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Steady-State Current-Voltage Relationship of the Na/K Pump in Guinea Pig Ventricular Myocytes

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ABSTRACT Whole-cell currents were recorded in guinea pig ventricular myocytes at ~36°C before, during, and after exposure to maximally effective concentrations of strophanthidin, a cardiotonic steroid and specific inhibitor of the Na/K pump. Wide-tipped pipettes, in combination with a device for exchanging the solution inside the pipette, afforded reasonable control of the ionic composition of the intracellular solution and of the membrane potential. Internal and external solutions were designed to minimize channel currents and Na/Ca exchange current while sustaining vigorous forward Na/K transport, monitored as strophanthidin-sensitive current. 100-ms voltage pulses from the −40 mV holding potential were used to determine steady-state levels of membrane current between −140 and +60 mV. Control experiments demonstrated that if the Na/K pump cycle were first arrested, e.g., by withdrawal of external K, or of both internal and external Na, then neither strophanthidin nor its vehicle, dimethylsulfoxide, had any discernible effect on steady-state membrane current. Further controls showed that, with the Na/K pump inhibited by strophanthidin, membrane current was insensitive to changes of external [K] between 5.4 and 0 mM and was little altered by changing the pipette [Na] from 0 to 50 mM. Strophanthidin-sensitive current therefore closely approximated Na/K pump current, and was virtually free of contamination by current components altered by the changes in extracellular [K] and intracellular [Na] expected to accompany pump inhibition. The steady-state Na/K pump current-voltage (I-V) relationship, with the pump strongly activated by 5.4 mM external K and 50 mM internal Na (and 10 mM ATP), was sigmoid in shape with a steep positive slope between about 0 and −100 mV, a less steep slope at more negative potentials, and an extremely shallow slope at positive potentials; no region of negative slope was found. That shape of I-V relationship can be generated by a two-state cycle with one pair of voltage-sensitive rate constants and one pair of voltage-insensitive rate constants: such a two-state scheme is a valid steady-state representation of a multi-state cycle that includes only a single voltage-sensitive step.

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INTRODUCTION

The Na/K pump is electrogenic because during a single cycle it transports unequal quantities of Na and K ions in opposite directions across the cell membrane, the excess charge movement appearing as membrane current. Normally, a single pump cycle, involving hydrolysis of one molecule of ATP, results in extrusion of 3 Na ions and uptake of 2 K ions and, hence, in the generation of an outward (hyperpolarizing) current (see reviews by Thomas, 1972*a*; Glynn, 1984). Evidence from snail neurons (Thomas, 1969), cardiac Purkinje fibers (reviewed in Gadsby, 1984), and squid axons (Rakowski et al., 1989) suggests that this Na/K transport stoichiometry of the pump remains constant over a relatively wide range of pump rates at various levels of intracellular [Na], extracellular [K], and membrane potential (for review, see De Weer et al., 1988*b*). Steady-state Na/K pump current therefore provides a convenient measure of the turnover rate of the Na/K pump cycle.

Membrane potential is expected to influence the pump rate on simple thermodynamic grounds. For a reversible Na/K pump reaction with fixed stoichiometry, in which 3 Na ions and 2 K ions are transported in opposite directions across the cell membrane and one molecule of ATP is hydrolyzed or synthesized during each complete cycle, an equilibrium potential, $E_{Na/K}$, can be defined, at which the free energy of hydrolysis of ATP, ΔG_{ATP}, equals the sum of the free energy changes associated with translocation of the 3 Na and 2 K ions, so that there is no net chemical reaction and thus no pump current (e.g., Chapman and Johnson, 1978; Tanford, 1981; De Weer et al., 1988*a*):

$$E_{Na/K} = \Delta G_{ATP}/F + 3E_{Na} - 2E_K \quad (1)$$

where F is Faraday's constant, and E_{Na} and E_K are the electrochemical equilibrium potentials for Na and K ions. Eq. 1 is independent of kinetic details of the Na/K pump reaction cycle. At potentials more negative than $E_{Na/K}$ the net reaction is driven backwards, so that the pump generates net inward current (De Weer and Rakowski, 1984; Bahinski et al., 1988; Rakowski et al., 1988), whereas positive to $E_{Na/K}$ the net reaction runs forwards, the pump generating outward current. Such thermodynamic considerations, however, can specify only the direction of the net reaction, not its rate. The voltage dependence of that turnover rate will be determined by kinetic properties of the reaction cycle, in particular, by the number and relative rates of the individual steps in the cycle that involve charge movement within the membrane field, because the transition rates of any such step must vary with membrane potential (e.g., Läuger and Stark, 1970; Hansen et al., 1981; Chapman et al., 1983; De Weer, 1984; Reynolds et al., 1985; Läuger and Apell, 1986). Nevertheless, the overall cycle rate will show voltage dependence only to the extent that voltage-sensitive transitions themselves rate-limit the cycle or control the concentration of intermediate entering a rate-limiting step (cf. De Weer et al., 1988*a*: Bahinski et al., 1988).

The present work was aimed at examining the influence of membrane potential on Na/K pump turnover rate by determining accurate steady-state current-voltage (I-V) relationships for the Na/K pump under conditions in which intracellular [ATP] and [Na], and extracellular [K], could be expected to remain approximately independent of the imposed changes of voltage. We isolated cells from guinea pig

ventricle, and recorded whole-cell currents (Hamill et al., 1981) using wide-tipped patch pipettes in combination with a device for exchanging the solution inside the pipette (Soejima and Noma, 1984). The wide tips facilitated rapid equilibration of the pipette solution with the cell interior. Intracellular (pipette) and extracellular solutions were designed to minimize ion channel currents and Na/Ca exchange current but sustain Na/K pump current. We detail here experimental conditions under which cardiotonic steroid-sensitive current provides an accurate measure of Na/K pump current, virtually free of contamination by nonpump currents altered by the [K] and [Na] changes at the cell membrane on inhibiting the pump. We find that, between −140 mV and +60 mV, the I-V relationship of the strongly activated forward-running Na/K pump has a sigmoid shape, and that pump current is steeply voltage dependent over the physiological range of diastolic membrane potentials. A preliminary report has been published (Gadsby et al., 1985). In the following paper (Nakao and Gadsby, 1989) we examine the influence of changes in intracellular [Na], extracellular [Na], and extracellular [K], on the Na/K pump I-V relationship.

MATERIALS AND METHODS

Cells and Solutions

Cells were isolated from guinea pig ventricles after partial digestion with collagenase as described by others (Isenberg and Klöckner, 1982; Matsuda et al. 1982). Adult guinea pigs (300–500 g) were anesthetized with pentobarbital (~50 mg/kg, i.p.) and then ventilated via a tracheotomy. The aortic arch was exposed and cannulated, the heart excised, and retrograde coronary perfusion begun, initially with normal Tyrode's solution containing 3 U/ml heparin, then for 3 min with nominally Ca-free (~1 μM free [Ca^{2+}]) Tyrode's, and then for ~15 min with low [Ca^{2+}] (~5 μM) Tyrode's solution containing 0.4–0.8 mg/ml collagenase (Type 1, Sigma Chemical Co., St. Louis, MO). The collagenase was then washed out of the heart with high [K] (~160 mM), nominally Ca-free, solution containing 0.5 mM EGTA, 10 mM KH_2PO_4, 25 mM KCl, 20 mM taurine, 10 mM oxalic acid, 80 mM L-glutamic acid, 5 mM pyruvic acid, 10 mM dextrose, and 10 mM Hepes, all adjusted to pH 7.4 with KOH (Isenberg and Klöckner, 1982; Bendukidze et al., 1985). All the above solutions were oxygenated and warmed to ~36°C. The resulting partially digested heart was cut open and stored at 4°C in the high [K], Ca-free solution.

Cells were transferred to the recording chamber attached to the mechanical stage of an inverted microscope (Diaphot, Nikon Inc., Garden City, NY) either directly, by agitating within the chamber a small fragment of digested ventricle held with fine forceps, or by pipette, as a cell suspension obtained by crudely mincing ventricle fragments, triturating them, and then filtering off debris with fine nylon mesh. Cells were allowed to settle on the glass coverslip bottom of the chamber for ~1 min before beginning superfusion with warmed Tyrode's solution containing (in millimolar): 145 NaCl, 5.4 KCl, 1.8 $CaCl_2$, 0.5 $MgCl_2$, 5.5 dextrose, 5 Hepes/NaOH (pH 7.4). All external solutions passed through water-jacketed glass heating coils connected to the two inputs of a two-position valve (HV-4-4, No. 86729, Hamilton Co., Reno, NV), one output of which flowed to the chamber via a ~5-cm length of narrow silastic tubing, and the other output flowed to waste. Temperature was monitored with a micro-thermistor bead (No. 32A130, Victory Engineering Corp., Springfield, NJ) positioned near the bottom of the chamber, close to the solution inflow, and was kept at ~36°C.

