An Effect of Voltage on Binding of Na\textsuperscript{+} at the Cytoplasmic Surface of the Na\textsuperscript{+}-K\textsuperscript{+} Pump*

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This work utilizes proteoliposomes reconstituted with renal Na\textsuperscript{+}-K\textsuperscript{+}-ATPase to study effects of electrical potential (40–80 mV) on activation of pump-mediated fluxes of Na\textsuperscript{+} or Rb\textsuperscript{+} (K\textsuperscript{+}) ions and on inhibitory effects of Rb\textsuperscript{+} ions or organic cations. The latter include guanidinium derivatives that are competitive Na\textsuperscript{+}-like antagonists (David, P., Mayan, H., Cohen, H., Tal, D. M., and Karlish, S. J. D. (1992) J. Biol. Chem. 267, 1141–1149). Cytoplasmic side-positive diffusion potentials significantly decreased the $K_{0.5}$ of Na\textsuperscript{+} at the cytoplasmic surface for activation of ATP-dependent Na\textsuperscript{+}-K\textsuperscript{+} exchange but did not affect the inhibitory potency of Rb\textsuperscript{+} (K\textsuperscript{+}) or any Na\textsuperscript{+}-like antagonist. Diffusion potentials did not affect activation of Rb\textsuperscript{+}-Rb\textsuperscript{+} exchange or binding of K\textsuperscript{+} (K\textsuperscript{+}) ions at the cytoplasmic surface and had only a minor effect on Rb\textsuperscript{+} activation at the extracellular surface. Previously, we proposed that the cation binding domain consists of two negatively charged sites, to which two K\textsuperscript{+} or Na\textsuperscript{+} ions bind, and one neutral site for the third Na\textsuperscript{+} (Glynn, I. M., and Karlish, S. J. D. (1990) Annu. Rev. Biochem. 59, 173–205). The present experiments suggest that binding of a Na\textsuperscript{+} ion in the neutral site at the cytoplasmic surface is sensitive to voltage. By contrast, binding of Rb\textsuperscript{+} ions at the extracellular surface of renal pumps appears to be only weakly or insignificantly affected by voltage. Inferences on the identity of the charge-carrying steps, based on experiments using proteoliposomes, are discussed in relation to recent evidence that dissociation of Na\textsuperscript{+} or association of K\textsuperscript{+} ions at the extracellular surface, represent the major charge-carrying steps.

The Na\textsuperscript{+}-K\textsuperscript{+} pump exchanges three Na\textsuperscript{+} for two K\textsuperscript{+} ions per molecule of ATP hydrolyzed. Because the pump is electrogenic, its rate must be affected by voltage. Voltage affects movements of charges, including those of transported ions, charged or polarized ligating groups, or other displacements of charges and dipoles associated with transport (Laüger, 1991a). The effects of voltage or charge movements have been investigated extensively in work with whole cells (Nakao and Gadsby, 1986; Lafaire and Schwartz, 1986; Bahinski et al., 1988; Gadsby et al., 1993; Rakowski, 1993) with membrane-bound Na\textsuperscript{+}-K\textsuperscript{+}-ATPase adsorbed to planar lipid bilayers (Fendler et al., 1985; Apell et al., 1987; Borlinghaus et al., 1987) or giant patches of whole cells (Hilgemann, 1994) or using electrochromic styryl dyes (Klodas and Forbush, 1988; Bühler et al., 1991; Stürmer et al., 1991; Stürmer and Apell, 1992; Heyse et al., 1994). We have described effects of diffusion potentials in reconstituted proteoliposomes (Karlish et al., 1985; Rephaeli et al., 1986a, 1986b; Goldshleger et al., 1987).

Early work led to one central conclusion concerning charge-carrying steps, namely that the conformational transition $E_2(2K\textsuperscript{+}) \rightarrow E_1$ is voltage-insensitive (Rephaeli et al., 1986a; Goldshleger et al., 1987; Bahinski et al., 1988; Stürmer et al., 1989), whereas the $E_1 \rightarrow P(3Na\textsuperscript{+}) \rightarrow E_2$ transition is voltage-sensitive (Karlish et al., 1985; Nakao and Gadsby, 1986; Rephaeli et al., 1986b; Goldshleger et al., 1987). Cation sites were suggested to contain two negative charges, transport of three Na\textsuperscript{+} ions being accompanied by movement of one positive charge, and no net charge movements accompanying transport of two K\textsuperscript{+} ions (Karlish et al., 1985; Goldshleger et al., 1987; Nakao and Gadsby, 1986). Two negatively charged sites could bind two K\textsuperscript{+} ions or two Na\textsuperscript{+} ions, and a neutral site could bind the third Na\textsuperscript{+} (Glynn and Karlish, 1990).

