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A Negative Slope in the Current-Voltage Relationship of the Na^+/K^+ Pump in *Xenopus* Oocytes Produced by Reduction of External $[K^+]$

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Summary. To investigate the voltage dependence of the Na^+/K^+ pump, current-voltage relations were determined in prophase-arrested oocytes of *Xenopus laevis*. All solutions contained 5 mM Ba^{2+} and 20 mM tetraethylammonium (TEA) to block K^+ channels. If, in addition, the Na^+/K^+ pump is blocked by ouabain, K^+-sensitive currents no larger than 50 nA/cm^2 remain. Reductions in steady-state current (on the order of 700 nA/cm^2) produced by 50 μM ouabain or dihydro-ouabain or by K^+ removal, therefore, primarily represent current generated by the Na^+/K^+ pump. In Na^+-free solution containing 5 mM K^+, Na^+/K^+ pump current is relatively voltage independent over the potential range from −160 to +40 mV. If external $[K^+]$ is reduced below 0.5 mM, negative slopes are observed over this entire voltage range. Similar results are seen in Na^+- and Ca^{2+}-free solutions in the presence of 2 mM Ni^{2+}, an experimental condition designed to prevent Na^+/Ca^{2+} exchange. The occurrence of a negative slope can be explained by the voltage dependence of the apparent affinity for activation of the Na^+/K^+ pump by external K^+, consistent with the existence of an external ion well for K^+ binding. In 90 mM Na^+, 5 mM K^+ solution, Na^+/K^+ pump current-voltage curves at negative membrane potentials have a positive slope and can be described by a monotonically increasing sigmoidal function. At an extracellular $[K^+]$ of 1.3 mM, a negative slope was observed at positive potentials. These findings suggest that in addition to a voltage-dependent step associated with Na^+ translocation, a second voltage-dependent step that is dependent on external $[K^+]$, possibly external K^+ binding, participates in the overall reaction mechanism of the Na^+/K^+ pump.

Key Words Na^+/K^+-ATPase · voltage dependence · *Xenopus laevis* · oocyte · current-voltage relationship

Introduction

The maintenance of electrochemical gradients for Na^+ and K^+ is essential for nearly all animal cells (*see*, e.g., De Weer, 1984; Glynn, 1984). This is achieved by the Na^+/K^+-ATPase: for each ATP molecule split, 3 Na^+ ions are transported outward and 2 K^+ ions inward during the forward pump cycle. Because of this unequal stoichiometry, the pump is electrogenic and the current generated is a measure of net pump activity. An important question for the understanding of the transport cycle is the identity of the steps in the pump cycle that involve movement of charge through the electrical field across the membrane. One approach to this question is to determine the voltage dependence of the overall transport rate by measuring the voltage dependence of pump current. The current-voltage (*I-V*) characteristics of the Na^+/K^+ pump have been analyzed in a variety of natural and reconstituted systems (*see* Passow, 1986; De Weer, Gadsby & Rakowski, 1988*a*,*b*).In all preparations studied thus far, it can be demonstrated that at normal extracellular $[Na^+]$, depolarization leads to stimulation of pump activity. This has been attributed to a step in the pump cycle associated with Na^+ translocation whose reverse rate constant is voltage-dependent (Nakao & Gadsby, 1986). K^+ translocation, on the other hand, is thought to be voltage insensitive (Goldshlegger et al., 1987; Rephaeli, Richards & Karlish, 1986; Bahinski, Nakao & Gadsby, 1988). For *X. laevis* oocytes, a second voltage-dependent step has been suggested (Lafaire & Schwarz, 1985, 1986). Under physiological conditions, in addition to the stimulation by depolarization, a reduction of pump current was observed at positive potentials. This negative slope in the *I-V* curve requires that for an unbranched pump cycle there are at least two voltage-dependent steps that are oppositely affected by the membrane field during each complete pump cycle. In contrast, Rakowski and Paxson (1988) found no evidence of a negative slope using a staircase pulse protocol designed to reveal contamination of Na^+/K^+ pump current by drift or hysteresis in the membrane *I-V* relationship. The aim of this paper is to resolve this apparent contradiction. For this purpose, artifactual currents that could explain a negative slope in the *I-V* relation of the Na^+/K^+ pump in

the oocytes of *X. laevis* have been minimized by blocking contaminating K^+ channel currents and Na^+/Ca^{2+} exchange current. Some of these results have been reported in abstracts (Rakowski, Paxson & LaTona, 1989*b*; Rakowski, Vasilets & Schwarz, 1990).

Materials and Methods

Oocytes

Females of the clawed toad *X. laevis* were anesthetized with 2 g/liter *m*-aminobenzoic acid ethyl ester methanesulfonate (MS222, Sandoz, Basel, Switzerland), and pieces of the ovary were removed. Oocytes arrested in the prophase of the first meiotic division (stages V and VI, Dumont, 1972) were selected after removal of enveloping tissue by treatment of the ovarian pieces with collagenase (0.6–0.8 U/ml in oocyte Ringer's solution, *see below*) and subsequent washing in Ca^{2+}-free solution. To increase the current generated by the Na^+/K^+ ATPase, intracellular $[Na^+]$ was elevated by incubating the cells for at least 2 hr at room temperature in Ca^{2+}- and K^+-free solution; intracellular Na^+ activity can be raised by this procedure to at least 30 mM, as determined by Na^+-selective microelectrodes (LaTona, 1990).

Electrophysiological Measurements

Voltage-clamp experiments were performed by conventional two-microelectrode techniques with technical details as described by Lafaire and Schwarz (1986). For determination of most *I-V* relationships, rectangular voltage pulses of varying amplitude and 500-msec duration were applied every 4 sec,and steady-state current was averaged during the last 100 msec.The holding potential between pulses was set to −60 mV. The experiments illustrated in Fig. 6 were peformed using the down-up-down staircase protocol described by Rakowski and Paxson (1988) from a holding potential of −40 mV. During all experiments, the experimental chamber was continuously perfused with test solution. After each solution change, at least two *I-V* curves were determined about 5 min apart. This allowed a correction to be made for current drift with time (*see* Results). All experiments were performed at room temperature (22°C).

