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Topical Review

Voltage Dependence of the Na/K Pump

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Introduction

Whether Na/K pump rate is influenced by membrane potential was still debated as recently as 1988 (De Weer, Gadsby & Rakowski, 1988). While it follows from first principles that the rate of net forward Na/K pumping must vanish with hyperpolarization as the thermodynamic reversal potential of the overall pump reaction is approached, it was not clear that this effect would be seen in the experimentally accessible range of membrane potentials or that the stoichiometry of the overall reaction would remain constant as the membrane is hyperpolarized. Simultaneous measurements of pump current and flux, however, have demonstrated that the ratio of pump-mediated Na efflux to current remains constant over a wide range of experimental conditions, including hyperpolarization. The reduction of pump current seen with hyperpolarization in solutions containing Na could, therefore, be confidently interpreted as a direct effect of membrane potential on forward pump rate (Rakowski, Gadsby & De Weer, 1989). Since this effect of membrane potential on pump rate is seen only in the presence of external Na, much attention has been focused on external Na deocclusion/reocclusion and the associated release/rebinding of Na ions as likely voltage-dependent steps. Several concise reviews have appeared that discuss this point, as well as the possible voltage dependence of other steps in the transport cycle and aspects of the structure and function of the Na/K pump (Karlish, 1988*b;* Apell, 1989; Apell, 1990; Glynn & Karlish, 1990; Horisberger et al., 1991; Bamberg et al., 1993; Glynn, 1993; Vasilets & Schwarz, 1993). This review will briefly summarize recent studies on the voltage dependence of the Na/K pump. A recurrent theme in recent work is the external access channel or ''ion well'' hypothesis. There are only two ways that net transport can be voltage dependent. Either a rate-limiting step in the transport cycle is itself voltage dependent or a voltage-dependent step controls the level of the enzyme intermediate entering the rate-limiting step. A step in the reaction cycle will be voltage dependent if charge is translocated through any part of the membrane field during that step. The traditional view has been that charge translocation accompanies conformational changes that occur during the reaction cycle. Recent work, however, has provided evidence that much of the charge translocation during the Na/K pump cycle is a simple consequence of the location of the external Na^+ and K^+ binding sites within the membrane field. This access channel or ''ion well'' hypothesis can account in large part for the voltage dependence of Na/K pump turnover rate under a variety of experimental conditions. Recent work in this area is briefly summarized here. More extensive reviews of ion translocation can be found in several excellent books and symposium volumes that discuss both the experimental and theoretical aspects of the mechanism of ion translocation by the Na/K pump (Kaplan & De Weer, 1991; Läuger, 1991; Scarpa, Carafoli & Papa, 1992; Bamberg & Schoner, 1994; Horisberger, 1994).

Key words: Na,K-ATPase — Membrane potential — Access channel — Ion well — Electrogenic pump current — Voltage dependence

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Electrogenic Pump Current is Voltage-sensitive

To accurately measure Na/K pump current by measuring the change in current produced by applying a blocking

agent (such as ouabain), it is crucial to demonstrate that changes in external [K]—expected to occur in a restricted diffusion space near the membrane when the pump is stopped—do not also cause a change in membrane current that could distort the estimate of the electrogenic current produced directly by the Na/K pump. Studies on isolated cardiac myocytes (Gadsby, Kimura & Noma, 1985; Gadsby & Nakao, 1989; Nakao & Gadsby, 1989) achieved sufficient reduction of passive K conductance by application of K-channel blockers that ouabain-sensitive current could be equated with Na/K pump current. This allowed measurement of Na/K pump current over a wide range of membrane potentials. The voltage dependence of pump current also has been measured in sheep Purkinje fibers (Glitsch, Krahn & Pusch, 1989; Glitsch, Krahn & Verdonck, 1989), and *Xenopus* oocytes (Lafaire & Schwarz, 1986; Rakowski & Paxson, 1988). In the presence of extracellular Na, forward-going pump current is decreased by hyperpolarization to an extent that depends on $[Na]_o$. This led to the suggestion of an external access channel in which Na binds. Hyperpolarization would be expected to raise the effective concentration of Na at its binding site and lead to increased Na rebinding thus decreasing the net rate of forward pumping.

