by $\alpha = \Delta C_M / C_M^0$ [15], where C_M^0 is the membrane capacitance in the absence of Na^+. By numerical simulations of the replacement circuit of Fig. 2B (at a frequency of 1.9 Hz, with $R_E = 150\ \Omega$, $Z_W = 0$, $R_M = 1\ M\Omega$, $R_I = 100\ k\Omega$, $C_I = 0.7$ μF, and $C_M = 1$ μF (in the absence of Na^+)) it was found that for an increase of $\Delta C_M / C_M^0$ in the range 0–0.5 the phase angle, $\Delta\varphi$, increased linearly from 0 to 7.5°. At 30.5 mM Na^+ the experimentally obtained $\Delta\varphi$ was 4.2 from which a dielectric coefficient $\alpha = 0.22$ is determined. This number has to be compared with proposals from other experiments in which α was determined indirectly by reasoning in the range of 0.25 for saturating Na^+ concentrations [6,10]. The agreement between this previously obtained dielectric coefficient by indirect methods and that determined directly in this presentation is very good. Earlier investigations of the effect of voltage on cytoplasmic Na^+ binding to ion pumps reconstituted in lipid vesicles led to a determination of a dielectric coefficient of 0.16 [13]. In experiments with solid supported membranes the total charge translocation of the ATP-dependent Na^+ translocation and the amount of charge moved by Na^+ binding could be determined. By comparison it was found that 30% of the total charge translocated may be attributed to Na^+ binding [8]. From this number, and taking into account that the release of all three Na^+ ions to the extracellular side is electrogenic [4,6,11], a dielectric coefficient for cytoplasmic Na^+ binding of the order of 0.3 may be estimated.

4. Conclusion

1. Optical experiments with Na,K-ATPase-containing membrane fragments based on the detection of changes of local electric fields by the styryl dye RH421 may be performed in solutions and with membrane fragments adsorbed to a transparent capacitative electrode applying the TIRF method. Both types of experiments were equivalent and provided the same information on substrate-dependent partial reaction of the ion pump.
2. Electric measurements in which a lock-in method was applied to membrane fragments coupled to a capacitative electrode allowed the determination of Na^+ concentration-dependent changes of the apparent capacity of the membrane fragments containing ion pumps in the E_1 state of the Na,K-ATPase. This indicated that Na^+ binding is electrogenic, in contrast to binding of other ions such as K^+.
3. The perfect agreement between the results of the RH421 experiments and the lock-in experiments provided strong evidence that both methods detect the same process of the ion pumps, namely electrogenic binding of Na^+ to its sites on the cytoplasmic face of the ion pump.
4. The absence of dielectric effects when ions other than Na^+ bind to the E_1 conformation of the Na,K-ATPase indicated that only the Na^+-specific binding step is electrogenic, i.e., binding of the third Na^+, since binding of the first two Na^+ ions is competitive with other ions and occurs at the same sites [12].
5. The 'dielectric depth' of the access channel (or ion well [1]) is about 25% within the membrane/protein dielectric. This does not necessarily mean that it is spatially 25% (or 7.5 Å of a 30 Å thick membrane) inside the bilayer.

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