To determine apparent K_m values for stimulation of the Na^+/K^+ pump by external $[K^+]$, pump current was fitted by the following form of the Hill equation:

$$I_p/I_{p\max} = [K^+]^n/(K_m^n + [K^+]^n) \quad (1)$$

where I_p is the magnitude of the Na^+/K^+ pump current at a given membrane voltage, K_m is the half-activating concentration for external $[K^+]$, and n is the Hill coefficient. Currents are either given in nA (measured *I-V* curves) or expressed as $\mu A\ cm^{-2}$ (difference *I-V* curves) assuming a spherical 1.2-mm diameter oocyte. Capacitance measurements indicate that the actual surface area is larger than that calculated for a smooth sphere by a factor of 3 to 4 (Vasilets et al., 1990).

Solutions

The composition of the oocyte Ringer solution used during dissection was (in mM): 110 NaCl, 3 KCl, 2 $CaCl_2$, and 5 morpholinopropane sulfonic acid (MOPS, adjusted to pH 7.6). The 90 Na^+ test solutions contained (in mM): 90 NaCl, varying KCl, 2 $CaCl_2$, 5 $BaCl_2$, 20 tetraethylammonium chloride (TEA), and 5 MOPS (pH = 7.6); to obtain a Na^+-free test solution, NaCl was replaced by tetramethylammonium chloride (TMA). The actual Na^+ and K^+ concentrations of test solutions were confirmed by flame photometry; in nominally Na^+- and K^+-free solutions, the contaminating level of the omitted cation was below 10 μM. The incubation medium for loading the oocytes with Na^+ had the following composition (in mM): 110 NaCl, 2.5 sodium citrate, and 5 MOPS (pH = 7.6).

Results

Correction of Difference Currents for Drift with Time

The chart record in Fig. 1*A* shows the time course of the holding current during a typical experiment in Na^+-free solution. The current record is interrupted by membrane *I-V* measurements (*a–k*); the capacitive current transients are off-scale at this gain. At least two *I-V*s were measured in each solution. At the holding potential of −60 mV in 0.25 mM $[K^+]$ solution (*a* and *b*) there was an outward holding current. Upon changing to K^+-free solution (*c–e*) the holding current became inward. A relatively rapid and large increase in holding current occurred upon changing to 5 mM $[K^+]$ solution (*f* and *g*). The current activated by K^+ was almost completely abolished by 50 μM dihydroouabain (DHO) (*h* and *i*). The removal of K^+ in the continued presence of DHO (*j* and *k*) had little effect on the holding current. Although the current record is quite stable under each of the above experimental conditions, there are slow drifts in holding current in each condition particularly, for example, after the change to K^+-free solution (*c–e*) and in the presence of DHO (*h–k*).

The reproducibility of *I-V* determinations is illustrated in Fig. 1*B*. All eleven *I-V* measurements from Fig. 1*A* are shown in *B*. The first determination in a particular solution is indicated by symbols, the second by connected straight line segments that, in most cases, pass through the symbols. The absolute magnitude of the current shift produced by changing between the most extreme conditions 5 mM $[K^+]$ and K^+-free solution, is seen to be about 25 nA in this oocyte and is relatively voltage independent as indicated by the parallel upward shift of the *I-V* curve upon addition of 5 mM K^+, corresponding to the outward current change between *e* and *f* in Fig. 1*A*. Before we accept that this parallel upward shift

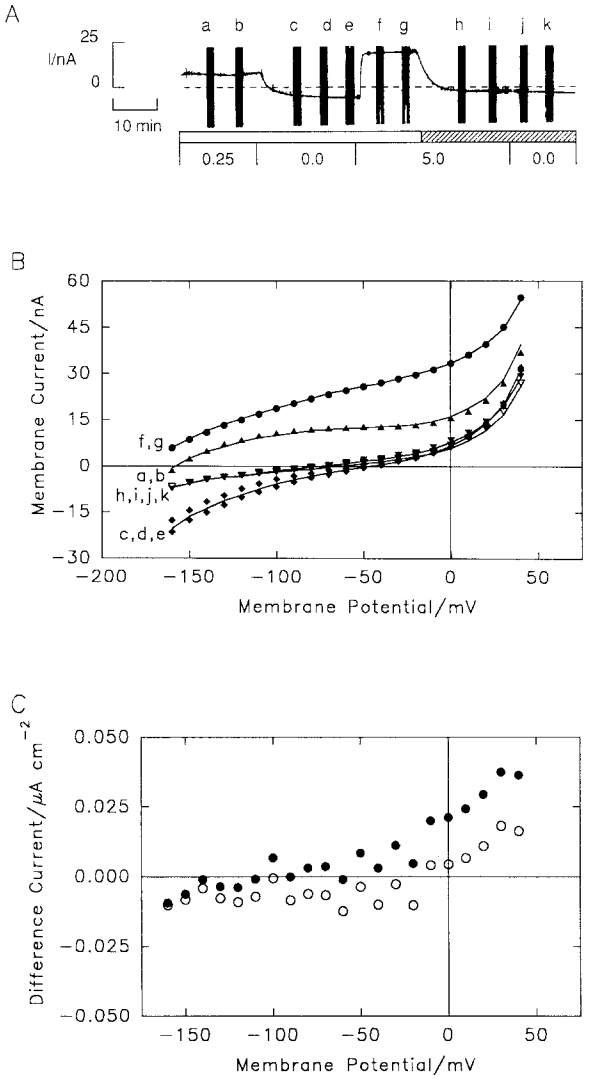

Fig. 1. Results from a typical experiment. (*A*) Chart record of holding current during an experiment in Na^+-free solution. The external $[K^+]$ was changed to different levels as indicated by the numbers (in mM). The letters indicate the various *I-V* curves that were measured. DHO (50 μM) was applied during the period of time indicated by the cross-hatched bar. (*B*) Voltage dependence of the steady-state membrane current. Symbols represent data obtained from the first (and last if more than two *I-V* relations were recorded) measurement after a change of solution. The solid lines are not fits to the data, but rather were obtained simply by connecting the data points obtained from the second measurement after a change of solution. Different symbols refer to different external $[K^+]$ solutions: (*f* and *g*) filled circles = 5 mM K^+, (*a* and *b*) filled triangles (up) = 0.25 mM K^+, (*c–e*) filled diamonds = 0 mM K^+, and downward-directed triangles represent currents in the presence of 50 μM DHO with (*h* and *i*) 5 mM $[K^+]$ (filled) or (*j* and *k*) K^+-free solution (open) symbols. The filled downward triangles obscure the open symbols at all but the most extreme voltages. The difference between these two conditions is shown more clearly in *C*. (*C*) Voltage dependence of membrane current sensitive to 5 mM external $[K^+]$ that persisted after inhibition of the Na^+/K^+ pump by 50 μM DHO. Filled circles represent difference currents between 5 and 0 mM $[K^+]$. Open circles represent the same data, but corrected for slow drift with time

of the *I-V* curve results from the activation of forward Na^+/K^+ pumping by addition of extracellular K^+, it must be demonstrated that there is no other significant source of K^+-sensitive current. This is shown by *I-V* records *h–k* obtained in the presence of DHO to eliminate Na^+/K^+ pump activity, which demonstrate that there is very little effect of removing 5 mM external K^+ when the Na^+/K^+ pump has been blocked by 50 μM DHO. The small residual K^+-sensitive current, shown more clearly in Fig. 1*C*, is discussed below.