The Steady-state Pump I-V Curve Has a Negative Slope in Na-free Solutions at Low External [K]

An early controversy concerning whether there is a region of negative slope in the steady-state Na/K pump *I-V* relationship (Lafaire & Schwarz, 1986; Rakowski & Paxson, 1988) was resolved when it became clear that both extracellular [Na] and [K] affect the shape of the pump *I-V* relationship (Rakowski et al., 1991). In Na-containing solutions the relatively large effect of a deep external ion well in which Na binds dominates the *I-V* behavior, leading to inhibition of pump rate as hyperpolarization drives the reaction in the direction of Na rebinding and reocclusion. This mechanism cannot operate in Na-free solutions. When external Na is absent and extracellular [K] saturating, the pump operates in a relatively voltage-independent manner. It has been clearly established that in the absence of external Na with external [K] below saturation, the pump current-voltage (*I-V*) relationship has a shallow negative slope (Rakowski et al., 1991). Here, hyperpolarization acts to increase the effective concentration of K at its external binding site and, therefore, to increase pump rate. The apparent affinity of K for its external transport site is increased by hyperpolarization. This suggests the existence of an outward-facing access channel for K.

The steady-state voltage dependence of pump current under different ionic conditions was investigated in detail by Sagar and Rakowski (1994) who provided a kinetic model of steady-state Na/K pumping that was sufficiently simple to allow the model parameters to be evaluated by a nonlinear least squares fit procedure. The only model parameters with a simple physical meaning at the microscopic level are the maximum current and the dielectric coefficients that can be obtained from the shift in the mid-point voltage of the *I-V* relationship upon changing external [Na] or [K]. The dielectric coefficient for a monovalent ion in an access channel represents the fraction of the total membrane potential (''well depth'') that is felt by the ion at its binding site (Läuger, 1991). The dielectric coefficients obtained from steady-state measurements are about 0.25 for K and 0.5 for Na (Rakowski et al., 1991; Sagar & Rakowski, 1994). All the other model parameters (except for the maximum current, reflecting pump density and maximal turnover rate) represent combinations of rate coefficients that cannot be separated using steady-state information. Similar data and conclusions have been reported by Schwarz and colleagues (Schwarz & Gu, 1988; Vasilets et al., 1991; Vasilets & Schwarz, 1992), who also investigated the voltage dependence of pump activation by various monovalent ions that can substitute for K at its external binding site (Omay & Schwarz, 1992).

Na/K Pump Turnover is Relatively Voltage Independent in Na-free, High-[K] Solution

It is of interest to determine the pump *I-V* relationship under conditions in which none of the postulated internal or external ion wells influence pump rate. This can be done by internal dialysis of squid giant axons with saturating concentrations of Na, and superfusion with Na-free but saturating [K] solutions. Under these conditions, the pump *I-V* has a very shallow positive slope (Rakowski et al., 1989), a feature also seen in Na-loaded oocytes (Rakowski et al., 1991; Sagar & Rakowski, 1994) and in cardiac myocytes (Nakao & Gadsby, 1989) and Purkinje cells (Bielen, Glitsch & Verdonck, 1991, 1992, 1993). These observations suggest that there is at least one step in the pump cycle whose voltage dependence does not arise from the presence of access channels. This is of particular interest since studies using electrochromic dyes (Stürmer et al., 1991; Stürmer & Apell, 1992) and a charge-pulse technique (Wuddel & Apell, 1995) have suggested that the inward- to outward-facing conformational change of the enzyme is slightly electrogenic and rate-limiting during forward Na transport (*see also*, Goldshlegger et al., 1987).