More recent studies provide strong evidence that dissociation of Na\textsuperscript{+} and binding of K\textsuperscript{+} ions at the extracellular surface are associated with movement of charge within the membrane dielectric (Lafaire and Schwarz, 1986; Rakowski et al., 1991; Vasilets et al., 1991; Bühler et al., 1991; Stürmer et al., 1991; Gadsby et al., 1993; Rakowski, 1993; Heyse et al., 1994). The findings imply that part of the trans-membrane potential drops along a high resistance access pathway or “ion well” connecting the sites to the extracellular medium, so that the probability of binding Na\textsuperscript{+} or K\textsuperscript{+} ions is affected by voltage (see Laüger and Apell (1986)). Indeed, the voltage-sensitive dissociation and association of cations via the extracellular ion well appear to constitute the major charge-carrying step of the cycle (Rakowski, 1993; Gadsby et al., 1993; Hilgemann, 1994; Heyse et al., 1994).

An aspect of the voltage sensitivity of the pump that has not been investigated intensively concerns cation binding at the cytoplasmic surface. In experiments using reconstituted vesicles, unlike those using whole cells, cation activation at the cytoplasmic surface can be studied conveniently due to the presence of pumps in an inside-out orientation. Previous work showed that the apparent affinity of Na\textsuperscript{+} for activation of ATP-dependent Na\textsuperscript{+}-K\textsuperscript{+} exchange, on inside-out oriented pumps, is increased by cytoplasmic side-positive diffusion potentials (Karlish et al., 1985; Goldshleger et al., 1987). It was suggested that voltage might affect Na\textsuperscript{+} binding at the cytoplasmic surface due either to an “allosteric” effect or to the existence of an ion well for Na\textsuperscript{+} at that surface (Goldshleger et al., 1987). The present study characterizes further effects of voltage on Na\textsuperscript{+} activation at the cytoplasmic side and on inhibition of Na\textsuperscript{+}-K\textsuperscript{+} exchange by different cations. A tool used for this purpose is a family of organic guanidinium derivatives, including pXBG,\textsuperscript{1} that act as competitive Na\textsuperscript{+}-like antagonists on renal Na\textsuperscript{+}-K\textsuperscript{+}-ATPase, inhibiting cation occlusion and ATPase activity and stabilizing the $E_1$ conformation (David et

\textsuperscript{1} The abbreviations used are: pXBG, para-xylylenebisguanidinium; EDA, ethylenediamine.
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al., 1992). When tested on the reconstituted vesicles, pXBG shows pronounced selectivity for the cytoplasmic surface (Or et al., 1993). It was of interest to establish whether diffusion potentials affect the interactions of pXBG and other Na\(^-\) like antagonists at the cytoplasmic surface.

We have also studied effects of diffusion potentials on (ATP + P\(_i\))-stimulated Rb\(^+\)-Rb\(^-\) exchange (Karlish et al., 1992), extending previous observations of Goldshleger et al. (1987). One can study the effects of voltage on activation by Rb\(^+\) at both cytoplasmic and extracellular surface and in the latter case compare observations with those on voltage effects on K\(^+\) activation of the pump in whole cells.

**EXPERIMENTAL PROCEDURES**

Na\(^+\)-K\(^-\)-ATPase (specific activity 12-18 units/mg of protein) was prepared from pig kidney, and ATPase activity and protein concentration were determined, as described by Jørgensen (1988). Prior to use, the enzyme was thawed and dialyzed overnight against 1000 volumes of 25 mM histidine and 1 mM EDTA, pH 7.0, and stored on ice.

Preparation of Reconstituted Vesicles—Soybean phospholipid vesicles were prepared as described by Goldshleger et al. (1990). Reconstitution of enzyme into phospholipid vesicles was done according to Karlish and Pick (1981). The standard reconstitution medium contained 0.8 mM EDTA, pH 7.0, and other ligands as given in the figure legends. After the reconstitution, the external medium was exchanged for an isotonic medium of choline-chloride, 25 mM Tris-HCl, pH 7.0 or 8.0, on small columns of Sephadex G-50 as described by Karlish (1988).

Measurement of Isotope Fluxes—Usually, vesicles and reaction mixture (25 \(\mu\)l each) were mixed together and incubated at 20-22 \(^\circ\)C for the time indicated in the figure legends (see Karlish 1988). Fluxes were stopped by the addition of 150 \(\mu\)l of ice-cold isotonic NaCl or RbCl solution. The suspension was then transferred to ice-cold Dowex columns, and trapped isotope was eluted into scintillation vials with 1-1.5 ml of ice-cold 0.2 M sucrose (Karlish, 1988). \(^{22}\)Na was measured by scintillation counting, and \(^{86}\)Rb was measured by its Cerenkov radiation. Fluxes were measured in duplicate tubes; differences between duplicates were less than 5%.

ATP-dependent \(^{22}\)Na uptake into K\(^+\)-loaded vesicles (Na\(^+\)-K\(^-\) exchange) was estimated as the difference in isotope uptake with or without 3 mM ATP. Concentrations of MgCl\(_2\), NaCl, and other ligands are indicated in the figure legends.