To correct for slow time-dependent drift, a polynomial was fitted to the change in current between successive *I-V* curves recorded before (*i–h*) and after (*k–j*) the removal of 5 mM K^+ in the presence of DHO. These two drift corrections were then weighted by the ratio of the time between each pair of control *I-V* curves in a given solution and the time between the *I-V* curves before and after the experimental change in solution. The total drift correction to be subtracted from the experimental difference *I-V* curve (*i–j*) was then computed by adding one-half of each of the time-weighted corrections before and after the experimental change. The corrected data are shown by the open symbols in Fig. 1*C*. This method of drift correction has the advantage in that it does not introduce additional random scatter in the data. The method has previously been used in experiments conducted in squid giant axons (Rakowski, Gadsby & De Weer, 1989).

Control Measurements of K^+-Sensitive Current in the Presence of DHO

The result of the subtraction of the steady-state *I-V* curve measured at *j* from the data in *i* (*i–j*) is shown by the filled circles in Fig. 1*C*. There is a small amount of, mostly outward, K^+-sensitive current. After correction for time-dependent drift, the residual current (open circles) does not exceed ± 0.025 $\mu A\ cm^{-2}$. The mean value of Na^+/K^+ pump current measured at 0 mV in Na^+free solutions in these experiments was 0.67 ± 0.04 $\mu A\ cm^{-2}$. The error that will result from neglecting nonpump K^+-sensitive current is, therefore, on average less than 4%.

A similar conclusion can be reached by considering the mean value of nonpump K^+-sensitive current measured in all experiments performed either in Na^+-free conditions (Fig. 2*A*) or in the presence of 90 mM external $[Na^+]$ (Fig. 2*B*). Over the entire range of voltages examined, the average nonpump, K^+-sensitive current is seen to be quite small (<0.05 $\mu A\ cm^{-2}$), except perhaps at extreme voltages where the increase in the slope of the underlying

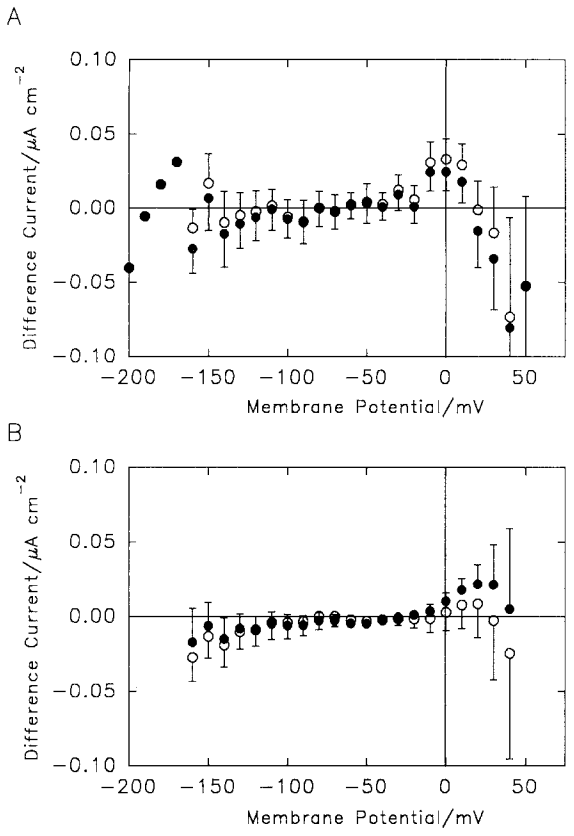

Fig. 2. Residual K^+-sensitive current in Na^+-free (*A*) and 90 mM Na^+ (*B*) solution in the presence of 50 μM DHO. (*A*) Average values of K^+-sensitive membrane current obtained as described in Fig. 1*C* from 12 experiments in Na^+-free solution (±SEM). Data points without error bars represent single determinations. (*B*) Average values of K^+-sensitive current as in *A* but in 90-mM Na^+ solution. The average of 10 experiments (±SEM) is shown. The open symbols in *A* and *B* represent data not corrected for drift while the filled symbols represent drift-corrected data