The Na/K Pump *I-V* Curve is Shifted Along the Voltage Axis as External [Na] and [K] are Changed

Voltage dependence of the apparent affinity for a transported ion has occasionally been taken as evidence that

the ion binding site is located within the membrane field. Such a conclusion is not necessarily warranted, since voltage dependence of any step in the transport cycle can, in principle, affect the apparent affinity for an activating ion. A definitive prediction of the ion well hypothesis is that changes in membrane potential are kinetically equivalent to changes in the concentration of the ion that binds within the well (Mitchell & Moyle, 1974; Läuger, 1991). When external [Na] is altered the midpoint voltage of the Na/K pump *I-V* relationship will shift along the voltage axis. The shift (ΔV) can be predicted by Eq. (1):

$$\Delta V = \frac{RT}{\delta F} \ln \frac{[Na]_2}{[Na]_1} \tag{1}$$

in which δ is the fraction of the membrane potential sensed by Na at its external binding site. This Nernst-like equation can be derived from the rapid equilibration of ions within an access channel according to a Boltzmann distribution (Läuger, 1991; Gadsby, Rakowski & De Weer, 1993; Sagar & Rakowski, 1994). Strong evidence supporting the existence of an access channel is that the *I-V* relationship undergoes an identical parallel shift along the voltage axis for each, say, doubling of ion concentration, the shape of the *I-V* curve remaining unaffected. This property of shifting according to a Nernst-like equation is, of necessity, common to all access channel models for steady-state pump current.

The Voltage Dependence of Steady-state Na/Na Exchange Provides Evidence for an External Access Channel

Strong evidence for an outward-facing access channel in the Na/K pump has been obtained by measuring unidirectional pump-mediated Na efflux under electroneutral Na/Na exchange conditions in squid axons (Gadsby et al., 1993). The shift in the mid-point voltage of the Na-efflux curve as external [Na] is varied from 400 to 200 to 100 mM is shown in Fig. 1. In an external access channel model only the Na reocclusion (reverse) rate coefficient (pseudo first order) is voltage dependent as a consequence of the effect of membrane potential on the equilibrium distribution of Na in the access channel. All kinetic models in which both a forward and a reverse rate coefficient are voltage dependent predict a flux *vs.* voltage relationship that is bell-shaped and approaches zero at either voltage extreme. If, on the other hand, only the forward rate coefficient is voltage dependent, depolarization should stimulate unidirectional Na efflux. Kinetic models consistent with the observation that Na efflux is increased by hyperpolarization to a saturating level are those in which only the reocclusion/rebinding rate coefficient is voltage dependent, as predicted for an

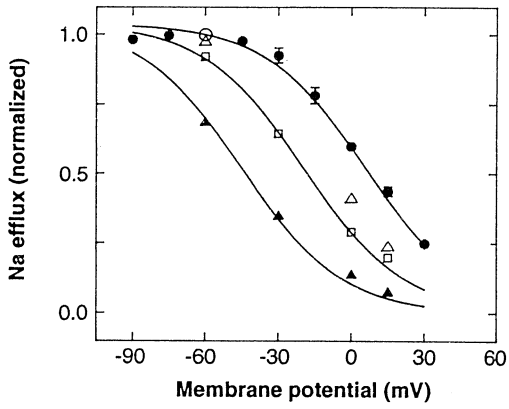

Fig. 1. Leftward shift of the voltage dependence of Na/Na exchange flux produced by reducing external [Na]. The voltage dependence of dihydrodigitoxigenin-sensitive Na efflux was determined under electroneutral Na/Na exchange conditions in internally dialyzed squid giant axons at 400 (filled circles), 300 (open triangles), 200 (open squares), and 100 (filled triangles) mM external [Na]. The open circle represents the value to which the data were normalized by repeated determinations in each axon at −60 mV and 400 mM external [Na]. The midpoint voltage of the theoretical curves fitted to the data shifted by 26 mV for each halving of external [Na]. This gives an estimate of the well depth for Na of 0.69. Figure from Gadsby et al. (1993) with permission.

access channel. A necessary qualification centers on the question of whether the saturating behavior would persist if even more extreme negative potentials were experimentally accessible. However, an estimate of the maximum degree of voltage dependence that could be associated with a forward-going transition in the electroneutral Na/Na exchange pathway can be made. The data available so far require that no more than 5–10% of the voltage dependence be associated with a forward transition rate coefficient (Gadsby et al., 1993). Because internal [Na] was maintained at saturating levels (50 mM) in these experiments, the results are not inconsistent with the proposal that internal Na binding and the E_1 to E_2 conformational change are weakly electrogenic (Wuddel & Apell, 1995) (*see below*).