\(^{86}\)Rb uptake into Rb\(^+\)-loaded vesicles (Rb\(^+\)-Rb\(^-\) exchange) was measured as described by Karlish (1988). (ATP + P\(_i\))-stimulated Rb\(^+\)-Rb\(^-\) exchange via inside-out oriented pumps was measured in the presence of 1 mM ATP and 10 mM P,(Tris) in the external medium. It was defined as the \(^{86}\)Rb influx that was inhibited by vanadate (550 \(\mu\)M) plus MgCl\(_2\) (2.2 mM) in the reaction medium. (ATP + P\(_i\))-stimulated Rb\(^+\)-Rb\(^-\) exchange via right-side-out oriented pumps was measured as a quabain-sensitive uptake into vesicles loaded with 1 mM ATP and 10 mM P,(Tris).

**Generation of Diffusion Potentials—** Vesicles were mixed either with ethanol (1% volume), ethanolic solutions of valinomycin (0.7-2 \(\mu\)M) or the Li\(^+\) ionophore AS701 (120-180 \(\mu\)M) (see Shanzer et al. (1992)). Usually, 55 \(\mu\)l of vesicle suspension were treated with ionophores, and duplicate samples of 25 \(\mu\)l were taken for measuring fluxes within 3 min. The magnitudes of the induced diffusion potentials were determined from the rates of passive influxes of \(^{22}\)Na or \(^{86}\)Rb, with and without added ionophores, using the constant field flux equation as described by Goldshleger et al. (1987). Equal volumes of vesicles (\(\pm\) ionophore) 38 \(\mu\)l were mixed, the reaction mixture lacking ligands and the pump-mediated flux (ATP and/or P) were mixed. Five 40-\(\mu\)l aliquots were withdrawn after 1-5 min, transferred to Dowex columns, and eluted into scintillation vials with 1.5 ml of ice-cold 0.2 M sucrose. The values of generated diffusion potentials were 40-80 mV.

Synthesis of pXBG—Synthesis of the hemi-sulfate salt is described in David et al. (1992).

Calculations—Data were fitted to theoretical equations by nonlinear regression analysis using the program Enzfitter (Elsevier-BIOSOFT) for IBM personal computer. The parameters are given as the values ± standard errors.

Materials—\(^{86}\)Rb and \(^{22}\)Na were obtained from DuPont NEN. Dowex 50W-X8 100 mesh H-form from Sigma or Fluka was converted to the Tris form. Sephadex G-50 (fine) was obtained from Pharmacia, and EDA was from Fluka. Ouabain, ATP (disodium salt), valinomycin, deoxy-cholate, and phosphatidyl-choline (from soybean) were purchased from Sigma. Choline-chloride was obtained from Fluka and was recrystallized from hot ethanol. Sodium vanadate (Ortho) was obtained from Fisher. Both vanadate and ATP were converted to Tris form by passage over columns of Dowex 50. The Li\(^+\) ionophore AS701 was a gift from Dr. A. Shanzer of the Weizmann Institute.

**RESULTS**

Effects of Voltage on Activation of ATP-dependent Na\(^+\)-K\(^-\) Exchange by Na\(^+\) Ions—We have looked at activation by Na\(^+\) of ATP-dependent \(^{22}\)Na uptake into K\(^+\)-loaded vesicles in the absence and the presence of cytoplasmic side-positive K\(^+\) diffusion potentials (40-80 mV). The experiment in Fig. 1 was done at pH 8.0, a pH at which Na\(^+\) antagonists inhibit with the highest affinity (David et al., 1992). The very low passive ATP-independent \(^{22}\)Na uptake in Fig. 1, linearly dependent on Na\(^+\) concentration, illustrates the high ratio of active to passive fluxes in these vesicles. The dependence of ATP-dependent Na\(^+\)-K\(^-\) exchange on cytoplasmic Na\(^+\) concentration is described adequately by empirical Hill equations (Karlish and Stein, 1985). Parameters for the curves in Fig. 1 are: \(V_{\text{max}} = 1.71 ± 0.03\) nmol of Na\(^+\)/min/10 \(\mu\)l of vesicles, \(K_{0.5} = 2.87 ± 0.29\) mM, \(n_h = 1.97 ± 0.10\) for \(\Delta V = 0\) mV (solid line) and \(V_{\text{max}} = 1.81 ± 0.08\) nmol of Na\(^+\)/min/10 \(\mu\)l of vesicles, \(K_{0.5} = 2.04 ± 0.17\) mM, \(n_h = 1.67 ± 0.07\) for \(\Delta V = 41\) mV (dashed line). Thus, the cytoplasmic side-positive diffusion potential significantly decreased the \(K_{0.5}\) of Na\(^+\), whereas \(V_{\text{max}}\) was unaffected. (Previously, stimulation of \(V_{\text{max}}\) was observed (Goldshleger et al., 1987). The difference may be due to a difference in pH of 7.0 or 8.0 in previous and present experiments, respectively (see "Discussion").) Analysis with a Hill equation causes the three Na\(^+\) affinities to be lumped into a single term (\(K_{0.5}\)), and the effect of voltage cannot be assigned to a specific Na\(^+\) site(s). There appears to be a small decrease in \(n_h\) (see also Table 2 in Goldshleger et al. (1987)), hinting at the possibility that the three Na\(^+\) affinities were not affected to the same extent.