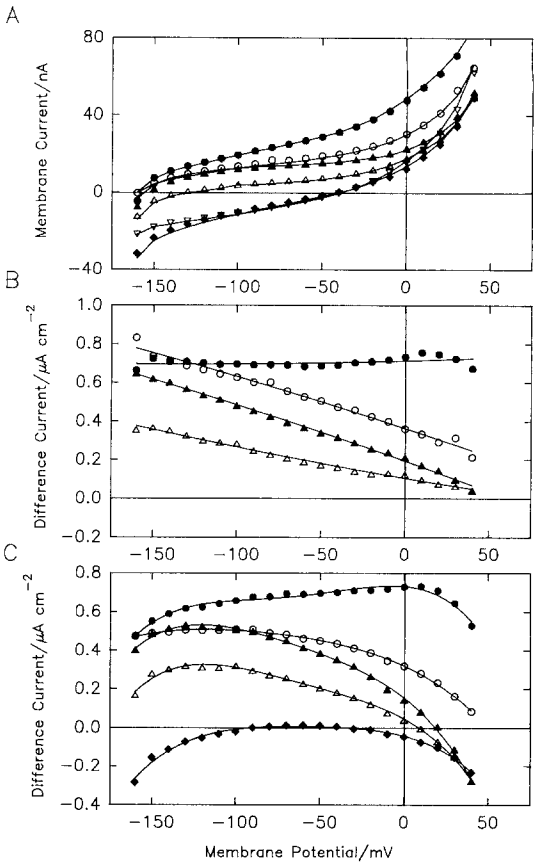

Fig. 3. Na^+/K^+ pump *I-V*s in Na^+-free solution. (*A*) Voltage dependence of steady-state membrane current in Na^+-free solution at different external $[K^+]$. Symbols represent data obtained from the first measurement after a change of solution. The solid lines connect data points obtained 5 min later in the same solution. Different symbols refer to different external $[K^+]$: filled circles = 5 mM $[K^+]$, open circles = 0.5 mM $[K^+]$, filled triangles (up) = 0.25 mM $[K^+]$, open triangles (up) = 0.1 mM $[K^+]$, filled diamonds = 0 mM $[K^+]$, and downward-directed open triangles represent measurements in the presence of 50 μM DHO in K^+-free solution. (*B*) Voltage dependence of Na^+/K^+ pump current at different external $[K^+]$. Pump currents were determined as the difference between total membrane current at the respective $[K^+]$ and in K^+-free solution. Different symbols refer to different external $[K^+]$: filled circles = 5 mM $[K^+]$, open circles = 0.5 mM $[K^+]$, filled triangles = 0.25 mM $[K^+]$, and open triangles = 0.1 mM $[K^+]$. The solid lines are polynomial fits to the data points. (*C*) Voltage dependence of pump current at different external K^+ concentrations as in *B*, but the currents obtained in the presence of DHO were used as the control condition. Symbols as in *B*. The filled diamonds represent the difference between the current measured in K^+-free solution and the same solution plus 50 μM DHO. The solid lines are polynomial fits to the data. All difference currents were corrected for drift

I-V relationship (see, e.g., Fig. 1*B*) results in an increase in the SE of the measurements. However, even at these extreme voltages, the difference currents are not statistically significantly different from zero ($P < 0.01$) so that on average no systematic error in the estimation of Na^+/K^+ pump current will result from neglecting nonpump K^+-sensitive currents under these experimental conditions.

Na^+/K^+ Pump *I-V* Relationship in Na^+-Free Solution

The results from an experiment similar to that of Fig. 1 are shown in Fig. 3. In this case several intermediate values of external $[K^+]$ have been examined. As in Fig. 1*B*, the *I-V* relationship in K^+-free solution (filled diamonds) undergoes a parallel upward shift of about 30 nA when Na^+/K^+ pump current is activated by 5 mM external $[K^+]$. This is more clearly shown by the drift-corrected K^+-sensitive difference current in Fig. 3*B*. The Na^+/K^+ pump current activated by 5 mM $[K^+]$ is nearly voltage

independent, in agreement with previous results in oocytes (Rakowski & Paxson, 1988), cardiac myocytes (Gadsby & Nakao, 1987; Nakao & Gadsby, 1989) and squid giant axons (Rakowski et al., 1989*a*). At low $[K^+]$, however, the K^+-sensitive difference *I-V* curves have a negative slope over the entire voltage range examined (as first observed by J.L.). The difference currents at very negative potentials approach those seen in 5 mM $[K^+]$ and decline towards zero at positive potentials. This behavior can be seen in the *I-V* curves in Figs. 1*B* and 3*A*; at intermediate $[K^+]$ the membrane current at very negative potentials approaches the value measured in 5 mM $[K^+]$, and at positive membrane potentials approaches the value measured in K^+-free conditions. This striking agreement at extreme voltages was consistently observed in these experiments, whenever the drift in holding current was small.

Ouabain-Sensitive Current Measured in Na^+-Free Solution

As indicated by the difference between the two-lowest *I-V* curves in Fig. 3*A*, we consistently observed an inward current blocked by ouabain or DHO in K^+-free solutions at voltages more negative than about -100 mV. Since the *I-V* measurements in the presence of ouabain or DHO were made at the end of the experiment and were usually widely separated in time from the *I-V* curves measured at various $[K^+]$, it was not routinely possible to make a correction for time-dependent drift in difference *I-V* curves derived from these data. However, if we calculate the ouabain-sensitive difference current (Fig. 3*C*), it is clear that at low $[K^+]$ the Na^+/K^+ pump current measured in this way shows a significant region of negative slope from -100 to 0 mV where the contribution of the ouabain-sensitive inward current is small.

Voltage Dependence of Apparent K_m for Activation by External $[K^+]$

The results shown in Fig. 4 were calculated by averaging the normalized difference *I-V* curves from all available experiments in Na^+-free solution. Data were normalized to the value of pump current measured at -100 mV. The mean values of the normalized pump current were then rescaled by the mean value of pump current at the normalizing voltage. These averaged data are similar to the result shown in Fig. 3*B* and also suggest that the degree of pump stimulation by extracellular K^+ depends on membrane potential. For a more complete analysis, pump

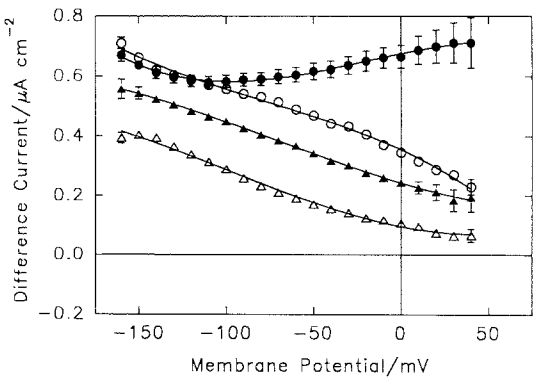

Fig. 4. Voltage dependence of average pump currents (±SEM) at different extracellular $[K^+]$ in Na^+-free solution: filled circles = 5 mM $[K^+]$ (nine determinations in eight oocytes), open circles = 0.5 mM $[K^+]$ (four determinations in four oocytes), filled triangles = 0.25 mM $[K^+]$ (nine determinations in eight oocytes), and open triangles = 0.1 mM $[K^+]$ (three determinations in three oocytes). All curves were corrected for time-dependent drift. Error bars are not shown when the SEM is smaller than the size of the symbol. The SEM was calculated based on normalized data. The data and SEM were rescaled by the mean value of pump current at the normalizing voltage (-100 mV). That is, the SEM represents the error associated with the shape of the curves rather than with the absolute magnitude of the data. The solid lines are polynomial fits to the data

currents determined at a fixed membrane potential were plotted *versus* $[K^+]$, and the Hill equation was fitted to the data (Eq. (1)). An average value of 1.28 ± 0.05 (±SEM) was obtained for the Hill coefficient, n, for the different membrane potentials. For subsequent determinations of apparent K_m values, a fixed value of $n = 1.3$ was used for all potentials.