Transient Current Provide a Direct Measure of Charge Translocation by the Na/K Pump

In contrast to the kinds of information that can be obtained from steady-state measurements, studies of kinetic transients can yield information directly related to the rate constants of individual transport steps. This is particularly the case if the experimental conditions are arranged so that only a limited set of partial reactions of the pump cycle can occur. Nakao and Gadsby (1986) found that ouabain-sensitive transient currents were elicited by voltage jumps applied to cardiac myocytes in the absence of K ions. One prediction of the access channel hypothesis developed above, that the reverse (rebinding/

reocclusion) rate coefficient should be strongly voltage dependent, whereas the forward (deocclusion/release) rate coefficient should be relatively voltage independent, was supported by those measurements. Similar observations have been made with the two-microelectrode (Rakowski, 1993), and cut-open (Holmgren & Rakowski, 1994), voltage clamp techniques in *Xenopus* oocytes. Rettinger et al. (1994) were able to obtain improved temporal resolution in oocytes with a patch-clamp technique, and Hilgemann (1994) was able to record two components of the transient currents using the giant patch voltage-clamp technique in myocytes, combined with high speed data acquisition. Very recent studies in squid giant axons (Wagg et al., 1996*a*,*b*) suggest that signals reflecting the sequential deocclusion and release to the exterior of the 3 Na ions can be resolved and that an additional ultrafast charge movement can be detected, likely related to the equilibration of ions within an (external) access channel. Interestingly, the slowest component of these pre-steady state currents associated with Na translocation, believed to reflect the major $E_1P \leftrightarrow E_2P$ conformational change, was recently found to be further slowed in N-terminal truncated Na/K pumps lacking the first 31 or 40 amino acids (Horisberger et al., 1994; Wang, Jassier & Horisberger, 1996), a finding consistent with earlier observations of a shift of the $E_1 \leftrightarrow E_2$ equilibrium towards E_1 in Na,K-ATPase clipped near the N terminus by trypsin and, hence, lacking the first 35 residues (Jorgensen & Karlish, 1980). This result is probably not due to a direct effect of the N-terminal truncation on the K binding site, but rather to an effect on the forward rate coefficient for external Na deocclusion.

Studies designed to detect charge movements associated with K translocation failed to find any transient current (Bahinski, Nakao & Gadsby, 1988). Nor were signals related to external K binding/occlusion found even when the external [K] was lowered to prevent saturation of external K binding sites (Gadsby et al., 1992). There are several possible explanations for this inability to observe transient currents related to K binding/occlusion including an unfavorable signal-to-noise ratio and inadequate temporal resolution. A recent abstract has reported detection of transient currents related to the external K binding/occlusion reaction step using the K congener Tl (Peluffo & Berlin, 1996).

Charge Translocation by the Na,K-ATPase Has Been Extensively Studied in Noncellular Systems

Charge moving steps in the Na/K pump cycle have been investigated in cell-free systems in which fragments of membranes rich in Na/K pumps are adsorbed to planar bilayers (Fendler et al., 1985; Borlinghaus, Apell & Läuger, 1987; Fendler, Grell & Bamberg, 1987; Fendler, Nagel & Bamberg, 1988; Fendler et al., 1988) or in which partially purified Na/K pumps are incorporated into proteoliposomes (Karlish, Rephaeli & Stein, 1985; Rephaeli, Richards & Karlish, 1986*a*,*b;* Goldshlegger et al., 1987; Karlish, 1988*a*). Results of these studies have contributed substantially to our understanding of the various electrogenic steps of the Na/K pump cycle, as discussed below.