The overall length and breadth of each (relatively flat) myocyte was measured with an eyepiece reticule and, for 80 cells represented here and in Nakao and Gadsby (1989), they averaged (±SEM) 126 ± 2 μm and 31 ± 1 μm, respectively. Fire-polished pipettes with tip diameters of ~5 μm, and tip resistances of ~1 MΩ (1.11 ± 0.02 MΩ, $n = 80$) when filled with Tyrode's solution, were used to form giga-ohm seals (monitored with 1-mV voltage steps applied to the inside of the pipette) between pipette tip and cell surface by applying gentle suction (~ −20 to −30 cm H_2O). Before rupturing the enclosed membrane patch with a brief pulse of more vigorous suction, the Tyrode's solution in the pipette was exchanged for K-free, Na-free, pipette solution containing (in millimolar): ~130 CsOH, 100 aspartic acid, 20 TEACl, 3 $MgCl_2$, 5.5 dextrose, 10 EGTA, 10 MgATP, 5 $Tris_2$-creatine phosphate, 5 pyruvic acid, 10 Hepes (pH 7.4). The apparatus used for exchanging the pipette solution has been described previously (Soejima and Noma, 1984; Sato et al., 1985). Briefly, release of a constriction device allowed negative pressure in the pipette to draw fluid from a miniature reservoir (volume, ~0.6 ml) through fine silastic tubing (0.02 inch I.D.; Dow Corning Corp., Midland, MI) and deliver it to within ~200 μm of the pipette tip via a narrow, tapered, polyethylene (PE) catheter. The catheter was pushed onto a 25-gauge stainless steel tube which passed through a silicone resin seal (Silastic Medical Adhesive Type A; Dow Corning Corp.) filling the upper arm of a four-way PE connector used to hold the pipette. This arrangement permitted optimal positioning of the catheter tip near the pipette orifice by sliding the steel tubing while observing the pipette tip under a dissecting microscope. Exchanging the ~25 μl volume between the catheter tip and the reservoir took ~1 min. The constriction device closed the silastic tube after completion of the exchange and during its transfer between reservoirs, which were kept isopotential by platinum wire bridges. When the membrane patch was ruptured, the wide pipette tip allowed rapid equilibration of the pipette solution with the cell interior (half time ≪1 min; see, e.g., Fig. 1, below) which could be monitored at −40 mV as an inward shift of holding current, due primarily to the loss of intracellular K (Matsuda and Noma, 1984; Sato et al., 1985).

The subsequent switching of the extracellular fluid to nominally Ca-free Tyrode's solution, identical in composition to the normal Tyrode's solution except for its divalent cation content which was 2.3 mM Mg, 2 mM Ba, and 0.2 mM Cd, caused a further reduction of membrane conductance and practically abolished time-dependent currents (see Fig. 1 *B*): the Ba blocked K channels, the Cd prevented Ca-channel currents (carried by Ba), and the lack of internal or external Ca prevented Na/Ca exchange current (Kimura et al., 1987). Na-free, Ca-free Tyrode's solution was made by equimolar replacement of all Na by *N*-methyl-D-glucamine (NMG), and K-free solution was made by substituting Na (or NMG) for K. A second pipette solution was made to contain 100 mM Na in place of 100 mM Cs, and these two pipette solutions were mixed to provide pipette Na concentrations, $[Na]_{pip}$, between 0 and 100 mM. All external and internal solutions had osmolalities close to 300 mOsmol/kg.

Strophanthidin (Sigma Chemical Co.) was added from a 0.5 M stock solution in dimethylsulfoxide (DMSO) and, in preliminary experiments, ouabain (Sigma Chemical Co.) was added from an aqueous 10^{-2} M stock solution.

Data Acquisition and Analysis

To minimize development of liquid junction potentials after changes in pipette solution or external solution, pipette and bath potentials were measured via miniature Ag/AgCl/3M KCl half cells (refreshed daily). For each pipette, the voltage clamp output was set to zero with the pipette tip in the bath when both contained Tyrode's solution. Unless the liquid junctions at the half cells were severely compromised by diffusional loss of KCl, their liquid junction potentials should have remained negligibly small. Moreover, because the measured liquid junction potential between Tyrode's and any pipette solution did not exceed 3 mV (assuming

a negligible junction potential between 3 M KCl and these solutions), true membrane potentials could be no more than 3 mV more negative than the measured values even if the 3 M KCl junction in the pipette were fully dissipated by diffusional exchange with Tyrode's solution. Junction potentials between the various external solutions used in these experiments did not exceed 1 mV. Accordingly, no corrections have been made to the data presented here.

The whole-cell voltage clamp amplifier was based on the design of Hamill et al. (1981) except that the head stage feedback resistor was 100 MΩ and command potentials were applied to the positive input (Ohara et al., 1983; Matsuda and Noma, 1984). Command voltage steps, typically 100 ms in duration, were generated using a stimulator (Digi-pulser, World Precision Instruments, Inc., New Haven, CT) and delivered to the clamp amplifier via a photoisolation coupler. After rupture of the cell membrane and application of the ion channel blockers, total cell capacitance (plus stray capacitance) was estimated from the area under the transient current elicited by a 10-mV step hyperpolarization; for 80 cells in this and the following work (Nakao and Gadsby, 1989), it averaged 183 ± 7 pF. The resistance in series with that capacitance was calculated from the time constant of a single exponential fitted to the decay of the transient current: the series resistance averaged 2.6 ± 0.1 MΩ (n = 80), of which up to ~60% could be compensated by summing a fraction of the clamp output to the command voltage. Analog voltage and current signals were archived on FM tape (Store 7DS, Racal Recorders Inc., Irvine, CA) and simultaneously low-pass filtered at 2 kHz (six-pole, Bessel; Frequency Devices, Haverhill, MA), then digitized on-line at 0.15-ms intervals (12-bit resolution; Data Translation 2801A, Marlboro, MA) and stored on the hard disk of a microcomputer (PC-XT or PC-AT, IBM Instruments Inc., Boca Raton, FL) for subsequent analysis. Data acquisition and analysis were controlled using routines written in ASYST (Asyst Software Technologies Inc., Rochester, NY). The steady current level at each test potential, determined by averaging 200 digitized current points (30 ms) near the end of each clamp pulse, was plotted against the test potential, measured in the same way from the corresponding voltage record, to yield steady-state membrane I-V relationships.

Unless otherwise indicated, mean values are given ± SEM.

RESULTS

Cardiotonic Steroid-sensitive Current

Na/K pump current was estimated as cardiotonic steroid-sensitive current as illustrated in Fig. 1. The low-speed chart record in Fig. 1 *A* shows the effects on the holding current (lower trace) of two brief exposures to 0.5 mM strophanthidin at the −40 mV holding potential. Application of strophanthidin when the pipette Na concentration was zero caused a negligibly small inward shift of holding current of <5 pA, a reasonable result because there should be no outward Na/K pump current in the absence of internal Na. After washing off the strophanthidin, raising $[Na]_{pip}$ to 50 mM caused a rapid (>90% complete within 40 s) outward shift of holding current of just over 200 pA, consistent with the expected strong activation of the Na/K pump by the high intracellular [Na]. This interpretation is supported by the ~200 pA inward current shift caused by the subsequent exposure to strophanthidin.

The six groups of vertical lines on the chart records of Fig. 1 *A* mark periods of application of 100-ms voltage pulses to membrane potentials between +60 mV and −140 mV. Fig. 1 *B* shows superimposed records of the resulting current changes elicited by the pulses to +40, 0, −60, and −100 mV, just before (*a*, *d*) and during (*b*, *e*) the exposures to strophanthidin, as well as the strophanthidin-sensitive currents (*a* − *b* and *d* − *e*) obtained by computer subtraction of current traces recorded in the presence of strophanthidin from those in its absence. The recorded currents (*a*, *b*, *d*, *e*) show rapid, poorly registered, capacity transients followed by smaller relaxations to the steady current levels. These small remaining time-dependent

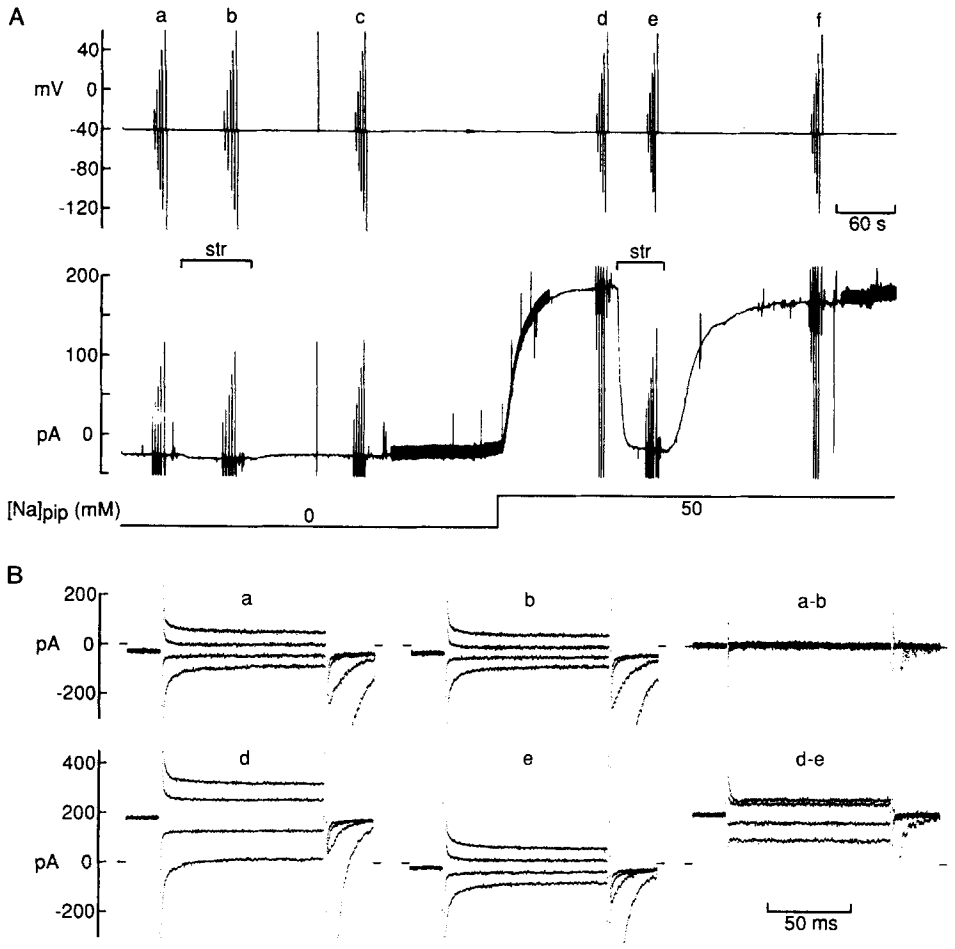

FIGURE 1. Determination of cardiotonic steroid-sensitive current. (*A*) Chart records of membrane potential (*upper trace*) and whole-cell current (*lower trace*). The line at the bottom indicates the approximate timing of the change in pipette [Na] from 0 to 50 mM. Bars over the current record mark periods of exposure to 0.5 mM strophanthidin. (*B*) Superimposed sample traces of currents elicited by 100-ms voltage pulses from −40 mV to +40, 0, −60, and −100 mV at the times (*a, b, d, e*) identified by the corresponding letters above the voltage record in *A*. The strophanthidin-sensitive currents (*a* − *b* and *d* − *e*) obtained by point-by-point subtraction of the appropriate digitized records are shown at the right. Cell capacitance, 207 pF; initial pipette resistance, 0.6 MΩ. The prominent tail currents in records *B*(*a, b, d, e*) are poorly controlled Na channel currents elicited after hyperpolarizing clamp pulses which removed inactivation: these currents were little altered by strophanthidin, so that strophanthidin-sensitive current tails were much smaller. The amount of Na estimated to enter the cell during those tails is expected to be negligible.