Figure 5*A* shows examples of the fit by the Hill equation for several selected potentials and clearly demonstrates that the apparent K_m for activation of the Na^+/K^+ pump by external K^+ increases with membrane depolarization. This behavior of Na^+/K^+ pump current in Na-free solution was predicted by the microscopic model of the Na^+/K^+ pump developed by Läuger and Apell (1986) (their Fig. 8). Figure 5*B* summarizes the voltage dependence of the apparent K_m values for all potentials within the investigated range. The data can be fit by the exponential function:

$$K_m = K_m(0)\exp(\alpha z FV/RT) \qquad (2)$$

with an apparent K_m at 0 mV of 0.46 mM, and an exponential steepness expressed as an effective charge for K^+ binding, αz, of 0.37 elementary charge. For a univalent ion binding site the parameter α is identical to the dielectric coefficient α'' defined by Eq. (7*b*) of Läuger and Apell (1986). The apparent K_m at 0 mV of 0.46 mM is comparable to

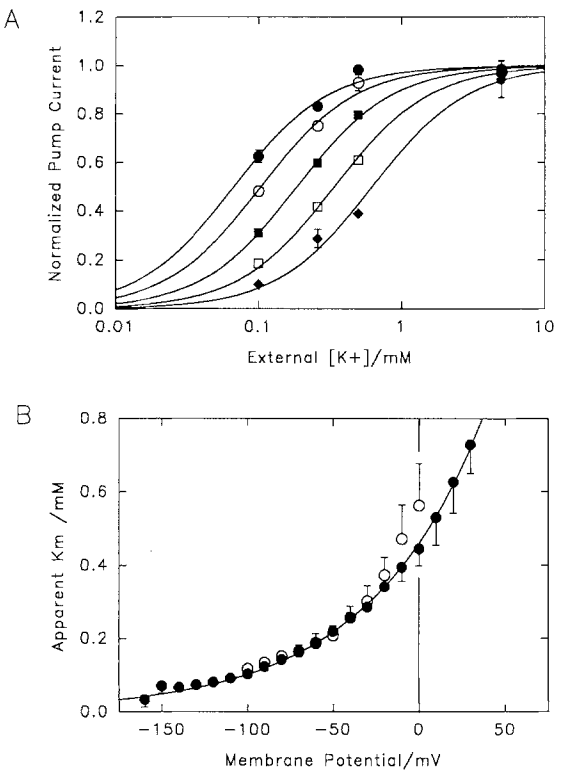

Fig. 5. Voltage dependence of the apparent K_m for activation of pump current by external $[K^+]$ in Na-free solution. Data represent average values from eight experiments. (*A*) Dependence of normalized pump current on extracellular $[K^+]$ for various membrane potentials (filled circles = −140 mV, open circles = −100 mV, filled squares = −60 mV, open squares = −20 mV, and filled diamonds = +20 mV). The solid lines represent fits of the Hill equation (Eq. (1)) to the data using an average Hill coefficient of $n = 1.3$. (*B*) Voltage dependence of the apparent K_m. Data were obtained from fits similar to those shown in Fig. 5*A* (±SEM, error bars not shown when SEM is smaller than the size of the symbol). The filled symbols represent data using the current measured in K^+-free solution as the control condition, while the open symbols represent data using measurements in the presence of DHO or ouabain as the control condition. The solid line represents the fit for the filled symbols by Eq. (2) with $K_m(0) = 0.459 \pm 0.003$ mM and $\alpha zF/RT = 0.0146 \pm 0.0002$ (that is, $\alpha z = 0.37$)

values found in other preparations: 0.45 mM in squid giant axon (−10 to −52 mV, Rakowski et al., 1989*a*), and 0.7 mM in reconstituted systems (Nagel et al., 1987).

Control Experiments in Na^+- and Ca^{2+}-Free Solution Containing 2 mM Ni^{2+}

Electrogenic Na^+/Ca^{2+} exchange cannot operate in its normal inward current mode in the absence of extracellular Na^+, but, exchange of internal Na^+ for external Ca^{2+} is possible. To rule out the possibility that starting or stopping the Na^+/K^+ pump could produce changes in internal $[Na^+]$ in a restricted diffusion space and thereby give rise to an artifactual current through reverse Na^+/Ca^{2+} exchange, we performed control experiments in Na^+- and Ca^{2+}-free solution containing 2 mM Ni^{2+}. These conditions should prevent Na^+/Ca^{2+} exchange and, since the ionic strength of the solution is unchanged, should not significantly affect the voltage dependence of Na^+/K^+ pump current. The results shown in Fig. 6 are similar to those obtained at normal external $[Ca^{2+}]$ and support the conclusion that the K^+-sensitive current recorded here represents Na^+/K^+ pump current. The *I-V* curves shown in Fig. 6*A–D* were obtained using the down-up-down staircase protocol (Rakowski & Paxson, 1988). Except for the single record *g* in 5 mM $[K^+]$, two down-up-down *I-V* curves were recorded about 5 min apart in each experimental condition. The *I-V* curves are plotted simply by connecting successive points by straight line segments and appear to be nearly smooth because of the small step size (5 mV). The data resulting from the down-up-down staircase for each *I-V* clearly demonstrate the absence of significant current hysteresis over the entire voltage range examined, and the superimposition of successive *I-V* curves demonstrates the absence of significant current drift. Similar results, almost free of hysteresis and drift, were obtained at normal $[Ca^{2+}]$ over this voltage range, although a voltage range only slightly more positive or negative elicited pronounced hysteresis. The K^+-sensitive difference currents plotted in Fig. 6*E*, meet all the objections previously raised by Rakowski and Paxson (1988): (*i*) there is no significant time-dependent hysteresis in the membrane *I-V* curves that would indicate nonsteady-state conditions, (*ii*) the difference currents have been corrected for the small amount of drift with time, and (*iii*) there is no significant K^+-sensitive current in the presence of ouabain, which demonstrates the absence of significant nonpump K^+-sensitive current (open symbols in Fig. 6*E*). In addition, the experiments were performed under conditions that should prevent Na^+/Ca^{2+} exchange. The K^+-sensitive difference currents have a magnitude and slope similar to those measured in 2 mM $[Ca^{2+}]$ (Figs. 3*B* and 4). The Na^+/K^+ pump *I-V* curve is voltage insensitive in 5 mM $[K^+]$ and clearly has a negative slope in 0.25 mM $[K^+]$. As previously noted in Figs. 1*B* and 3*A*, there is a small ouabain-sensitive inward current component in K^+-free solution (filled diamonds, Fig. 6*E*), possibly representing a reverse mode of operation of the Na^+/K^+ pump, but since