Four Steps in the Pump Cycle are Electrogenic

Although most of the voltage dependence of the steady-state pump *I-V* relationship can be explained by the presence of external access channels for Na and K ions, experiments performed under conditions designed to isolate partial reactions of the cycle have provided evidence suggesting that at least four steps of the pump cycle are voltage dependent. These steps are: (1) cytoplasmic Na binding, (2) the inside- to outside-facing (E_1 to E_2) conformational change, (3) extracellular release of Na, and (4) extracellular K binding. Release of the last two Na ions and the binding of two K ions in the external access channel of the enzyme appear to involve ion movement in opposite directions but over comparable (or identical) well depths (Wuddel & Apell, 1995). Therefore, the electrogenic contributions of these two steps approximately cancel over the complete forward Na/K pump cycle. In the case of uncoupled Na efflux (Garrahan & Glynn, 1965; Garrahan & Glynn, 1967) (Fig. 2), the dielectric coefficient γ, associated with the return step $P\text{-}E_2$ to E_1 has not been measured experimentally, but based on the requirement that the sum of the dielectric coefficients for the uncoupled export of 3 Na in each cycle equals 3, γ is predicted to have a value of 1.8. These partial reactions are all shown in Fig. 2 with their associated dielectric coefficients (α, β, δ, γ). An electrostatic model of the Na/K pump cycle is shown in Fig. 3 in which the dielectric coefficients are associated with specific ion translocation steps.

The intracellular binding of Na has been investigated in proteoliposomes and in membrane fragments adsorbed onto lipid bilayers (Goldshlegger et al., 1987; Stürmer et al., 1991; Heyse et al., 1994; Or, Goldshleger & Karlish, 1996). The $E_1P \leftrightarrow E_2P$ conformational transition and subsequent release of Na occur during electroneutral Na/Na exchange. Experimental conditions for Na/Na exchange can be achieved simply by working under K-free conditions and, therefore, these steps have usually been investigated without separating them. Both transient (Nakao & Gadsby, 1986; Borlinghaus, Apell & Läuger, 1987; Läuger & Apell, 1988; Stürmer et al., 1991; Stürmer & Apell, 1992; Heyse et al., 1994; Wuddel & Apell, 1995) and steady-state methods (Gadsby et al., 1993; Sagar & Rakowski, 1994) have been employed in investigating the Na occlusion/deocclusion, unbinding and release steps. Both steady-state (Rakowski et al.,