currents seem largely unrelated (but see below) to activity of the Na/K pump because they were recorded in the presence of strophanthidin (*b*, e) as well as in its absence (*a, d*). Thus, with no Na in the pipette, the strophanthidin-sensitive currents (Fig. 1 *B*, *a* − *b*), were approximately zero at all potentials and at all times. With 50 mM $[Na]_{pip}$, on the other hand, there was a substantial strophanthidin-sensitive current (Fig. 1 *B*, *d* − *e*) which, in the steady

state, was ~250 pA in amplitude at both +40 and 0 mV, but ~200 pA at −40 mV (the holding potential, as already described), 165 pA at −60 mV, and only ~90 pA at −100 mV. This voltage dependence of steady-state, strophanthidin-sensitive current is illustrated more clearly by the I-V relationships in Fig. 2.

Note that the steady changes in strophanthidin-sensitive current elicited by the voltage steps from −40 mV (Fig. 1 *B*, *d* − *e*) are all preceded by rapid current relaxations which

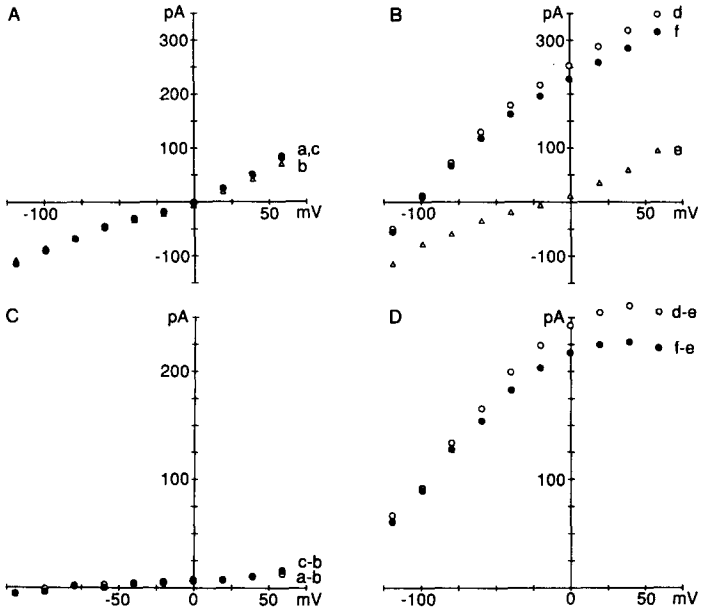

FIGURE 2. Steady-state I-V relationships from the experiment of Fig. 1. In all panels, the letters beside the I-V plots identify the data sampling periods from Fig. 1 *A*. (*A*) Steady levels of whole-cell currents recorded at 0 mM $[Na]_{pip}$ before (○), during (△), and after (●) strophanthidin. (*B*) Whole-cell currents at 50 mM $[Na]_{pip}$, with and without strophanthidin: symbols as in *A*. (*C*) Strophanthidin-sensitive currents at 0 mM $[Na]_{pip}$ determined by subtracting steady current levels in strophanthidin from those obtained before (*a* − *b*, ○) or after (*c* − *b*, ●) strophanthidin. (*D*) Strophanthidin-sensitive currents at 50 mM $[Na]_{pip}$: symbols analogous to those in *C*.

reflect pre–steady-state kinetics of the Na/K pump reaction cycle (see Fig. 4 *B*, inset, and Discussion; cf. Nakao and Gadsby, 1986; Bahinski et al., 1988).

Voltage Dependence of Cardiotonic Steroid-sensitive Current

Fig. 2 shows I-V plots from the experiment of Fig. 1. The upper graphs show steady levels of whole-cell current determined immediately before (○), during (△), and just after (●) the applications of strophanthidin at either 0 mM $[Na]_{pip}$ (Fig. 2 *A*) or 50 mM $[Na]_{pip}$ (Fig. 2 *B*). The lower graphs show the corresponding strophanthidin-sensitive I-V relationships at 0 mM $[Na]_{pip}$ (Fig. 2 *C*) or 50 mM $[Na]_{pip}$ (Fig. 2 *D*) obtained by subtracting steady current levels in strophanthidin from those determined either just before (○) or just after (●) exposure to strophanthidin. With Na-free pipette solution these two subtracted I-V relationships are identical (Fig. 2 *C*),

both showing practically zero strophanthidin-sensitive current at all potentials. The strophanthidin-sensitive I-V relationships at 50 mM $[Na]_{pip}$ (Fig. 2 *D*), however, are both of large amplitude and both have the same shape, the second curve seeming to be a slightly scaled-down version of the first one. If strophanthidin-sensitive current is simply Na/K pump current (an assumption tested below), one interpretation of this result could be that recovery from the strophanthidin was incomplete. Whereas it is difficult to rule out such an explanation in this particular instance, in many cases the holding current recovered more fully on quickly washing out the strophanthidin (see, e.g., Fig. 1 of Gadsby and Nakao, 1986; Figs. 1, 4–6, 9, 11, of Nakao and Gadsby, 1989). Moreover, in most cells a slow decline of holding current occurred over tens of minutes, in the absence of strophanthidin, correlated with a corresponding reduction of strophanthidin-sensitive current. Such phenomena seem to reflect a gradual loss of functional pumps from the cell surface and are described in a later section.

Control Tests for Nonspecific Effects of Strophanthidin and DMSO

The definition of Na/K pump current as cardiotonic steroid-sensitive current requires that, under the specified experimental conditions, neither the cardiotonic steroid nor its vehicle have any direct effect on membrane currents other than that generated by the Na/K pump, and that no other currents change as an indirect consequence of pump inhibition. Although these requirements were shown to hold, at least approximately, for the conditions of our preliminary experiments (Gadsby et al., 1985), we now routinely use >10-fold higher concentrations of strophanthidin, together with the solvent DMSO.

Measurement of the full Na/K pump current signal necessitates complete inhibition of the pump with a maximally effective concentration of cardiotonic steroid. Preliminary dose-response data for strophanthidin were obtained using cells exposed to 5.4 mM K, 150 mM Na external solution, and 33–100 mM Na pipette solution, by determining the relative size of the steady inward shift of holding current at −40 mV caused by a brief application of 20 μM to 1 mM strophanthidin, in comparison with the current shift caused by full pump inhibition either by application of 0.5 mM strophanthidin in K-free external solution (four cells) or complete withdrawal of internal Na (two cells). The combined results can be approximated by a portion of a theoretical curve, assuming that binding of a single steroid molecule fully inhibits one Na/K pump, and they suggest that half-maximal inhibition occurs at ~20 μM strophanthidin at 150 mM $[Na]_o$. Similar preliminary data obtained in five cells exposed to Na-free, 5.4 mM K external solution, and up to 5 mM strophanthidin, suggest that half-maximal pump inhibition requires ~150 μM strophanthidin at zero $[Na]_o$. Ouabain is a more potent inhibitor but washes off much more slowly (cf. Fig. 1 of Gadsby et al., 1985), results from three cells (0.01–100 μM ouabain) indicating half-maximal inhibition by roughly 2 μM ouabain at 150 mM $[Na]_o$. Because of its rapid on and off time courses (e.g. Fig. 1 *A*) we used strophanthidin in all experiments reported here; we used 0.5–2 mM at 150 mM $[Na]_o$, which should provide ~96–99% pump inhibition, and we used 2 mM at $[Na]_o$ levels <150 mM, which should yield ≥93% inhibition.