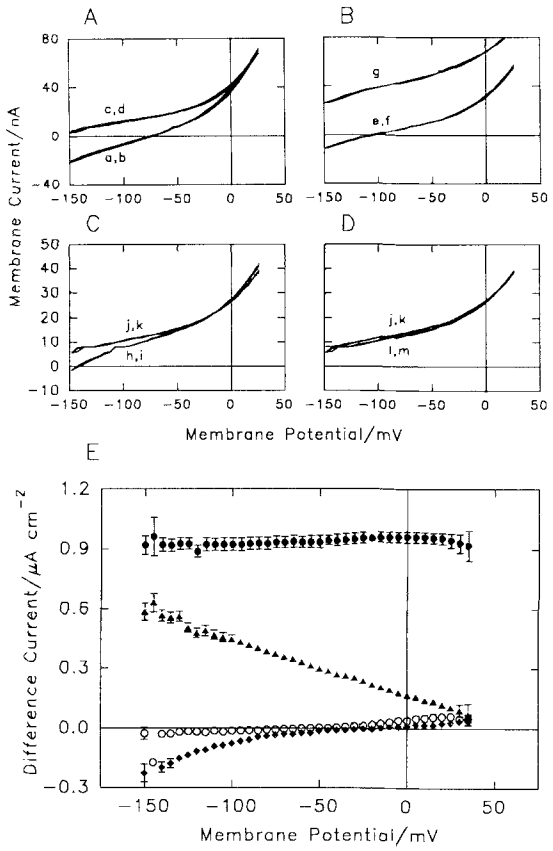

Fig. 6. *I-V* measurements in Ca^{2+}- and Na^+-free solution in the presence of 2 mM [Ni^{2+}]. (*A* and *B*) Membrane currents measured at various external [K^+] (*a* and *b* = 0; *c* and *d* = 0.25; *e* and *f* = 0, *g* = 5 mM). (*C* and *D*) Membrane currents measured in K^+-free solution (*h* and *i*), in K^+-free solution during exposure to 100 μM DHO (*j* and *k*), and in 5 mM [K^+] in the presence of DHO (*l* and *m*). The down-up-down staircase protocol described by Rakowski and Paxson (1988) was performed from a holding potential of −40 mV. Two *I-V* curves were determined about 5 min apart in each experimental condition except for 5 mM K^+ in *B*. (*E*) Difference currents calculated from eight experiments similar to that shown in *A–D*. Filled circles represent Na^+/K^+ pump current in 5 mM [K^+], and filled triangles = 0.25 mM [K^+]. The open circles represent the residual K^+-sensitive current measured as the difference between currents in 5 mM [K^+] with DHO and in K^+-free conditions with DHO. The filled diamonds represent the difference between currents in K^+-free solution and the same solution with DHO. The variability of pump current magnitude in these experiments was quite small. This allowed averaging without normalization for variations in pump current magnitude. The values shown in Fig. 6*E* are simply mean values ±SEM of all measurements under these conditions. SEM not shown when smaller than the size of the symbol. All difference currents have been correct for drift

the experiments were performed in Na^+-free solution some other ion is required to substitute for Na^+. As will be discussed below, this ouabain-sensitive inward current component is not seen in 90 mM external [Na^+].

Voltage Dependence of Pump Current in High [Na^+]

The data presented in the previous figures have demonstrated that in Na^+-free solution Na^+/K^+ pump current declines with depolarization when external [K^+] is low. This suggests that a voltage-dependent step in the reaction cycle, different from the Na^+-translocating step, governs the transport cycle in Na-free solutions when external [K^+] is below saturation. The observations can be described by a voltage-dependent apparent K_m for stimulation of the Na^+/K^+ pump by external K^+. A negative slope should also be detectable at physiological extracellular [Na^+] when [K^+] is below saturation. To find the appropriate conditions, we performed experiments at various [K^+] in 90-mM Na^+ solution. Figure 7*A* shows examples of *I-V* curves obtained at different external [K^+] (5, 1.3 and 0 mM). As in Fig. 3*A*, the first *I-V* measurement is shown by the symbols and the second *I-V* in a given solution is shown as connected straight line segments. The two lowest curves that are nearly superimposed represent current with the Na^+/K^+ pump blocked (K^+-free solution without or with 50 μM ouabain). In contrast to the experiments in Na^+-free solution, application of ouabain has nearly no effect on membrane current persisting in K^+-free solution over the whole potential range (*see also* diamonds in Fig. 7*B*). The *I-V* curves obtained in K^+-free solution were used as the control condition for determining the pump current since the effect of external K^+ application is rapidly reversible and those difference *I-V* curves could be corrected for slow drift. At 5 mM external [K^+], the difference current shows the well-established increase with membrane depolarization (Fig. 7*B*). There is only a hint of a negative slope at positive potentials. When the external [K^+] is reduced to 1.3 mM, however, the *I-V* curve in Fig. 7*A* asymptotically approaches the control curve. The difference curve (Fig. 7*B*), therefore, has a negative slope at positive potentials.

The same result was obtained in other experiments summarized in Fig. 8. The data were averaged by a normalization procedure similar to that used in Fig. 4*A* except that 0 mV was used as the normalizing voltage. The characteristics seen in the experiment of Fig. 7 persist in the average curves: (*i*) ouabain has no significant effect on the membrane current in K^+-free solution, (*ii*) in 5 mM K^+, the pump *I-V* curve shows saturation at positive potentials, and (*iii*) in 1.3 mM K^+, an *I-V* curve with a maximum at about 0 mV is obtained. Considering the error in the difference measurements, the fact that the data obtained in 1.3 mM K^+ cross the zero-current axis at about −150 mV should not be taken as evidence that this is the Na^+/K^+ pump reversal potential un-