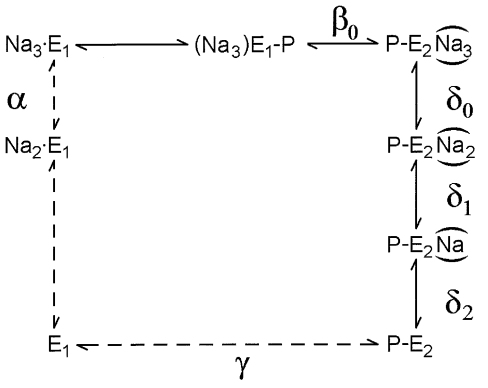

Fig. 2. Simplified reaction cycle of the Na,K-ATPase in the absence of extracellular Na and K showing the four steps that are thought to be electrogenic. The solid lines show reaction steps that occur after an ATP concentration jump under K-free conditions with saturating internal [Na]. These steps also occur in response to a voltage jump under similar conditions. The extent of the occlusion reaction, Na_3E_1 to $(Na_3)E_1$-P, depends on [ATP]/[ADP]. The dielectric coefficient thought to be associated with the binding of the third internal Na ion to a neutral site (α) is estimated to be 0.25 (Heyse et al., 1994). The conformational change, $(Na_3)E_1$-P to P-E_2Na_3, is thought to have a dielectric coefficient (β_0) of 0.1. Unbinding and release of Na to the outside can be divided into steps for each ion. The greatest electrogenicity is associated with the unbinding and release of the first Na (γ_0 = 0.65). The unbinding and release of the second and third Na are less electrogenic (δ_1 = 0.1 to 0.2, and δ_2 = 0.1 to 0.2) (Wuddel & Apell, 1995). The release of the last two Na ions is associated with dielectric coefficients comparable to that estimated from the voltage dependence of external K binding (0.27) (Rakowski et al., 1991). Since the binding of 2 K ions and release of the last 2 Na ions move charge in opposite directions, the electrogenicity of these processes approximately cancels in the normal forward pump cycle. Figure from Wuddel and Apell (1995) with permission.

1991; Vasilets et al., 1991; Sagar & Rakowski, 1994) and transient methods (Bahinski et al., 1988; Stürmer et al., 1991; Peluffo & Berlin, 1996) have been applied to the study of the voltage dependence of extracellular K binding. There are no published experimental results on the voltage dependence of electrogenic uncoupled Na efflux.

Cytoplasmic Binding of Na is Voltage-sensitive

The possibility that binding of Na to its internal transport site is voltage dependent has not been studied extensively in cellular preparations (*but see,* Nakao & Gadsby, 1989). Internal [Na] is typically kept at saturating levels either by Na-loading or by internal dialysis. This has the advantage of producing maximally activated pump currents and eliminating any possible contribution of voltage-dependent changes in the level of activation by Na at its internal binding site. Such effects, however, could be important, particularly if they were subject to regulation by intracellular second messenger systems. Several studies that have been done in vesicles in which membrane potential is estimated by indirect methods have suggested that binding of Na at the internal face of the Na/K pump is weakly voltage sensitive (Karlish et al., 1985; Goldshlegger et al., 1987). Recent work on proteoliposomes indicated that the establishment of cytoplasmic-side positive potentials increased the apparent affinity for Na activation of Na/K exchange but did not affect Rb/Rb exchange (Or et al., 1996). These results support the conclusion from data showing that internal binding of probably the third Na ion produces a change in the fluorescence signal of an electrochromic dye (RH421), whereas binding of K or K congeners does not (Stürmer et al., 1991; Heyse et al., 1994; Schwappach et al., 1994). On the other hand, little effect on the shape of the steady-state *I-V* curve was seen in *Xenopus* oocytes in which the internal [Na] was monitored with a Na-selective microelectrode (LaTona, 1990). Small shape changes, consistent with a weak voltage dependence of internal Na binding, were noted in cardiac myocytes in the presence, but not in the absence, of external Na (Nakao & Gadsby, 1989). Since the effect of voltage on internal Na binding appears small (estimated dielectric coefficient 0.1 to 0.25), it is not surprising that the effect of internal voltage-dependent Na binding on the shape of the *I-V* curve is not easily quantified when not separated from the much larger effect of voltage on external Na deocclusion and release. Further steady-state, as well as transient, studies would seem worthwhile in which internal [Na] is accurately maintained, membrane potential measured directly, and pump current or flux measurements are made in external Na-free solutions to avoid complications from Na rebinding.

The E1 to E2 Conformational Change Appears to be Relatively Voltage-insensitive

Early work supported the general conclusion that K translocation steps in the pump cycle are voltage insensitive (Rephaeli et al., 1986*b;* Goldshlegger et al., 1987; Bahinski et al., 1988; Stürmer et al., 1989). On the other hand, several lines of evidence suggested that one or more steps in the Na half of the pump cycle are strongly voltage dependent (Karlish et al., 1985; Nakao & Gadsby, 1986; Rephaeli et al., 1986*a;* Goldshlegger et al., 1987). Attention has been focused on the deocclusion and release steps in the Na half cycle as the major charge translocating steps. Perhaps one of the more surprising results of recent work has been the conclusion that the major inside- to outside-facing conformational change (E_1 to E_2) is not very voltage sensitive and, therefore, not directly responsible for a substantial charge translocation through the membrane (Heyse et al., 1994; Hilgemann, 1994; Wuddel & Apell, 1995). Instead, it appears that the large charge movement occurs only after the ion binding sites gain access to the external solution,