To attain such high concentrations, strophanthidin was added from a 0.5 M stock

solution in DMSO, resulting in DMSO concentrations up to 0.4% by volume. Fig. 3 *A* shows that even 0.5% DMSO had no effect on steady-state membrane current under the conditions of our experiments. Because the cell of Fig. 3 *A* was exposed to 5.4 mM $[K]_o$ and equilibrated with 50 mM $[Na]_{pip}$, this result implies that DMSO affected neither pump current nor "background" current unless it had exactly offsetting effects on both components. That unlikely possibility is ruled out by the results in Fig. 3 *B* which show that when the pump was first inhibited by exposure to

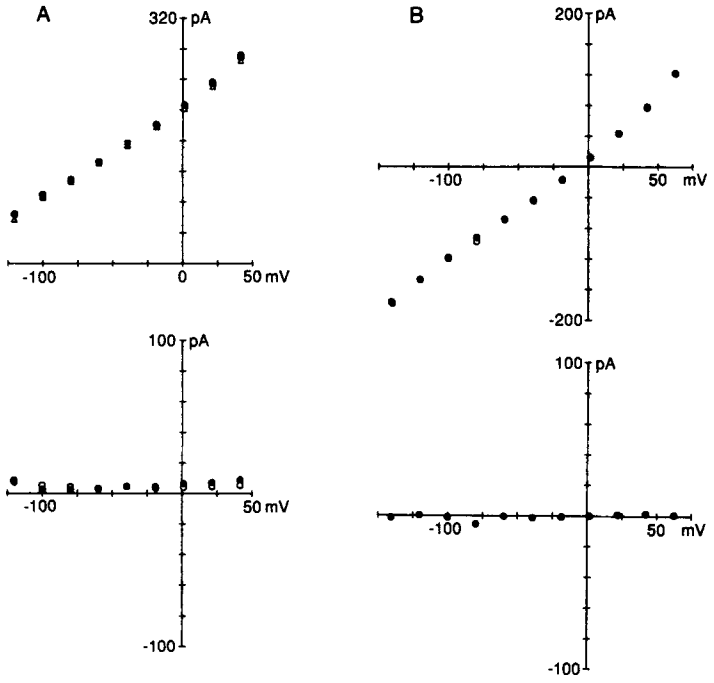

FIGURE 3. Control tests for effects of 0.5% DMSO on whole-cell currents in the presence (*A*) or absence (*B*) of Na/K pump current. (*A*) Upper graph shows steady levels of whole-cell currents before (○), during (△), and after (●) exposure to DMSO at 5.4 mM $[K]_o$, 0 mM $[Na]_o$, and 50 mM $[Na]_{pip}$. Lower graph shows, on an expanded current scale, DMSO-sensitive current obtained by subtracting current levels in DMSO from those before (○) or after (●) DMSO. Cell capacitance, 177 pF; initial pipette resistance, 1.1 MΩ. (*B*) Upper I-V curve shows current levels before (○) and during (●) exposure to DMSO for a cell in K-free, 150 mM Na solution, and equilibrated with 25 mM Na pipette solution. Lower graph shows DMSO-sensitive current on an expanded current scale. Cell capacitance, 155 pF; initial pipette resistance, 1.3 MΩ.

K-free solution, 0.5% DMSO was without effect on the remaining components of membrane current. Any effects of applying up to 2 mM strophanthidin must therefore be attributed solely to the steroid, and not to the accompanying DMSO.

A further question is whether the high concentrations of strophanthidin had any direct effects other than inhibition of the Na/K pump. The I-V relationships in Fig. 4, obtained in two different kinds of experiments, argue strongly that they didn't.

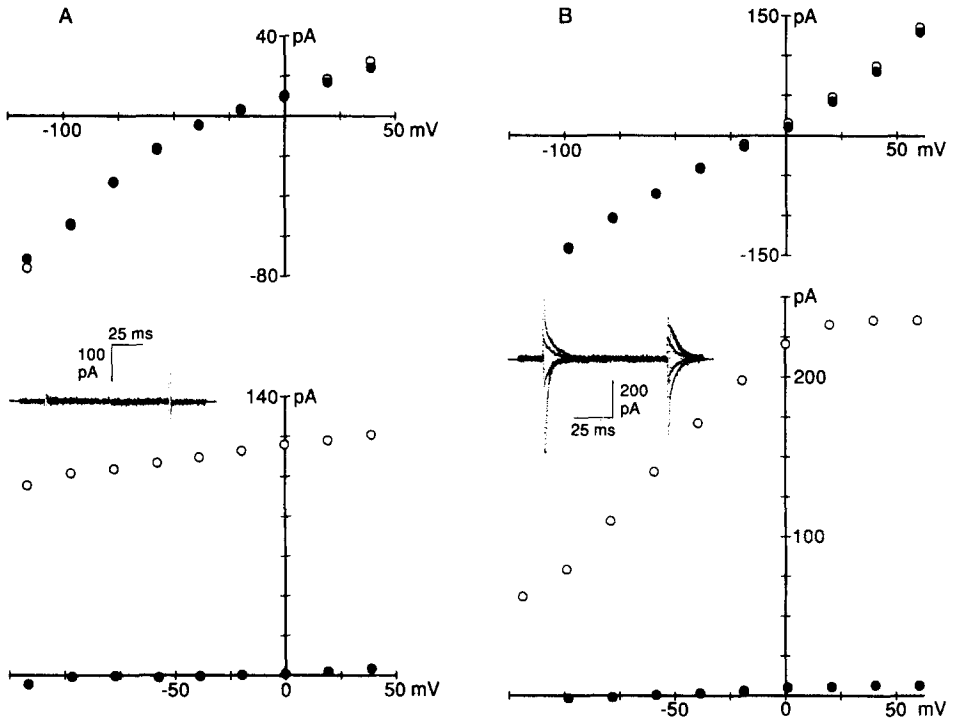

FIGURE 4. Control tests for nonspecific effects of strophanthidin. (*A*) Upper graph shows steady level of whole-cell current in the absence (○) and presence (●) of 2 mM strophanthidin at 5.4 mM $[K]_o$, 0 mM $[Na]_o$, and 0 mM $[Na]_{pip}$, and the lower I-V plot shows the steady levels of the resulting strophanthidin-sensitive current (●). Inset shows superimposed traces of the corresponding strophanthidin-sensitive current for the pulses to +40, 0, −80, and −120 mV from the holding potential of −40 mV. For comparison, the lower graph also shows the level of strophanthidin-sensitive current obtained later in the same cell after raising $[Na]_{pip}$ to 50 mM (○). The weak voltage dependence of that $[Na]_{pip}$-dependent, strophanthidin-sensitive current reflects the weak voltage dependence of the Na/K pump rate in the absence of external Na (see Nakao and Gadsby, 1989). Cell capacitance, 165 pF; initial pipette resistance, 1.1 MΩ. (*B*) Upper I-V plots show steady-state whole-cell currents in the absence (○) or presence (●) of 0.5 mM strophanthidin, at 0 mM $[K]_o$, 150 mM $[Na]_o$, and 50 mM $[Na]_{pip}$. The steady levels of strophanthidin-sensitive current (●), obtained by subtraction, are shown below: the inset shows superimposed traces of the corresponding strophanthidin-sensitive currents for the pulses from 0 to +60, +20, −20, −60, and −100 mV, and although steady currents are practically zero for all pulses, large transient currents at the beginning and end of each pulse demonstrate that voltage steps induce charge displacements mediated by Na-loaded pump molecules when they are prevented from completing the normal Na/K transport cycle by the absence of external K (Nakao and Gadsby, 1986). Again for comparison, the steady-state strophanthidin-sensitive current determined in the same cell at 5.4 mm $[K]_o$ (○) is shown, revealing the characteristic sigmoid voltage dependence of the Na/K pump rate at high external [Na]. Cell capacitance, 218 pF; initial pipette resistance, 1.0 MΩ.

The strategy was to apply strophanthidin under conditions in which the Na/K pump was prevented from generating steady net current, either by removing Na ions from both sides of the cell membrane (but leaving external K) as in the experiment of Fig. 4 *A*, or by withdrawing extracellular K ions (but leaving internal and external Na) as in Fig. 4 *B*. In both cases the upper graphs show the whole-cell I-V curves in the absence (○) and presence (●) of strophanthidin, and the lower graphs show the strophanthidin-sensitive currents (● and insets) obtained by subtraction. For comparison, the lower graphs also show the relatively large strophanthidin-sensitive currents (○) determined in the same two cells after activating the Na/K pump by restoring either 50 mM $[Na]_{pip}$ (Fig. 4 *A*), or 5.4 mM $[K]_o$ (Fig. 4 *B*). These results make it clear that if the pump was first inhibited by other means, subsequent application of 0.5–2 mM strophanthidin had no effect on the remaining components of steady membrane current over the entire voltage range examined. We conclude that under the conditions of our experiments strophanthidin affected steady-state membrane current only by inhibiting the Na/K pump.

The transient, strophanthidin-sensitive currents in the inset of Fig. 4 *B* reflect transient voltage-dependent charge displacements generated by the Na/K pump, which become particularly evident when its normal transport cycle is interrupted by removal of external K ions, and pump activity is thereby largely restricted to Na translocation steps (see Discussion; cf. Nakao and Gadsby, 1986; Bahinski et al., 1988).

Tests for Current Shifts due to Pump-mediated Changes in Ion Concentration

A more subtle problem is that of distinguishing the current change caused directly by pump inhibition from any consequent, indirect current changes. The most likely source of such indirect effects is spatial redistribution of the pumped ions, Na and K, due to the changes in their net fluxes and to the presence of diffusion barriers both inside (pipette tip; cf. Pusch and Neher, 1988; Oliva et al., 1988) and outside (e.g. T-tubular system; cf. Gadsby et al., 1977) the cell. Thus, during vigorous pumping in the forward direction, e.g., at 50 mM $[Na]_{pip}$ and 5.4 mM $[K]_o$, some depletion of K at the external surface of the cell membrane and of Na at its internal surface must be expected, whereas when the pump is stopped, the steady-state concentrations just outside and just inside the cell should closely approximate those in the bath and pipette, respectively (assuming that nonpump net fluxes of Na and K are small). Both extracellular and intracellular equilibration occurred sufficiently rapidly (see Fig. 1 *A*) for that steady state to have been approached during the brief (1–2 min) periods of pump inhibition by strophanthidin, so that changes in pump rate can be presumed to have been accompanied by changes in external [K] and in internal [Na].