In the current series of experiments, the range of values of the diffusion potentials, calculated by passive flux measurements, was 40-80 mV. These values are significantly smaller than the diffusion potentials generated in our previous experiments (120-180 mV) calculated from flux data and compatible, within error, with the expected Nernst potentials (Fig. 3 of Goldshleger et al., 1987). However, we now know that Nernst potentials were somewhat overestimated, due to the fact that a nominally K\(^+\)-free medium with 0.15 mM K\(^+\) added actually contained about 0.9 mM K\(^+\). A calculated Nernst potential of 184 mV would be revised down to 141 mV. The reason for the lower size of potentials in the present work is unknown. There is one source of uncontrollable variability between different batches of soybean phospholipid, namely the intrinsic conductance of the membrane (Goldshleger et al., 1990). Possibly, the intrinsic conductance was higher in the present series of experiments, causing the K\(^+\) diffusion potential to be partially short-circuited.

Effects of Voltage on Inhibition of ATP-dependent Na\(^+\)-K\(^-\) Exchange by Competitive Na\(^+\)-like Antagonists and Rb\(^+\) Ions—Figs. 2 and 3 present results of experiments to determine whether a potential has an effect on the affinity of a relatively potent Na\(^+\)-like antagonist, pXBG. The rate of ATP-dependent Na\(^+\)-K\(^-\) exchange at a low Na\(^+\) concentration (100 \(\mu\)M) was determined at increasing concentrations of pXBG, in the absence or the presence of a cytoplasmic side-positive diffusion potential (75 mV). At 100 \(\mu\)M Na\(^+\), the ratio of ATP-dependent Na\(^+\)-K\(^-\) exchange was 10-fold. The rates of ATP-dependent Na\(^+\)-K\(^-\) exchange increased in the absence of pXBG were 6.9 and 13.3 pmol of Na\(^+\)/min/10 \(\mu\)l of vesicles, without or with the potential, respectively. Measured rates at each concentration of inhibitor (11) are presented as a percentage (P) of the measured control flux (i.e. with or without valinomycin). Pres-
Presentation of the results in this way illustrates the degree of superimposition of the curves. The equation 
$$P = \frac{P_O \times K_i}{(1) + K_i}$$
was used to obtain best fit parameters for $K_i$, the apparent inhibition constant, and also $P_O$, the uninhibited control, a value with experimental errors similar to that of the other points. Best fit parameters are: $P_O = 98.9 \pm 2.1\%$ and $K_i = 758 \pm 55\ \mu M$ for $\Delta \psi = 0$ (solid line) and $P_O = 103.0 \pm 3.0\%$ and $K_i = 668 \pm 63\ \mu M$ for $\Delta \psi = 75\ \text{mV}$ (dashed line). Evidently, pXBG inhibited the flux along a hyperbolic curve, and the diffusion potential did not significantly alter its affinity. At a limitingly low Na$^+$ concentration (e.g. $100\ \mu M$), the calculated apparent affinity of pXBG should be close to its real binding affinity\footnote{In an experiment in which Na$^+$ concentration was $180\ \mu M$ instead of $100\ \mu M$, the apparent affinity of pXBG was unaltered. Thus the requirement that cytoplasmic Na$^+$ must be limitingly low is satisfied by the} in

![Graph 1: Activation of ATP-dependent Na$^+$-K$^+$ exchange by Na$^+$ without (○) and with (●) a cytoplasmic side-positive diffusion potential. Vesicles loaded with 182 mM KCl were treated with 0.73 µM valinomycin or 1% ethanol. The reaction medium contained 25 mM Tris-Cl, pH 8.0, 0.5–25 mM NaCl and $^{22}\text{Na} (7 \times 10^6 \text{cpm/sample})$, and 3 mM MgCl$_2$ with or without 3 mM ATP. Choline-chloride was used to adjust the ionic strength to 200 mM. $^{22}\text{Na}$ fluxes were measured for 60 min. ○-ATP is the measured $^{22}\text{Na}$ uptake into vesicles in the absence of ATP.}