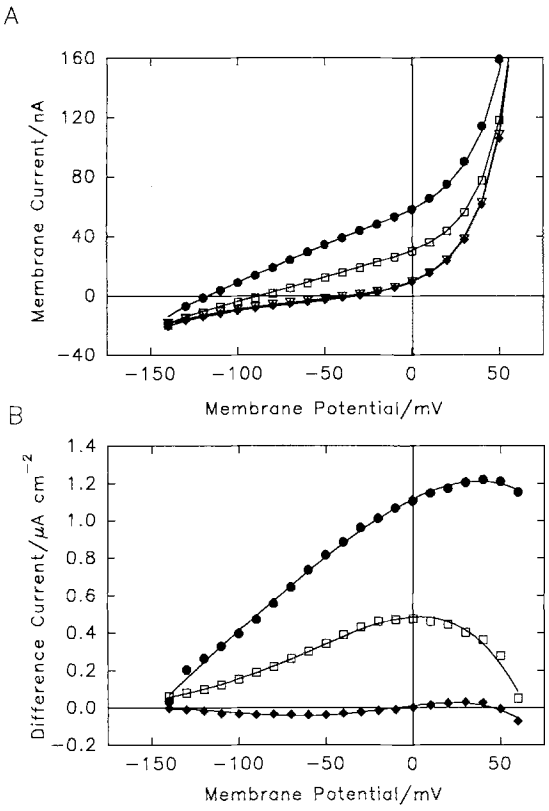

Fig. 7. Pump *I-V* relationships in 90 mM Na^+. (*A*) Voltage dependence of steady-state membrane current determined using the pulse protocol from a holding potential of −60 mV. Symbols represent data obtained from the first measurement after a change of solution. The solid lines connect data points obtained 5 min later. Different symbols refer to different external $[K^+]$: filled circles = 5 mM $[K^+]$, open squares = 1.3 mM $[K^+]$, filled diamonds = K^+-free solution, and downward-directed open triangles represent currents in the presence of 50 μM DHO in K^+-free solution. Note that in contrast to Na^+-free conditions, the currents in K^+-free solution with and without DHO nearly coincide, and the open triangles are obscured. (*B*) Voltage dependence of pump current at 5 (filled circles) and 1.3 (open squares) mM external $[K^+]$. Pump currents were determined as the difference between total membrane current at the respective K^+ concentration and in K^+-free solution. The filled diamonds represent the difference between currents measured in K^+-free solution and in K^+-free solution plus 50 μM DHO. All curves were corrected for slow drift with time. Solid lines are polynomial fits to the data

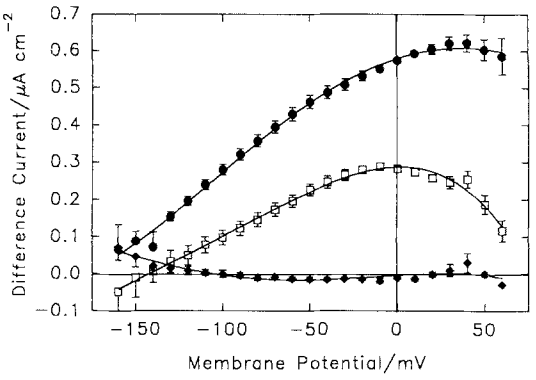

Fig. 8. Voltage dependence of averaged Na^+/K^+ pump current (±SEM) at 5 mM (filled circles) (13 determinations in 8 oocytes) and 1 mM (open squares) (14 determinations in 8 oocytes) external $[K^+]$ in solution containing 90 mM Na^+. Filled diamonds represent the ouabain-sensitive current that remains in extracellular K^+-free solution (seven determinations in seven oocytes). Solid lines are polynomial fits to the data. As in Fig. 4*A* the error bars represent the SE of the shape of the curve rather than the total SE including variations in magnitude. Error bars are not shown when SEM is smaller than the size of the symbol. All currents have been correct for drift

der these conditions. The presence of a sharp downturn in the *I-V* curve in the vicinity of −150 mV makes it difficult to support previous claims of Na^+/K^+ pump current reversal at extreme negative voltages (Béhé & Turin, 1984; Wu & Civan, 1990).

Discussion

Voltage sensitivity of the current generated by the Na^+/K^+ pump reflects voltage-dependent steps in the reaction cycle. These might be rate limiting themselves, or might determine pump rate by controlling the level of enzyme intermediate that enters a rate-limiting step. Stimulation of pump currents by depolarization can be attributed to a voltage-dependent step associated with Na^+ translocation. There is considerable evidence that the translocation of Na^+ involves movement of charge through the membrane field. Direct evidence is provided by the experiments of Nakao and Gadsby (1986) in cardiac myocytes showing that strophanthidin-sensitive charge movement can be recorded under Na^+/Na^+ exchange conditions. Presteady-state transient currents elicited by ATP concentration jumps and optical signals related to conformational changes of Na^+/K^+ pump molecules have a similar time course (Stürmer, et al., 1989), suggesting that the conformational change or release of Na^+ is accompanied by charge movement through the membrane field.

In contrast to the evidence that Na^+ translocation is voltage dependent, it is generally thought that K^+ translocation is not. Goldshlegger et al. (1987) reported that ATP-activated Rb^+/Rb^+ exchange is not affected by membrane potential. Gadsby, Bahinski and Nakao (1989) found no evidence for pump-mediated charge movement under K^+/K^+ exchange conditions. Fendler et al. (1985) also did not detect stationary or transient pump currents under K^+/K^+ exchange conditions in response to the release of caged ATP, and Stürmer et al. (1989) did not detect transient pump currents under Na^+-free conditions despite the presence of fluorescence changes

thought to reflect conformational changes of the Na^+/K^+ pump. These observations provide strong evidence that conformational changes involved in K^+ translocation are voltage insensitive and do not involve charge movement through the membrane field.

The observation of a negative slope in the current-voltage relationships at positive potentials for the endogenous as well as the expressed *Torpedo* pump in *Xenopus* oocytes (Lafaire & Schwarz 1986; Schwarz & Gu, 1988) suggested, however, that there are special conditions where a second voltage-dependent step becomes rate determining. Since pump current is measured as a difference, the possibility of artifacts due to the subtraction procedure has been raised (Rakowski & Paxson, 1988). The experiments described in this paper were designed to address these objections. The results give clear evidence for the presence of a negative slope in the Na^+/K^+ pump *I-V* in Na-free solution when external $[K^+]$ is below saturation.

As discussed by Apell (1989), previous evidence that K^+ translocation is voltage insensitive (Bahinski et al., 1988; Stürmer et al., 1989) does not rule out the possibility that external K^+ binding and release are voltage dependent. In the absence of external Na^+, the apparent K_m for activation of Na^+/K^+ pump current by external K^+ is quite low (Fig. 5). Most of the available information on the voltage dependence of Na^+/K^+ pump current and presteady-state charge movement has been obtained at external $[K^+]$ well above half-saturation where it is unreasonable to expect that effects of membrane potential on K^+ binding would be seen since external K^+ binding sites are nearly fully occupied. We have explored this low $[K^+]$ range and found clear evidence for a negative slope in the Na^+/K^+ pump *I-V* relationship in the absence of external Na^+. We have also provided evidence that a negative slope can be observed in the presence of external Na^+ when the membrane potential is made sufficiently positive and external $[K^+]$ is reduced below the half-saturation value for these conditions. These results confirm the existence of a negative slope under experimental conditions similar to those previously used by Lafaire and Schwarz (1986).