We demonstrated previously that steady-state membrane current was rendered practically insensitive to changes in extracellular [K] between 1 and 10 mM, by replacing internal K with Cs, and adding 20 mM TEA to internal solutions and 0.9 mM Ba to external solutions (see Fig. 3 *B* of Gadsby et al., 1985). Even under those conditions, however, lowering $[K]_o$ from 5.4 mM to zero caused small but measurable current changes at large negative potentials (Fig. 5 *A*). Fig. 5 *B* shows that at 5.4 mM $[K]_o$, raising the external [Ba] from 0.9 to 2 mM further reduced steady

inward current at negative voltages, but there was no additional effect at 4 mM [Ba]. In the presence of 0.5 mM strophanthidin and 2 mM Ba, membrane current (Fig. 5 *C*, upper graph) seemed virtually insensitive to changes in $[K]_o$ between 5.4 mM (○) and zero (●). The lower graph in Fig. 5 *C* shows the $[K]_o$-dependent current (●) obtained by subtraction and also shows, for comparison, the $[K]_o$-dependent current

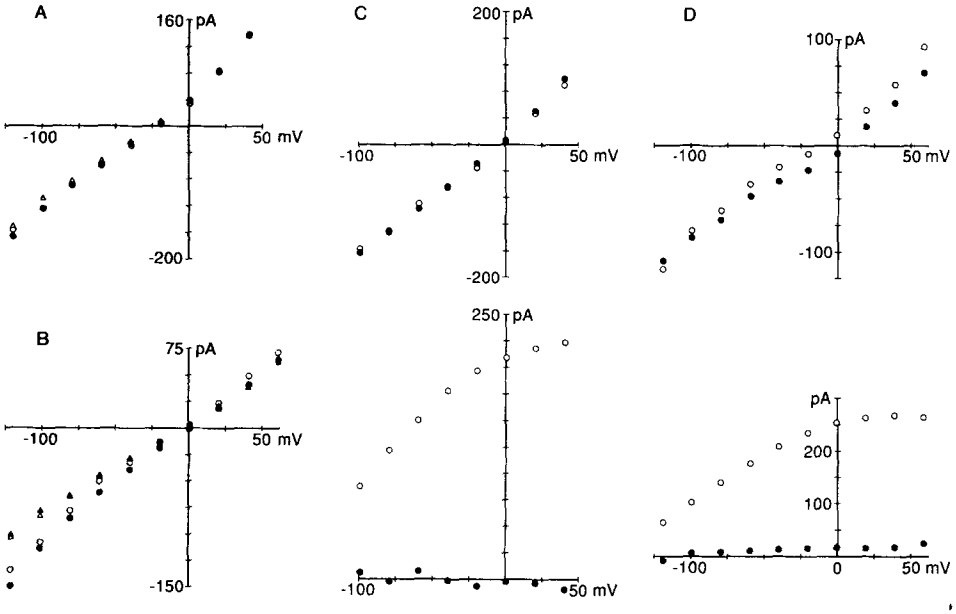

FIGURE 5. Control tests for effects of changes in $[K]_o$ or $[Na]_{pip}$ on nonpump currents. (*A*) Steady-state, whole-cell I-V curves in a cell exposed to 0.9 mM Ba, 2 mM strophanthidin, 0 mM $[Na]_o$, and with 0 mM $[Na]_{pip}$, at 5.4 mM $[K]_o$ before (○) and after (●) switching to 0 mM $[K]_o$ (△). A small $[K]_o$-dependent current is evident at large negative potentials. Cell capacitance, 169 pF; initial pipette resistance, 1.3 MΩ. (*B*) Steady-state, whole-cell I-V curves at 0 mM $[Na]_{pip}$ during exposure to 5.4 mM $[K]_o$, 145 mM $[Na]_o$, and first 0.9 mM Ba (○), then 2 mM Ba (△), 4 mM Ba (▲), and finally 0.9 mM Ba again (●). Cell capacitance, 152 pF; initial pipette resistance, 1.1 MΩ. (*C*) Upper I-V plot shows steady whole-cell currents at 5.4 mM $[K]_o$ (○) and at 0 mM $[K]_o$ (●) in a cell exposed to 2 mM Ba, 0.5 mM strophanthidin, and 150 mM $[Na]_o$, and with 50 mM $[Na]_{pip}$; lower graph shows the resulting $[K]_o$-dependent current (●) determined by subtraction, as well as the $[K]_o$-dependent current in the absence of strophanthidin (○), obtained from the same cell. Cell capacitance, 218 pF; initial pipette resistance, 1.3 MΩ. (*D*) I-V plots of whole-cell current levels (*upper graph*) in the presence of 5.4 mM $[K]_o$, 150 mM $[Na]_o$, and 0.5 mM strophanthidin, at 0 mM $[Na]_{pip}$ (●) and at 50 mM $[Na]_{pip}$ (○). Lower graph shows the corresponding $[Na]_{pip}$-dependent currents (determined by subtraction) in the presence of strophanthidin (●) and, for comparison, the $[Na]_{pip}$-dependent currents obtained from the same cell in the absence of strophanthidin (○). Same cell as in Fig. 1.

from the same cell in the absence of strophanthidin (○). Because the $[K]_o$ at the cell surface must always have been substantially greater than zero during forward pumping and could not have exceeded the bath concentration, 5.4 mM, in the presence of strophanthidin (because the pipette [K] was nominally zero), any contamination

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by $[K]_o$-sensitive current of the Na/K pump I-V relationship determined using strophanthidin (e.g., Fig. 2 *D*), must have been much smaller than the [K]-dependent current shown by the solid circles in the lower graph of Fig. 5 *C* and therefore negligible.

The influence of pump-mediated changes in intracellular [Na] on the background current was probably also small. The I-V relationships in the upper graph of Fig. 5 *D* were obtained in the presence of strophanthidin, at 0 $[Na]_{pip}$ (●) and at 50 mM $[Na]_{pip}$ (○). The difference current is plotted in the lower graph (●) which also shows, for comparison, the current increment caused by the same change in $[Na]_{pip}$, in the same cell, but in the absence of strophanthidin (○). With strophanthidin, raising $[Na]_{pip}$ at the expense of $[Cs]_{pip}$ caused a small outward shift of background membrane current, larger at positive potentials, indicating the existence of some strophanthidin-insensitive current pathway capable of distinguishing between Na and Cs ions. At 50 mM $[Na]_{pip}$, because the internal [Na] at the cell periphery must have been much greater than zero during pumping, the change in that [Na] on inhibiting the pump, and hence the resulting change in background current, must have been much smaller than that in Fig. 5 *D* which (at 0 mV) amounted to only ~6% of the amplitude of Na/K pump current estimated as strophanthidin-sensitive current. In 10 cells, the similarly weakly voltage-dependent current shift elicited by raising $[Na]_{pip}$ from 0 to 50 mM in the presence of strophanthidin amounted to 12 ± 1% of the strophanthidin-sensitive current (at 0 mV) determined in the same cells at 50 mM $[Na]_{pip}$.

We conclude from these control measurements that, under the particular conditions of our experiments, the strophanthidin-sensitive I-V relationship closely approximates the true Na/K pump I-V relationship (probably to within <5%). We shall therefore refer to strophanthidin-sensitive current as Na/K pump current.

Shape of the Steady-state Na/K Pump I-V Relationship

Fig. 6 *A* shows a typical example of the Na/K pump I-V relationship when the pump is strongly activated by 50 mM $[Na]_{pip}$, 10 mM $[ATP]_{pip}$, and 5.4 mM $[K]_o$ (at 150 mM $[Na]_o$). Fig. 6 *B* shows average (±SD) results from six cells. Despite the scatter of the averaged data, it is clear that the pump I-V curve is sigmoid in shape, with the steepest slope occurring between −50 and −100 mV and with less steep regions both at more positive and at more negative potentials. This voltage dependence of outward Na/K pump current cannot be attributed to any voltage dependence of strophanthidin action, by whatever mechanism, because in the experiment of Figs. 1 and 2, for example, a practically identical I-V relationship to that in Fig. 2 *D* (○) was obtained by subtracting currents recorded with Na-free pipette solution from those with 50 mM $[Na]_{pip}$, all in the absence of strophanthidin, as is illustrated in Fig. 5 *D* (lower graph, ○). In addition, subtraction of currents recorded in K-free external solution from those recorded at 5.4 mM $[K]_o$, again in the absence of strophanthidin, yielded analogous I-V curves (e.g., Fig. 5 *C*, lower graph, ○) showing the same strongly voltage-dependent current, which must have been pump current because no significant $[K]_o$-dependent current was seen in the presence of strophanthidin (e.g., Fig. 5 *C*, lower graph, ●). Fig. 6 *C* shows the average data from Fig. 6 *B* replotted after scaling them by 55 s^{-1}, the estimated mean turnover rate at +40 mV (see