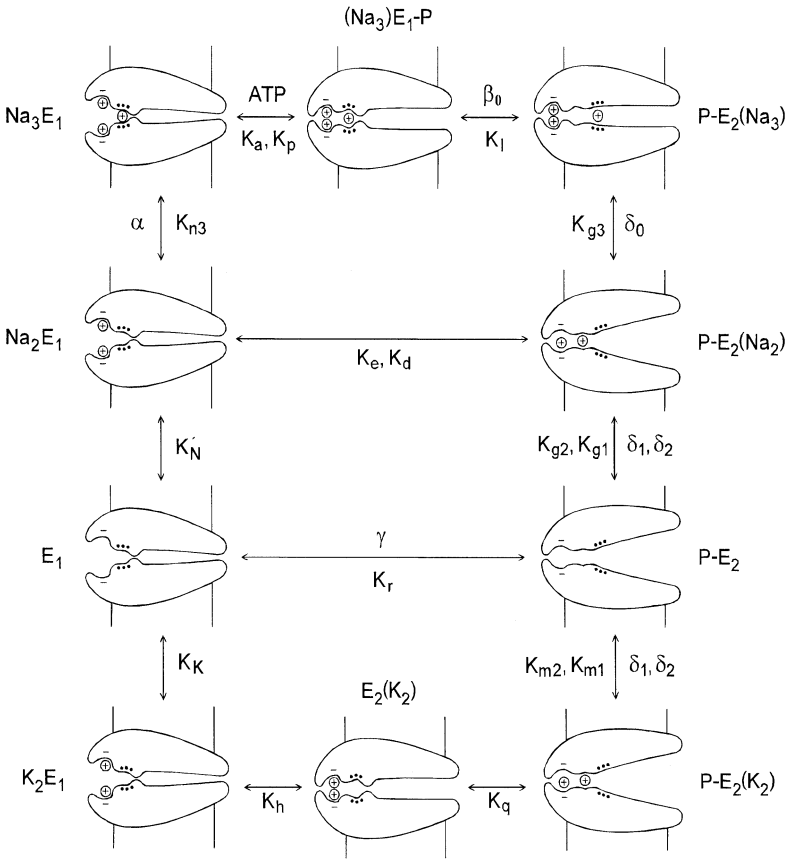

Fig. 3. Electrostatic model of the mechanism of Na and K translocation by the Na,K-ATPase cycle based on estimated values of dielectric coefficients. Starting from the inward-facing unloaded enzyme, E_1, the postulated reaction steps are as follows: (1) equilibrium binding of two internal Na ions to negatively charged sites (E_1 to Na_2E_1), (2) electrogenic binding of the third Na ion to an uncharged site within a shallow internal ion well (Na_2E_1 to Na_3E_1), (3) phosphorylation of the enzyme and occlusion of the bound Na ions (Na_3E_1 to $(Na_3)E_1$-P), (4) the major inward- to outward-facing conformational change of the enzyme ($(Na_3)E_1$-P to P-$E_2(Na_3)$), (5) unbinding of the first Na ion within a high-field access channel followed by its equilibration with the bulk solution (P-$E_2(Na_3)$ to P-$E_2(Na_2)$), (6) unbinding and release of the second and third Na ions to the external solution (P-$E_2(Na_2)$ to P-E_2), (7) entry and binding of two K ions from the external medium over about the same dielectric distance as the last two Na ions (P–E_2 to P–$E_2(K_2)$), (8) occlusion of the two K ions within the membrane accompanied by dephosphorylation of the enzyme (P-$E_2(K_2)$ to $E_2(K_2)$), (9) deocclusion of the bound K ions accompanied by a major outward- to inward-facing conformational change ($E_2(K_2)$ to K_2E_1), (10) unbinding and release of the K ions from their charged binding sites at the internal face of the enzyme (K_2E_1 to E_1). The pathway directly from P–E_2 to E_1 can occur in the absence of K and external Na and allows electrogenic, uncoupled Na efflux. Steps 2, 4, 5, 6, and 7 are thought to be electrogenic. The principal charge translocating step of the forward pump cycle is step 5. Figure from Wuddel and Apell (1995) with permission.