![Graph 2: Inhibition of ATP-dependent Na$^+$-K$^+$ exchange by pXBG without (○) and with (●) a cytoplasmic side-positive diffusion potential. Vesicles loaded with 260 mM KCl were treated with 2 µM valinomycin or 1% ethanol. The reaction medium contained 25 mM Tris-Cl, pH 7.0, 0.1 mM NaCl and $^{22}\text{Na} (8.2 \times 10^5 \text{cpm/sample})$, 0–3 mM pXBG, and 3 mM MgCl$_2$ with or without 3 mM ATP. Choline-chloride was used to adjust the ionic strength to 277 mM. $^{22}\text{Na}$ fluxes were measured for 2 min.]
the prevailing conditions (Equation 3 in the "Appendix"). The dotted line shows the curve that could be predicted ($K_i = 416 \mu M$) if the voltage raised the affinity of pXBG to the same extent as at a Na⁺ site, calculated as 1.82-fold for Fig. 3 (see "Discussion"). The data can also be presented as an enhancement ratio ($V_{\text{V}+\text{valinomycin}}/V_{\text{V}+\text{EtOH}}$, at varying concentrations of pXBG (Fig. 3). The enhancement ratio is defined as the quotient of the ATP-dependent 22Na influx with an imposed diffusion potential to that observed without a potential (control). The presentation of data in this way offers a qualitative and clear distinction between two mutually exclusive alternatives. If the affinity of the inhibitor is affected by voltage, the enhancement ratio falls hyperbolically from the initial value toward 1 as the concentration of the inhibitor increases (Equation 8 in the "Appendix"). If the binding affinity is unaffected by voltage, the enhancement ratio remains constant at all inhibitor concentrations (Equation 7 in the "Appendix"). $V_{\text{V}+\text{valinomycin}}/V_{\text{V}+\text{EtOH}}$ for pXBG is constant (1.82), and fluctuations about the average value are randomly distributed (Fig. 3). Similar results were obtained at pH 8.0 (data not shown).

We have also looked at the effects of voltage on inhibition by three additional Na⁺-like antagonists and Rb⁺ ions, which act as product inhibitors. Table I summarizes the effects of cytoplasmic side-positive diffusion potentials on the apparent affinities of pXBG, guanidinium, EDA, and Rb⁺ ions. Evidently, there was no effect of potential on the affinities of any Na⁺-like antagonist or of Rb⁺ ions. This is reflected in the constancy of enhancement ratios for guanidinium, EDA, and Rb⁺ (1.78, 1.89, and 1.63, respectively; data not shown).

In conclusion, cytoplasmic side-positive diffusion potentials increase apparent Na⁺ affinity at the cytoplasmic surface but have no effect on the apparent affinities of Rb⁺ (K⁺) and the Na⁺-like antagonists tested.

**Effect of Voltage on Activation of Rb⁺-Rb⁺ Exchange by Rb⁺**

ionophore AS701 (Shanzer et al., 1983) is used to induce Li⁺ diffusion potentials in vesicles containing Li⁺ ions (Goldshleger et al., 1987).  

**Effects of potential on apparent affinities of Na⁺ and Na⁺-like antagonists at the cytoplasmic side of Na⁺-K⁺-ATPase**

The values for the affinities of Na⁺ and pXBG were taken from Figs. 1 and 2, respectively. Corresponding values for guanidinium and EDA were obtained from experiments done as described in the legend to Fig. 2, except that the medium contained either 0–50 mM guanidinium or 0–3 mM EDA, respectively. Conditions of the experiment with Rb⁺ as a competitor were the following: vesicles were prepared with 100 mM KCl and 150 mM LiCl. The external medium contained 25 mM Tris-Cl, pH 7.0, 0.5 mM NaCl, and 117 mM Na (5.2 x 10⁵ cpm/sample), 0–30 mM RbCl, 25 μM LiCl, and 3 mM MgCl₂ with or without 3 mM ATP. Ionic strength was adjusted to 270 mM with choline-chloride. Diffusion potentials were induced with 160 μM AS701, and 22Na fluxes were measured for 1 min.

![Fig. 3.](image)

**Fig. 3. Lack of effect of pXBG on stimulation of ATP-dependent Na⁺-K⁺ exchange by a cytoplasmic side-positive diffusion potential.** Data are taken from the experiment in Fig. 2. The solid line is the mean enhancement ratio (1.82). The dashed line obeys the equation $V_{\text{V}+\text{valinomycin}}/V_{\text{V}+\text{EtOH}} = 1 + [\text{pXBG}]k/K_i$, where $K_i = 755 \mu M$.

**Table I**

| Cation      | Apparent affinity | $\Delta \varepsilon > 0$ | $\Delta \varepsilon = 0$ |
|-------------|-------------------|--------------------------|--------------------------|
| Na⁺         | 2.87 ± 0.29 μM    | 2.04 ± 0.17 μM           |
| pXBG        | 758 ± 55 μM       | 668 ± 63 μM              |
| Guanidinium | 23.3 ± 3.1 μM     | 19.6 ± 2.4 μM            |
| EDA         | 151 ± 29 μM       | 167 ± 28 μM              |
| Rb⁺         | 3.42 ± 0.02 μM    | 3.9 ± 0.8 μM             |
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**DISCUSSION**

Fig. 5 presents a model of charge transfer combining several elements. First, the cation binding domain consists of two negatively charged sites, to which two K\(^+\) or two Na\(^+\) ions bind, and one neutral site for the third Na\(^+\) (Goldshleger et al., 1987; Nakao and Gadsby, 1986; Glynn and Karlsh, 1990). Second, the neutral site lies in a shallow cytoplasmic well. Third, binding or dissociation of cations in a deep extracellular access well constitutes the major charge-carrying steps (Lafaire and Schwarz, 1986; Rakowski et al., 1991; Vasilets et al., 1991; Gadsby et al., 1993; Rakowski, 1993; Heyse et al., 1994; Hilgernann, 1994).