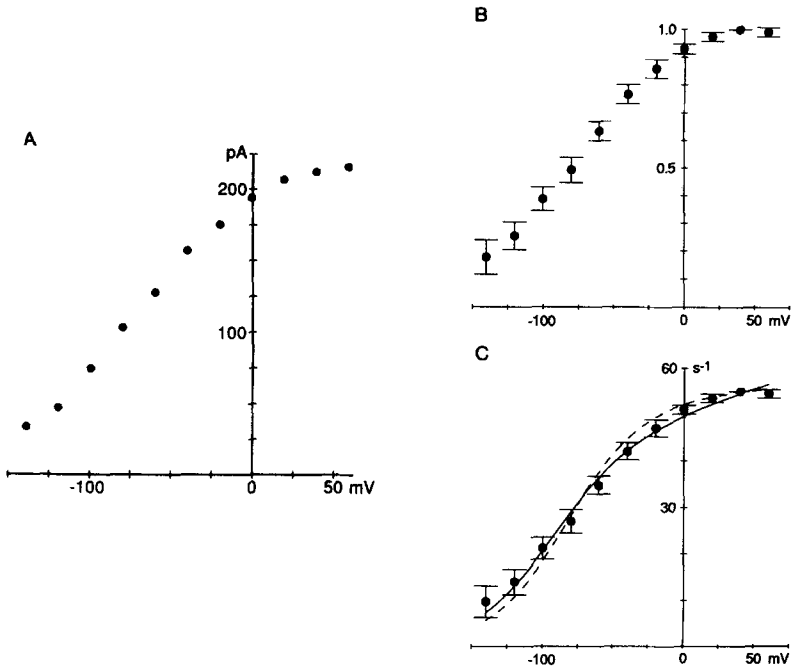


FIGURE 6. Shape of the steady-state Na/K pump I-V relationship. (*A*) Representative result from a single cell at 50 mM $[Na]_{pip}$, 150 mM $[Na]_o$, and 5.4 mM $[K]_o$, obtained by subtracting I-V data recorded after ~30 s of exposure to 0.5 mM strophanthidin from the average of I-V curves determined ~10 s before that exposure and after ~2 min of washout; in those two curves, whole-cell current differed by ≤15 pA over the entire voltage range; cell capacitance, 191 pF; initial pipette resistance, 1.1 MΩ. (*B*) Average (±SD) of steady-state strophanthidin-sensitive I-V relationships determined in six cells under the same conditions as in *A*. To reduce variability due to differences in, e.g., cell size or density of functioning Na/K pumps, the amplitude of strophanthidin-sensitive current at each membrane potential was first normalized with respect to its size at +40 mV for each cell, and the resulting ratios were then averaged over the six cells. Mean (±SD) cell capacitance, 162 ± 30 pF; initial pipette resistance, 1.1 ± 0.2 MΩ. (*C*) Data from *B*, scaled by the mean turnover rate at +40 mV, 55 s^{-1} (estimated from mean current amplitude of 1.1 μA/μF, see Discussion). The curves show two nonlinear least-squares fits (using an iterative technique based on the Gauss-Newton method, implemented in ASYST software; fit tolerance, 0.01%) of the two-state scheme described in the Discussion, which yields a turnover rate = $(\alpha c - \beta d)/(\alpha + \beta + c + d)$, the rate constants c and d being voltage independent while $\alpha = \alpha^0 \cdot \exp[\delta VF/RT]$, and $\beta = \beta^0 \cdot \exp[-(1-\delta)VF/RT]$, where α^0 and β^0 give the values of the voltage-sensitive rate constants at 0 mV. With d fixed at 0, and δ fixed at 0.1 (see text), best-fit values for the parameters α^0, β^0, and c, were 104, 6, and 101 s^{-1} (*solid curve*). Dashed curve shows the best fit to the same data if only β^0 is allowed to vary (yielding β^0 = 24 s^{-1}), with other parameters fixed at α^0 = 490 s^{-1}, c = 61 s^{-1}, d= 0 s^{-1}, and δ = 0.1 (best-fit values from 1.5 mM $[Na]_o$ data of Nakao and Gadsby, 1989).

Discussion). The smooth curves drawn through the points in Fig. 6 *C* show two least-squares fits to a simplified pseudo two-state scheme for the Na/K pump reaction cycle, as discussed below (cf. Hansen et al., 1981; De Weer, 1984; Bahinski et al., 1988).

Outward Na/K pump current appears to approach a maximal level at positive

voltages and, at least up to +60 mV, there was no convincing evidence of a negative slope in the pump I-V curve, of the kind reported for dihydroouabain-sensitive I-V relationships of Na-loaded *Xenopus* oocytes (Lafaire and Schwarz, 1985, 1986; Schweigert et al., 1988). The average pump current at +40 mV was 183 ± 27 pA (±SD) for the six cells of Fig. 6 *B* whose total capacitance averaged 162 ± 12 pF, yielding a mean pump current density of 1.1 ± 0.1 μA/μF.

Apparent "Rundown" of Na/K Pump Current

In most experiments in which the compositions of internal and external solutions were kept constant, we nevertheless observed a gradual and variable decline in the size of the strophanthidin-sensitive current, which occurred over tens of minutes.

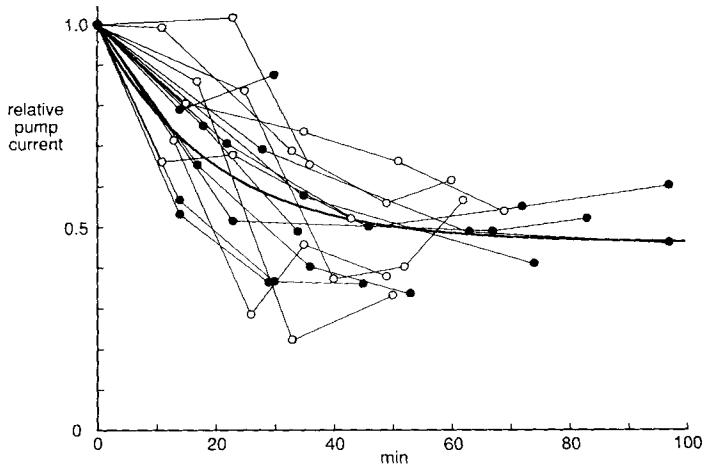


FIGURE 7. Rundown of Na/K pump current during whole-cell recording with wide-tipped pipettes. The points show the relative amplitude of Na/K pump current at 5.4 mM $[K]_o$, 50 mM $[Na]_{pip}$, and at 0 mV, with respect to its initial amplitude when first measured shortly after switching to 50 mM [Na] pipette solution, plotted against lapsed time from that initial measurement. Open circles show results from seven cells at 150 mM $[Na]_o$ and solid circles are from nine cells at 0 mM $[Na]_o$. The bold curve is the best fit (nonlinear, least squares) single exponential to all the points; it has a time constant of 20 min, and eventually decays to 46% of its initial amplitude.

Fig. 7 shows, for 16 cells, the relative amplitude of pump current measured at 0 mV, plotted against the time after its first determination, to give an indication of the time course of this "rundown" and of its variability from cell to cell. The rundown usually seemed incomplete, some 30–60% of the initial pump current persisting after more than an hour. Although the combined data are scattered, the time course of the pump current decline can be reasonably well approximated by an exponential decay to ~50% of its initial value with an average time constant of 20 min (bold curve, Fig. 7).

Fig. 8 shows the effect of this rundown on pump I-V relationships obtained at various times from two cells exposed to 150 mM $[Na]_o$ and 5.4 mM $[K]_o$, and with 50 mM $[Na]_{pip}$. The pump I-V relationships in Fig. 8 *A* were determined at 17, 33, and 67 min, and those in Fig. 8 *B* at 21, 45, and 57 min after breaking into the cell,

and though they vary in amplitude they seem to have the same shape. This apparent scaling effect is confirmed by the normalized plots of the same data (lower graphs), which yield virtually superimposible I-V relationships and so indicate that pump current is changed in amplitude, but not in its voltage dependence, with time during the experiment.

Repeated measurements of the time constant of decay of cell membrane capaci-

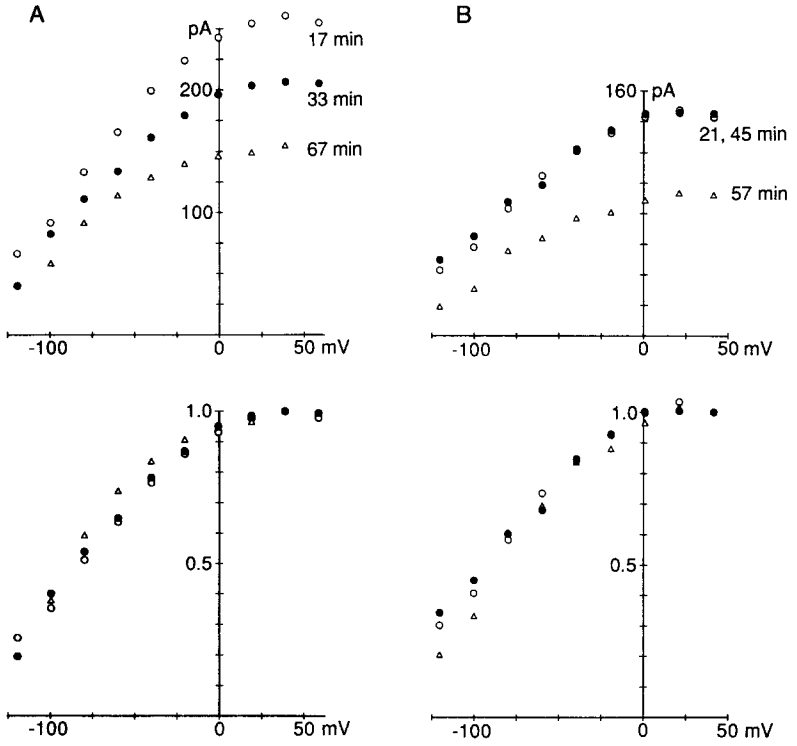


FIGURE 8. Rundown of Na/K pump current is associated with a simple scaling of the steady-state Na/K pump I-V relationship. (*A*) Upper graph shows pump I-V curves determined in the cell of Fig. 1, at 50 mM $[Na]_{pip}$, 150 mM $[Na]_o$, and 5.4 mM $[K]_o$, at the indicated times after rupture of the cell membrane and initiation of cell dialysis. $[Na]_{pip}$ was raised from 0 mM to 50 mM ~2 min (see Fig. 1) before the first determination (○). For each I-V curve, pump current at the various membrane potentials was normalized with respect to its amplitude at +40 mV, and the resulting three normalized pump I-V relationships are shown, using the same symbols, in the lower graph. (*B*) Steady-state pump I-V curves as in *A*, but in the cell of Fig. 3 *A*. $[Na]_{pip}$ was raised from 0 to 50 mM ~7 min before the first pump I-V curve (○) was obtained. Lower graph shows the same I-V curves, but normalized as in *A*.

tance currents, and occasional switches to pipette solutions containing higher concentrations of Na, ATP, or creatine phosphate all suggested that the rundown does not usually reflect progressive obstruction of the pipette tip and hence impaired diffusional delivery of substrates to the Na/K pumps. On the contrary, the effect seemed to be associated with vigorous dialysis of the cell contents and seemed not to occur in intact cells because the initial amplitude of the pump current shortly after

starting intracellular dialysis with high pipette [Na] was always roughly the same (i.e., ~1 μA/μF), irrespective of whether the cell had previously been lying superfused in the chamber for a few minutes or several hours. The simple scaling down of pump current with time, demonstrated in Fig. 8, seems most easily interpreted as indicating a gradual loss of some fraction of functional pump molecules, by some as yet unknown mechanism (see below).