and ions at the binding sites rapidly equilibrate with the bulk solution. Recent studies using a charge pulse technique to impose changes in membrane potential on membrane fragments containing Na/K pumps adsorbed to lipid bilayers conclude that the E_1 to E_2 conformational change is detectable and electrogenic, but that the dielectric coefficient is only on the order of 0.1 (Wuddel & Apell, 1995). This small effect of membrane potential on the conformational transition may, nevertheless, be responsible for the shallow positive slope of the steady-state Na/K pump *I-V* curve measured in Na-free solution at saturating extracellular [K].

Deocclusion Followed by Release of the First Na ion to the Outside is the Major Charge Carrying Step in the Pump Cycle

One of the most exciting developments in the field of active transport of ions is the ability to resolve the unbinding/release process for each of the three transported Na ions. This major advance has become possible because of technical improvements in voltage-clamp techniques (e.g., the giant patch method) and the availability of ultrafast analog-to-digital converter systems that operate with sampling frequencies above 1 MHz. Hilgemann (1994) was able to record two components of presteady state current mediated by the pump under Na/Na exchange conditions in giant patches of cardiac membranes at 37°C. Because of the inside-out geometry of the giant patch the pump-mediated currents were obtained as difference currents based on sensitivity to the presence of cytoplasmic Na and ATP. A slow relaxation process with a time constant on the order of 1 msec and a dielectric coefficient of 0.74 appeared to correspond to the unbinding and release of the first Na ion, rate limited by the major $E_1P \leftrightarrow E_2P$ conformational change. This was preceded by a temporally unresolved fast transient charge movement with a dielectric coefficient of 0.26 that is thought to reflect the release of the last two Na ions. Wudell and Apell (1995) have recently developed a method of applying voltage steps to membrane fragments containing Na,K-ATPase adsorbed on lipid bilayers by applying a large charge pulse to the bulk solution

at 20°C. This less-direct method for generating jumps of membrane voltage, nevertheless, produced an estimate for the deocclusion rate of the first Na ion on the order of 1400 sec^{-1}, following an $E_1P \leftrightarrow E_2P$ conformational change of only 25 sec^{-1}. The dielectric coefficient for release of the first Na ion was estimated to be 0.65, and that for each of the fast (700–4000 sec^{-1}) release steps for the remaining two Na ions was 0.1–0.2. Recently, we have been able to take advantage of the excellent temporal resolution possible with internally dialyzed, voltage-clamped squid giant axons to record at least three ouabain-sensitive components of pre-steady state transient current associated with deocclusion and release of the three Na ions. At least two components have relaxation rates that are resolved, and an additional ultrafast component remains beyond our present resolution (Wagg et al., 1996*a*,*b*). We believe that the two readily resolvable components of the transient current reflect the sequential deocclusion and release of the first Na ion, followed by deocclusion and release of one or both of the next two Na ions, signaled by passage of the released Na ions, through outward-facing access channels. The ultrafast component that cannot yet be temporally resolved is thought to represent the equilibrium redistribution of ions within the access channel.

Note Added in Proof

Uncoupled Na efflux has been examined in a recent paper (Apell, H.-J., Roudna, M., Corrie, J.E.T., Trentham, D.R. 1996. Kinetics of the phosphorylation of Na, K-ATPase by inorganic phosphate detected by a fluorescence method. *Biochemistry* **35:**10922–10930). Pure uncoupled Na efflux was not observed. In the absence of K, Na efflux was thought to be accompanied by proton influx (as a congener of K).

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