Voltage-sensitive Binding of a Na\(^+\) in the Neutral Site at the Cytoplasmic Surface—The data on Na\(^+\)-K\(^+\) exchange (Figs. 1-3 and Table I) indicate that although voltage affects activation by Na\(^+\) ions at the cytoplasmic surface, inhibition by the Na\(^+\)-like antagonists (pXBG, guanidinium, and EDA) and Rb\(^+\) ions is unaffected by voltage. The lack of apparent effect of voltage on Rb\(^+\) binding at the cytoplasmic surface is confirmed by the Rb\(^+\)-Rb\(^+\) exchange experiment on inside-out oriented pumps (Fig. 4A). The lack of effect of voltage inhibition by Rb\(^+\) ions or by Na\(^+\)-like antagonists suggests most simply that only one of the three Na\(^+\) sites, the neutral site, is affected by voltage. The effect of voltage seems to be exerted on the binding of a Na\(^+\) (see Appendix) and can be explained by invoking the existence of a cytoplasmic ion well (Fig. 5). The two charged sites, to which two Rb\(^+\) or two Na\(^+\) ions or the Na\(^+\)-like antagonists bind (Or et al., 1993), would be located outside the ion well, and therefore binding at these sites is unaffected by voltage. This concept is depicted by the E\(_1\) (3Na\(^+\)) forms in Fig. 5 (top line). E\(_1\) (3Na\(^+\)) is an extra phosphorylated form with three occluded Na\(^+\) ions as proposed recently in a two-step model of cation occlusion (Or et al., 1993).

Consider the scheme in the ‘Appendix’ describing Na\(^+\) activation of ATP-dependent Na\(^+\)-K\(^+\) exchange assuming a fixed coupling ratio\(^3\) and competitive inhibition of the exchange by Na\(^+\)-like antagonists. Equation 2 indicates that at low Na\(^+\) concentrations of Na\(^+\)\(_{eff}\), the rate of Na\(^+\)-K\(^+\) exchange is governed only by the rate constant of phosphorylation (a) and by the sodium binding constant (K\(_{Na}\), K\(_{K}\), and K\(_{j}\)). Because a is known to be voltage-insensitive (Borlinghaus et al., 1987), stimulation of the rate of Na\(^+\)-K\(^+\) exchange at low Na\(^+\) concentrations by voltage (Fig. 1) indicates that voltage

\(^3\) At low Na\(^+\) concentrations, the coupling ratio of 3Na\(_{eff}\)/2K\(_{eff}\) may be reduced to 1Na\(_{eff}\)/K\(_{eff}\) (Polvani and Blosstein, 1989; Goldshleger et al., 1990). Equations for a scheme assuming a variable coupling ratio are similar to those in the “Appendix,” except that the voltage-sensitive Na\(^+\) dissociation constant must be K\(_j\).
affects a Na\(^+\) binding constant(s) (Goldshleger et al., 1987). As inferred above, only one binding constant appears to be affected by voltage. The value of the enhancement ratio \((V_{\Delta \psi}/V_{\Delta \psi=0})\) provides a measure of that effect of voltage. \(\delta\) is the fraction of trans-membrane voltage experienced by the Na\(^+\) in its passage down the high resistance access path and can be calculated from Equation 6. From four experiments at pH 7.0, the average value of \(V_{\Delta \psi}/V_{\Delta \psi=0}\) was 1.77 ± 0.06 for a calculated \(\Delta \psi = 59 \pm 9\) mV. The estimated value of \(\delta\) is 0.24. A previous value of \(\delta \sim 0.1\) in Goldshleger et al. (1987) was somewhat underestimated, due to the overestimate of the Nernst potential referred to under "Results." Using the observed value for \(V_{\Delta \psi}/V_{\Delta \psi=0}\) of 2.4 in Goldshleger et al. (1987) and a revised value of 141 mV rather than 184 mV, we calculate \(\delta = 0.16\). Thus, a range of values 0.16 < \(\delta\) < 0.24 is the best estimate of this parameter from all proteoliposome experiments, past and present.

A cytoplasmic ion well for Na\(^+\) has also been proposed on the basis of a finding that Na\(^+\) but not K\(^+\) or other congener ions induce a change in RH421 fluorescence signal on renal Na\(^+\)-K\(^+\)-ATPase, indicating movement of positive charge into the membrane dielectric (Laüger, 1991b; Stürmer et al., 1991; Heyse et al., 1994). A dielectric coefficient of 0.25 was calculated for binding of Na\(^+\) at the cytoplasmic side (Heyse et al., 1994), similar to the range of values estimated from the proteoliposome experiments.