DISCUSSION

Voltage Dependence of Na/K Pump Rate

Over the past three decades, attempts to demonstrate the expected slowing of the Na/K pump on membrane hyperpolarization were mostly unsuccessful, probably for a number of reasons (reviewed in De Weer et al., 1988*a*) including the limited voltage range studied (e.g., Hodgkin and Keynes, 1955; Lederer and Nelson, 1984), possible inclusion of contaminating voltage-dependent currents or fluxes with those attributed to the Na/K pump (e.g., Brinley and Mullins, 1974; Isenberg and Trautwein, 1974; Eisner and Lederer, 1980; Glitsch et al., 1982), or use of Na-free external solution (e.g., Zade-Oppen et al., 1979) which attenuates voltage sensitivity of the pump (see Fig. 4; Béhé and Turin, 1984; Gadsby and Nakao, 1987; Rakowski and Paxson, 1988; Rakowski et al., 1989). When some degree of appropriate voltage sensitivity was found (e.g., Marmor, 1971; Thomas, 1972*b*; Hasuo and Koketsu, 1985), it was not possible to rule out alternative mechanisms, in particular, reduction of external [K] due to an inward shift of passive net K flux during the membrane hyperpolarization. Recent voltage-clamp studies using oocytes (Turin, 1984; Lafaire and Schwarz, 1985, 1986; Eisner et al., 1987; Schweigert et al., 1988; Rakowski and Paxson, 1988), in which possibly contaminating K currents are naturally small (Lafaire and Schwarz, 1986; Schweigert et al., 1988), or squid axons (Rakowski et al., 1989) or cardiac myocytes (Gadsby et al., 1985; Stimers et al., 1986; Mehrke et al., 1987; Glitsch et al., 1989), in some cases during exposure to K channel blockers, have mostly but not always (Glitsch et al., 1987) demonstrated a substantial decline of cardiotonic steroid-sensitive current with hyperpolarization.

If the existence and direction of the voltage sensitivity of outward Na/K pump current are no longer in doubt, the form of that voltage dependence, which is important for guiding the selection of appropriate kinetic schemes for the Na/K pump reaction cycle, remains controversial. The accuracy of the shape of the pump I-V relationships illustrated in Fig. 6 is supported by a number of control experiments, which ruled out nonspecific effects of (*a*) up to 2 mM strophanthidin (Fig. 4) and (*b*) up to 0.5% DMSO (Fig. 3 *B*), and (*c*) showed that background (i.e., non-pump) current was insensitive to changes in external [K] (Fig. 5, *B* and *C*), and that (*d*) a change in internal [Na] much larger than could possibly occur on stopping the pump had a relatively small effect on strophanthidin-insensitive current (Fig. 5 *D*). The voltage dependence of strophanthidin-sensitive current was shown to be that of Na/K pump current and not that of strophanthidin action.

Simultaneous measurements of Na/K pump current and pumped ^{22}Na efflux in voltage-clamped, internally dialyzed squid giant axons have revealed that both measures were similarly voltage dependent, both at high and low $[Na]_o$, demonstrating

that the pump's transport stoichiometry is unaffected by changes in membrane potential (Rakowski et al., 1989). Because the same should hold for cardiac cells (see Gadsby, 1984), the Na/K pump I-V curves presented here should reflect the voltage dependence of the pump turnover rate (see, e.g., Fig. 6 *C*). The simplest interpretation of that roughly sigmoid voltage dependence is that at positive potentials, where the pump rate is approximately constant, a voltage-independent step rate-limits the pump reaction cycle, whereas at moderately negative potentials, a step with voltage-sensitive transition rates either limits the cycle rate directly, or controls the concentration of enzyme intermediate entering the rate-limiting step (e.g., Hansen et al., 1981; De Weer, 1984; Läuger and Apell, 1986; De Weer et al., 1988*a*; Bahinski et al., 1988).

Voltage-sensitive Step(s)

Several lines of evidence presently suggest that Na translocation involves a voltage-dependent step. (*a*) The cycling rate of reconstituted Na/K pumps in membrane vesicles was not accelerated by a positive membrane potential set by ionophores at low [ATP], when K translocation should be rate limiting, but was accelerated at high [ATP] when Na translocation was thought to be "at least partially rate limiting" (Karlish et al., 1985; Goldshlegger et al., 1987). (*b*) With fluorescein-labeled enzyme, a positive potential accelerated a fluorescence change thought to reflect the major conformational transition associated with Na translocation (Rephaeli et al., 1986*b*). (*c*) Voltage steps applied to cardiac cells elicited transient charge movements via the Na/K pump (Nakao and Gadsby, 1986) when it was limited to Na translocation by exposure to zero $[K]_o$ (Fig. 4 *B*, *inset*), and the transients required internal and external Na and ATP. (*d*) Similar Na-dependent transient currents were generated by photolytic release of ATP from caged ATP in the absence of K by Na/K pumps in membrane fragments attached to planar lipid bilayers (Fendler et al., 1985, 1987; Borlinghaus et al., 1987). The consensus from these various experiments is that Na translocation involves net charge movement through the membrane field and so occurs at a rate that is voltage dependent.

An important related question is whether the pump reaction cycle includes more than one voltage-sensitive step. Unequivocal demonstration of a region of negative slope in the pump I-V relationship would provide strong evidence for an additional voltage-dependent step in which net charge movement occurs in the opposite sense, because in that case large voltages that accelerate one of the steps must retard the other (e.g., Chapman et al., 1983; De Weer, 1984, 1986; Lafaire and Schwarz, 1985, 1986; Reynolds et al., 1985; Läuger and Apell, 1986). Negative slope regions of the pump I-V curve have been reported for the backward-running pump in squid giant axon (De Weer and Rakowski, 1984) and the forward-running pump in *Xenopus* oocytes (Lafaire and Schwarz, 1985, 1986; Schweigert et al., 1988). However, more recent measurements in squid axons (De Weer et al., 1988*b*; Rakowski et al., 1988) incorporating additional voltage-clamp circuitry to eliminate stray currents flowing from the axon ends, and K channel blockers to diminish pump-mediated variation of K current, have shown the reverse pump current (like that in cardiac cells; Bahinski et al., 1988) to increase monotonically with hyperpolarization with no evidence of a negative slope (Rakowski et al., 1988). Moreover, although K currents

are generally small in *Xenopus* oocytes, a $[K]_o$-sensitive voltage-dependent outward current exists (Fig. 12 of Lafaire and Schwarz, 1986) which, if augmented by extracellular K accumulation on pump inhibition, would contribute a negative slope to the cardiotonic steroid-sensitive I-V relationship at positive potentials (cf. De Weer et al., 1988*a*). Additional increments of voltage-dependent outward current might arise on pump inhibition if Na accumulates just beneath the cell membrane and enhances, e.g., outward Na/Ca exchange current (probably sensitive to $[Ba]_o$; see Schweigert et al., 1988) and consequently, perhaps, Ca-activated Cl current (Miledi and Parker, 1984). It is worth mentioning that whereas strophanthidin-sensitive current seems to saturate near 0 mV in cardiac myocytes dialyzed with Cs-containing fluid, as described here (e.g., Figs. 2, 4–6, and 8), after switching to K-containing pipette solution a negative slope appears in the strophanthidin-sensitive I-V relationship at positive potentials (Bahinski and Gadsby, 1988). However, under those conditions (in contrast to the results in Fig. 5 *C*), a $[K]_o$-sensitive but strophanthidin-insensitive outward current is seen at potentials more positive than −20 mV, similar to that suggested above to possibly contribute to contamination of the positive limb of steroid-sensitive I-V curves in oocytes. Obviously, the apparent negative slope in the pump I-V curve of oocytes needs cautious reexamination (e.g., Rakowski and Paxson, 1988) because it now constitutes the sole piece of evidence supporting the existence of more than one voltage-sensitive step in the Na/K pump cycle.

The apparent saturation of outward pump current at positive potentials (present experiments) and of inward (reverse) pump current at negative potentials, in both squid axon (De Weer et al., 1988*b*; Rakowski et al., 1988) and cardiac cells (Bahinski et al., 1988), is consistent with the pump cycle including only one voltage-dependent step. Also consistent with that idea is the voltage dependence of the quantity of charge moved during the transient pump currents elicited in the absence of K (e.g., Fig. 4 *B*), which was reasonably well approximated by a Boltzmann distribution appropriate for the movement of a single positive charge across the entire membrane field (Nakao and Gadsby, 1986), sufficient to account for a complete 3Na/2K transport cycle. Further support comes from several recent results indicating that K translocation by the pump is not voltage-sensitive: (*a*) Karlish and co-workers found no effect of an imposed positive potential on Na/K pump rate in vesicles at very low [ATP], when K translocation should have been rate-limiting (Karlish et al., 1985; Goldshlegger et al., 1987); (*b*) they also saw no effect of the positive potential on Rb/Rb exchange (Goldshlegger et al., 1987), and (*c*) with fluorescein-labeled enzyme, the positive potential affected neither the transition rates nor the equilibrium distributions between the E_1K and E_2K conformations (Rephaeli et al., 1986*a*); (*d*) transient pump currents have now been recorded in cardiac cells under conditions of forward Na/K pumping (e.g., Fig. 1 *B*), backward pumping (Bahinski et al., 1988), and Na/Na exchange (Fig. 4 *B*; Nakao and Gadsby, 1986), but not under conditions appropriate for K/K exchange (Bahinski et al., 1988).