Difference Currents Obtained by Subtraction of Current in K^+-Free Solution

We used the data obtained in K^+-free solution for subtracting nonpump currents from total membrane current. This procedure assumes that under K^+-free conditions the pump is completely inhibited. This assumption is fairly well met in 90-mM Na^+ solution as judged by the lack of response to ouabain or DHO (Figs. 7*B* and 8), but in K^+- and Na^+-free solution we consistently observed an inwardly directed ouabain-sensitive current at potentials more negative than about -100 mV (Figs. 1*B* and 3*A*). Since there is no evidence that ouabain directly affects any transport system other than the Na^+/K^+ pump, we suggest that this curent is related to a mode of pumping that persists under these conditions. One speculation is that, in Na^+- and K^+-free solution, the pump operates in a reversed mode transporting some Na^+ congener inwards in exchange for K^+.

Controls for Contributions to Membrane Current by Na^+/Ca^{2+} Exchange

In heart muscle, the possibility that blocking the Na^+/K^+ pump could affect currents produced by the Na^+/Ca^{2+} exchanger has been raised (Gadsby & Nakao, 1989). Our control experiments in solutions without added Ca^{2+}, but with 2 mM Ni^{2+} to prevent Na^+/Ca^{2+} exchange, showed that reduction of extracellular K^+ still produces a negative slope in the pump *I-V* curve (Fig. 6).

Voltage Dependence of the Na^+/K^+ Pump in Na^+-Free Solution

As has been demonstrated previously in squid axon (De Weer, Rakowski & Gadsby, 1987; Rakowski et al., 1989*a*), heart muscle (Nakao & Gadsby, 1989), and also in oocytes (Rakowski & Paxson, 1988; Schweigert, Lafaire & Schwarz, 1988), removal of extracellular Na^+ results in a reduced voltage dependence of the Na^+/K^+ pump. Voltage-independent pump current in Na^+-free solution is observed when external $[K^+]$ is well above the saturating concentration for stimulation of Na^+/K^+ transport by external K^+ (5 mM). At lower $[K^+]$ depolarization reduces Na^+/K^+ pump current which can be explained by a voltage-dependent apparent affinity for K^+ activation of the pump. The potential dependence of the apparent K_m (Fig. 5*B*) can be described by an effective charge of about $+0.4$ of an elementary charge that moves inward in the electrical field during K^+ binding. A simple explanation of this result that is consistent with data suggesting that K^+ translocation is voltage insensitive (Bahinski et al., 1988; Stürmer et al., 1989), is that the binding sites for K^+ are located within an external vestibule of the pump molecule (ion well) so that K^+ ions must cross part of the membrane field to reach their binding sites

(Läuger & Apell, 1986). Stürmer et al. (1990) have recently reported that fluorescence changes measured in membrane fragments noncovalently labeled with amphiphilic styryl dyes indicate that the occlusion of external K^+ may involve the migration of K^+ into an ion well. If ouabain inhibits the Na^+/K^+ pump cycle without affecting external K^+ migration within such an ion well, ouabain-sensitive transient currents would not include charge movement of ions within the well. Another possible explanation of our results is that the membrane potential affects the distribution of various conformational states of the transport protein and by this means modulates the interaction of K^+ ions with their binding sites.

Results in Cardiac Myocytes

Nakao and Gadsby (1989) have also investigated the behavior of the Na^+/K^+ pump *I-V* relationship at reduced extracellular $[K^+]$ in Na^+-free conditions. In contrast to the results reported here they found that the *I-V* relationship seemed to be simply scaled down as $[K^+]$ was reduced (their Fig. 10). We have no completely satisfactory explanation of this difference in experimental findings. The difference may, in part, be explained by technical difficulties resulting from the very low value of the apparent K_m for external $[K^+]$ in Na^+-free conditions (0.22 ± 0.03 mM at 0 mV) in cardiac myocytes compared to the somewhat higher value (0.459 ± 0.003 mM) in *Xenopus* oocytes (Fig. 5), and from the small amount of data obtained from myocytes at sufficiently low extracellular $[K^+]$. There is an indication at high external $[Na^+]$ that a negative slope can be seen at +40 mV when external $[K^+]$ is reduced to 1.0 mM (Fig. 9 of Nakao & Gadsby, 1989), but the large size of the voltage increments used (20 mV) makes it difficult to determine the precise shape of the *I-V* curve. The difference might also result from a shift of the curves to more positive voltages in guinea pig cardiac myocytes compared to *Xenopus* oocytes, or some other species difference. Schwarz and Vasilets (1990) have recently reported that the voltage dependence of the K_m for activation by external $[K^+]$ is different in the endogenous Na^+/K^+ pump compared to the Na^+/K^+ pump expressed in the oocytes by injection of cRNA from *Torpedo* electroplax and that the voltage dependence can be modulated by stimulation of protein kinases.

Pump *I-V* Relationships under Various Ionic Conditions

The apparent discrepancy concerning the existence of a negative slope in the *I-V* relationship of the Na^+/K^+ pump described in the Introduction has now been resolved. The voltage dependence of the current generated by the Na^+/K^+ pump in *X. laevis* oocytes strongly depends on the following experimental conditions:

1) In Na^+-free solutions with saturating external $[K^+]$ neither the voltage-dependent step associated with Na^+ translocation nor the external K^+-binding step are rate determining. Voltage-independent steps are rate limiting, and the Na^+/K^+ pump behaves as a rheogenic (constant-current) transporter.

2) If the Na^+-translocating step is made rate determining by increasing extracellular $[Na^+]$, the pump *I-V* monotonically increases with depolarization and saturates at positive potentials.

3) If in Na^+-free solution the K^+-binding step is made rate determining by reducing extracellular $[K^+]$ below saturation, pump current decreases monotonically with depolarization.

4) With 90 mM Na^+ and 1.3 mM K^+ present, both voltage-dependent steps contribute to the shape of the pump *I-V* curve. At negative potentials, depolarization stimulates the overall transport rate by aiding the Na^+-dependent step, while at positive potentials when Na^+ translocation is no longer rate determining, the pump transport rate is apparently reduced by electrostatic inhibition of K^+ binding.

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