Other Charge Movements Accompanying Na\(^+\) and K\(^+\) Movements—The most recent evidence, obtained from measurements of rapid charge movements in cardiac membranes (Hilgemann, 1994) or renal Na\(^+\)-K\(^+\)-ATPase (Heyse et al., 1994; Wuddel and Apell, 1995), is that the major charge-carrying step of the cycle is dissociation of the first Na\(^+\) in the step \(E\text{,}_P(3\text{Na}^+) \rightarrow E\text{,}_P(2\text{Na}^+)\) (dielectric coefficient, 0.65). The rate of dissociation is very rapid, in the microsecond time scale (Hilgemann, 1994) or 1400 s\(^{-1}\) (Wuddel and Apell, 1995). Dissociation of the other two Na\(^+\) ions and association of two K\(^+\) ions in the steps \(E\text{,}_P(2\text{Na}^+) \rightarrow E\text{,}_P\) and \(E\text{,}_P \rightarrow E\text{,}_P(2\text{K}^+)\), respectively, carry much less charge (dielectric coefficient, 0.1–0.2). In Fig. 5 (bottom line), dissociation of the first Na\(^+\) is therefore depicted as occurring in a narrow channel, down which most of the electric field drops, and dissociation of the other two Na\(^+\) ions or association of two K\(^+\) ions as occurring in a wider "low field" channel (as proposed by Hilgemann (1994) and Wuddel and Apell (1995)).

The evidence that the major charge movement accompanies rapid Na\(^+\) dissociation raises two questions concerning interpretation of proteoliposome experiments. First, is the conformational transition \(E\text{,}_1 \rightarrow P(3\text{Na}^+) \rightarrow E\text{,}_P(3\text{Na}^+)\) voltage-sensitive? Previously we proposed that a 20–30% acceleration of \(V_{\text{max}}\) of Na\(^+\)-K\(^+\) exchange by a cytoplasmic positive potential is due to acceleration of \(E\text{,}_1 \rightarrow P(3\text{Na}^+) \rightarrow E\text{,}_P\) (Goldshleger et al., 1987). The turnover rate of the cycle, 15–30 s\(^{-1}\) at 20°C ( Forbes, 1984; Steinberg and Karlish, 1989), is partly limited by the rate of \(E\text{,}_P(3\text{Na}^+) \rightarrow E\text{,}_P(3\text{Na}^+)\) and partly limited by that of \(E\text{,}_2(2\text{K}^+) \rightarrow E\text{,}_1\), which is voltage-insensitive (rates are in the range 15–60 s\(^{-1}\) at 20°C, tabulated in Heyse et al. (1994)). In the present experiments done at pH 8.0 and not at 7.0, \(V_{\text{max}}\) was not affected by voltage (Fig. 1), possibly because \(E\text{,}_1 \rightarrow P(3\text{Na}^+) \rightarrow E\text{,}_P\) is not rate-limiting at pH 8.0 and above (Forbush and Klodos, 1991). In a proteoliposome experiment, dissociation of the Na\(^+\) accompanying \(E\text{,}_P(3\text{Na}^+) \rightarrow E\text{,}_2P(2\text{Na}^+)\) could be expected to be greatly accelerated by the voltage. However, voltage-dependent acceleration of active \(2^2\) Na uptake in a unidirectional flux mode is not explained by such an effect, because even the unaccelerated rate of Na\(^+\) dissociation (1400 s\(^{-1}\)) is much too fast to limit the rate of the cycle. Wuddel and Apell (1995) have concluded recently that \(E\text{,}_P(3\text{Na}^+) \rightarrow E\text{,}_P(3\text{Na}^+)\) does contribute to charge movement but only weakly (dielectric coefficient, <0.1). Evidently, weak voltage sensitivity of the conformational transition \(E\text{,}_P(3\text{Na}^+) \rightarrow E\text{,}_P(3\text{Na}^+)\) is not incompatible with strong voltage sensitivity of dissociation of Na\(^+\) in \(E\text{,}_P(3\text{Na}^+) \rightarrow E\text{,}_P(2\text{Na}^+)\).

Second, is the very small or insignificant effect of the diffusion potential observed on activation of Rb\(^+\)-Rb\(^+\) exchange by Rb\(^+\) at the extracellular surface (Fig. 4B) compatible with the evidence for the extracellular cation access pathway? The answer appears to be affirmative, provided that dissociation of the last two Na\(^+\) ions and binding of two K\(^+\) ions is only weakly voltage-sensitive, as predicted by Hilgemann (1994) and Wuddel and Apell (1995). Charge movements and voltage dependence of activation by extracellular K\(^+\) were not observed with native cardiac myocytes (Bahinski et al., 1988; Nakao and Gadsby, 1989), but in other systems, notably Xenopus oocytes, with endogenous pumps or expressed Torpedo pumps, much larger effects of voltage on apparent affinity of extracellular K\(^+\) have been observed (Laüfer and Schwarz, 1986; Rakowski et al., 1991; Vasilets et al., 1991). The quantitative contributions of K\(^+\) binding to the overall voltage sensitivity appear to vary between different species, physical conditions, physiological states (Vasilets and Schwartz, 1992), or isoforms of the pump \(\alpha\) and \(\beta\) subunits (J. Isser et al., 1994).