Rate Limiting Step(s)

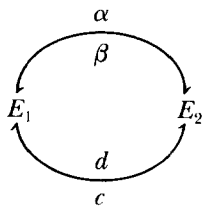
Using a high-speed sampling technique, Forbush (1985) showed that on sudden photolysis of caged ATP within right-side-out kidney membrane vesicles exposed to external K, a transient burst of ^{22}Na efflux preceded its steady-state rate. The result

demonstrated that phosphorylation of the pump, Na translocation, and Na release all precede the rate-limiting step in the Na/K transport cycle. Karlish and Kaplan (1985) arrived at the same conclusion after finding a pre–steady-state burst of ^{22}Na influx into inside-out vesicles at 0°C on suddenly exposing them to ATP. An analogous, transient burst of pump current preceded the establishment of steady-state current across lipid bilayers with attached pumps, when caged ATP was photolyzed in the presence of Na and K (Fendler et al., 1985; Borlinghaus et al., 1987), as expected if Na translocation is obligatorily coupled to charge translocation. Fig. 1 *B* (records *d* – *e*) shows that transient pump currents also preceded attainment of the new steady-state levels of pump current after steps of voltage (see also Bahinski et al., 1988). These results all argue that, under conditions of strong activation by ATP and Na, the voltage-dependent step, i.e., Na translocation (and/or release to the exterior), precedes the rate-limiting step. They therefore lead to the conclusion that the Na/K pump rate is voltage dependent (Fig. 6) not because the rate-limiting step is voltage-sensitive, but because the voltage-sensitive step controls the concentration of the enzyme intermediate entering the rate-limiting step.

An important difference between the transient pump currents elicited by voltage steps (Figs. 1 *B* and 4 *B*) and the transient bursts of current or ^{22}Na efflux initiated by a step of [ATP], is that the transients induced by voltage steps rise virtually instantaneously (within the limits of our measurements, ~1 ms; see Bahinski et al., 1988), whereas the [ATP]-jump transients show a distinct rising phase followed by a three- to eightfold slower falling phase (Forbush, 1984, 1985; Borlinghaus et al., 1987; Apell et al., 1987; Fendler et al., 1987) indicating that, in those cases, both charge translocation and ^{22}Na release are preceded by other steps. This difference is expected, however, because a voltage jump can directly affect only a charge-translocating step, and so an instantaneous current rise is then expected (cf. Hansen et al., 1983).

Kinetic Analysis of the Na/K Pump I-V Relationship

Hansen et al. (198) have shown that for purposes of interpreting steady-state pump I-V relationships, any multistate single cycle containing one voltage-sensitive step can be represented by a reduced pseudo two-state cycle (cf. De Weer, 1984, 1986; Bahinski et al., 1988):



characterized by forward (α) and backward (β) empirical voltage-dependent rate constants, and forward (*c*) and backward (*d*) lumped voltage-independent empirical rate constants which are algebraic combinations of all voltage-independent rate constants of the original cycle. Assuming that one charge is translocated per cycle, and allowing for an asymmetrical Eyring barrier in the charge transit pathway (Nakao

and Gadsby, 1986), the voltage-dependent rate constants are given by $\alpha = \alpha^0 \cdot \exp[\delta VF/RT]$, and $\beta = \beta^0 \cdot \exp[-(1-\delta)VF/RT]$, where α^0 and β^0 are the rate constants at 0 mV, V, F, R, and T have their usual meanings, and δ represents the fraction of the membrane potential that drops between the cytoplasm and the energy barrier to charge transit (cf. Läuger and Apell, 1986). All four empirical rate constants are smaller than the real rate constants of the corresponding multistate cycle by one of two so-called reserve factors (always ≥ 1), which are combinations of the real voltage-independent rate constants and which take account of enzyme sequestered in the multiple voltage-independent reactions (Hansen et al., 1981). Despite its apparent simplicity, such a two-state scheme can easily account for I-V relationships shaped like those in Fig. 6 (Hansen et al. 1981; De Weer, 1984, 1986). Although a complete I-V relationship, from saturating-outward to saturating-inward current, is needed to determine all four empirical rate constants, the conclusion that the Na/K pump cycle includes only a single voltage-dependent step suggests that some limited analysis in terms of the pseudo two-state cycle might be instructive.

The saturating forward pump current at positive potentials is proportional to c, and the current at 0 mV is proportional to $(\alpha^0 c - \beta^0 d)/(\alpha^0 + \beta^0 + c + d)$, the proportionality constants being identical and containing Faraday's constant, the number of charges moved in the voltage-dependent transition, the pump site density, and the product of the two reserve factors (Hansen et al., 1981). But because the pump current amplitude at 0 mV is close to the saturating level (Fig. 6), $c \simeq (\alpha^0 c - \beta^0 d)/(\alpha^0 + \beta^0 c + d)$, so that $\alpha^0 \gg \beta^0 + c + d$, indicating that the voltage-dependent step is not rate limiting, at least near 0 mV. A value of $\sim 60\ s^{-1}$ for c is suggested by the saturating current level at positive potentials (see below), and the highly reversible, voltage-dependent charge movements seen when the pump is limited to Na translocation steps (at zero $[K]_o$; Nakao and Gadsby, 1986) argue that β^0 cannot be negligible. However, the large negative reversal potential for pump current (e.g., Fig. 6) means that the product of the backward rate constants must both be extremely small, because the reversal potential of the cycle is given approximately by $60 \cdot \log_{10}(\beta^0 d/\alpha^0 c)$ mV (Hansen et al., 1981). Thus, although the sigmoid shape of the pump I-V curve warns against extrapolation, Fig. 6 indicates that the reversal potential must be < -180 mV under the conditions of these experiments, so that $\alpha^0 c/\beta^0 d > 10^3$. Therefore, d must be extremely small and is assumed, for present purposes, to be 0 (consistent with the absence of inward Na/K pump currents under any of the conditions of the present experiments; Nakao and Gadsby, 1989). Because, in addition, the decay rates of transient pump currents at zero $[K]_o$ indicate an asymmetrical voltage dependence for α and β, with only $\sim 10\%$ falling on α (cf. De Weer, 1990), we have set $\delta = 0.1$. With those constraints, the best fit values for α^0, β^0, and c, from Fig. 6 *B*, are 104, 6, 101 s^{-1}. That fitted curve, however, shows less tendency to saturate at positive potentials than the pump current generally does (Figs. 2, 4–6, and 8). Moreover, an acceptable fit is also obtained (dashed curve, Fig. 6 *C*) using values of $\alpha^0 = 490\ s^{-1}$, and $c = 61\ s^{-1}$, borrowed from a fit to I-V data at nearly 0 $[Na]_o$ (Fig. 3 in Nakao and Gadsby, 1989): the fit gave $\beta^0 = 24\ s^{-1}$. Evidently, a limited portion of a pump I-V curve, determined under a single set of conditions, yields disappointingly little information in the way of rate constants.

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Nevertheless, the ratio of the forward-to-backward rate constants is apparently large in both voltage-dependent and voltage-independent limbs of the cycle, and this indicates expenditure of considerable energy in both limbs.

Maximum Turnover Rate

From measurements of transient pump currents (e.g., Fig. 4 *B*), the maximum quantity of movable charge is ~20 nC/μF (Nakao and Gadsby, 1986; Bahinski et al., 1988) so that if a single charge (1.6×10^{-19} C) moves per pump, the pump site density is $\sim 12 \times 10^{10}$ sites/μF (equivalent to 1,200 μm^{-2}, assuming membrane capacitance to be 1 $\mu F \cdot cm^{-2}$; cf. Lobaugh and Lieberman, 1987). Because the saturating outward pump current in these experiments is 1.1 $\mu A/\mu F$ (Fig. 6 *B*), the corresponding turnover rate is estimated to be ~55 s^{-1} (cf. Bahinski et al., 1988). In the following paper (Nakao and Gadsby, 1989) we show that this rate is increased by 13% at saturating internal [Na], and by a further 30% at saturating external [K], so that the maximum turnover rate of the Na/K pump in guinea pig myocytes at 36°C appears to be ~81 s^{-1}. This maximum turnover rate is about half that of partially purified Na,K-ATPase from pig kidney (~167 s^{-1} at 37°C; e.g., Jørgensen, 1980) but only slightly lower than a value of 93 s^{-1} recently estimated from pumped K fluxes in Na-loaded, cultured myocytes from chick heart (Lobaugh and Lieberman, 1987).

Rundown of Pump Current

The variable rundown of pump current seems not to be due to inadequate supply of substrates via intracellular dialysis but probably reflects loss of functional pumps, because we have noticed a similar slow decline (during long experiments) in the total quantity of the movable charge associated with transient pump currents in cells exposed to K-free fluid (Nakao, M., and D. C. Gadsby, unpublished observations). In preliminary tests, addition of the protease inhibitors leupeptin or aprotinin to the pipette solution did not prevent the rundown (cf. Chad and Eckert, 1985), although we have no evidence that the inhibitors reached the cytoplasmic face of the cell membrane. Possibly, the process responsible for the rundown might be related to that mediating down-regulation of Na/K pumps in intact cells which, however, has a half time of ~3 h in cultured chick muscle (Lobaugh and Lieberman, 1985; Wolitsky and Fambrough, 1986), or it might reflect speeded degradation of pumps, believed normally to involve endocytosis.

If the rapid outward shift of holding current on suddenly raising $[Na]_{pip}$ (Fig. 1 *A*; time constant, ~20 s) reflects the time course of diffusional exchange of Na ions between the pipette and cell interior (Pusch and Neher, 1988; Oliva et al., 1988), then the much slower time course of rundown (Fig. 7; time constant, ~20 min) might reflect washout of an intracellular component essential for the integrity of about half of the Na/K pumps. If so, that component behaves as if it has an apparent diffusion coefficient roughly 60-fold lower than that of a Na ion, which could indicate (Pusch and Neher, 1988) that the molecule is large, possibly 100–200 kD, and/or subject to intracellular binding.

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