Structural Implications—The evidence for high resistance...
access pathways supports biochemical evidence that cation occlusion sites are buried within the membrane domain (see Karlish et al. (1990) and Capasso et al. (1992)). As illustrated in Fig. 5, the deep access pathway and weak voltage sensitivity of $E_1 P(3Na^+)$ imply also that transport involves small gating movements of the protein (moving barrier models) rather than large movements of the sites with bound cations (moving carrier models).

APPENDIX

In the following analysis, we derive steady-state rate equations for the activation of ATP-dependent Na$^+$-K$^+$ exchange by Na$^+$ ions and competitive inhibition by Na$^+$-like antagonists. We consider the alternatives that the inhibition constant ($K_i$) is or is not affected by voltage. The scheme assumes that three Na$^+$ ions must bind at the cytoplasmic sites to initiate the cycle,$^3$ and binding of one inhibitor molecule suffices to block the cycle. The Na$^+$-K$^+$ exchange is considered unidirectional due to the absence of reaction products, Pi, ADP, extracellular Na$^+$ ions, and cytoplasmic K$^+$ ions. All reaction rate constants are pseudo-first order, because concentrations of all substrates except sodium are constant and saturating. Ion binding reactions are considered to be in rapid equilibrium (Reaction 1). By applying steady-state assumptions, mass action, and the method of Cha (1968), the steady-state rate expression for Na$^+$ activation of the Na$^+$-K$^+$ transport cycle is obtained:

$$V = \frac{aE_i[Na]^3}{K_i[Na]^2 + K_i[Na] + K_i(K_i(1 + \frac{[I]}{K_i}))} + aR[Na]^3$$

(Eq. 1)

At very low Na$^+$ concentration Equation 1 is reduced to:

$$V = \frac{aE_i[Na]^3}{K_i[K_i(1 + \frac{[I]}{K_i})]} [Na] \rightarrow 0$$

(Eq. 2)

At limitingly low Na$^+$, inhibition of Na$^+$-K$^+$ exchange by competitive Na$^+$-like antagonists is hyperbolic:

$$V_i = \frac{K_i}{K_i + [I]}$$

(Eq. 3)

Thus as Na$^+$ concentration approaches zero, the apparent inhibition constant approaches the real binding affinity of the inhibitor.

The dissociation constant of a cation in an ion well is assumed to be affected by voltage according to Equation 4 (Läuger, 1991a):

$$K_{3a} = K_{3a=0} \exp\left(\frac{-ZF\Delta \phi}{RT}\right)$$

(Eq. 4)

$\delta$ refers to the fraction of the voltage experienced by the ion, and the other symbols have their usual meaning.

The dependence of the enhancement ratio on the concentra-

tion of the inhibitor can be derived from Equation 2.

$$V_{3b}/V_{3b=0} = \frac{(K_i[K_iK_i]_{3a=0}(1 + \frac{[I]}{K_i[K_i]})}{(K_i[K_iK_i]_{3a=0}(1 + \frac{[I]}{K_i[K_i]})}$$

(Eq. 5)

In the following we assume that of the three Na$^+$ dissociation constants at the cytoplasmic side, only one is affected by voltage. The affinity of the inhibitor ($K_i$) may or may not be affected by voltage. Upon substitution of Equation 4 into Equation 5 we obtain:

$$V_{3b}/V_{3b=0} = \frac{1 + \frac{[I]}{K_i[K_i]}}{\sigma(1 + \frac{[I]}{K_i[K_i]})} \left\{ \sigma = \exp\left(\frac{-ZF\Delta \phi}{RT}\right) \right\}$$

(Eq. 6)

If the binding affinity of the inhibitor is not affected by voltage ($K_{i(3a=0)} = K_{i(3a=0)}$), then the enhancement ratio is constant and independent of inhibitor concentration:

$$V_{3b}/V_{3b=0} = \frac{1 + \frac{[I]}{K_{i(3a=0)}}}{\sigma(1 + \frac{[I]}{K_{i(3a=0)}})}$$

(Eq. 7)

However, if the inhibitor binds inside an ion well, $K_i$ is affected by voltage as described by Equation 4. In this case the enhancement ratio is not constant but varies with the inhibitor concentration:

$$V_{3b}/V_{3b=0} = \frac{1 + \frac{[I]}{K_{i(3a=0)}}}{\sigma(1 + \frac{[I]}{K_{i(3a=0)}})}$$

(Eq. 8)

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