Pre-Steady-State Transient Currents Mediated by the Na/K Pump in Internally Perfused *Xenopus* Oocytes

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ABSTRACT Pre-steady-state transient currents have been investigated in the vegetal pole of *Xenopus* oocytes using the open-oocyte vaseline-gap technique of Taglialatela, Toro, and Stefani (*Biophysical Journal.* 61:78–82, 1992). Voltage pulses 40 ms in duration were made from a holding potential of −40 mV to command potentials over the range −160 to + 60 mV in increments of 20 mV. Current records (averaged 20X; sampled every 200 μs) in the presence of dihydroouabain (DHO) or absence of external Na^+ (Na_o) were subtracted from current records obtained under Na/Na exchange conditions, i.e internally perfused with 50 mM Na^+, 5 mM ATP, and 5 mM ADP (K^+-free) and externally superfused with 100 mM Na^+,K^+-free solution. Transient currents were dependent on intracellular Na^+ and nucleotides, and diminished by activation of forward pumping; they were also reduced by 10 μg ml^{-1} of oligomycin B applied to the external solution. These properties of the pre-steady state currents are consistent with the Na/K pump operating in its electroneutral Na/Na exchange mode. The voltage dependence of the DHO- and Na_o-sensitive transient currents was analyzed using a pseudo two-state model in which only the rate coefficient for Na_o-binding/reocclusion is voltage-dependent (Rakowski, R.F. 1993. *J. Gen. Physiol.* 101:117–144). The apparent valence of the charge moved during the on ($z_{q\text{-}on}$) and off ($z_{q\text{-}off}$) of the pulse were 0.96 ± 0.05 and 0.95 ± 0.05 for Na_o-sensitive, and 1.10 ± 0.07 and 0.85 ± 0.06 for DHO-sensitive transient currents, respectively. The total amount of charge moved (Q_{tot}) and the mid-point voltage of the charge distribution (V_q) were 230 ± 15 pC and −56.2 ± 5.1 mV, and 268 ± 34 pC and −67.0 ± 7.6 mV for Na_o- and DHO-sensitive transient currents, respectively. The apparent valence (z_k) and the voltage at which the forward and backward rates are equal (V_k) obtained from the relaxation rates were 0.80 ± 0.05 and −129.3 ± 10.0 mV, and 0.86 ± 0.10 and −135.1 ± 9.0 mV for the Na_o- and DHO-sensitive pre-steady state currents, respectively. The values of the parameters were not statistically significantly different between the Na_o- and DHO-sensitive transient currents. Excluding the first 600 μs after the onset of a voltage step which was not temporally resolved, transient currents showed no indication of a rising phase. These results support the idea that charge translocation occurs within an external access channel at a rate that is governed by a voltage-dependent binding/reocclusion process and a voltage-independent deocclusion/unbinding process.

INTRODUCTION

Kinetic information about the steps in the Na/K pump cycle that translocate charge can be obtained from current relaxation experiments. The pump, which is initially in a steady state, is perturbed by a sudden change of an external parameter such as substrate concentration or voltage. After the perturbation the occupancy of intermediate states of the enzyme will approach a new steady-state distribution. The pre-steady-state current associated with the relaxation gives information on the charge translocating steps of the pump cycle (Läuger and Apell, 1988; Läuger, 1991).

Nakao and Gadsby (1986) recorded strophanthidin-sensitive transient currents in ventricular myocytes in response to imposed voltage jumps. These transient currents were recorded with the whole-cell patch-clamp technique using wide-tipped pipettes and K^+-free pipette (internal) and external solutions, which should limit the Na/K pump to operate in its Na/Na exchange mode. Depolarizing voltage jumps from a holding potential of −40 mV produced strophanthidin-sensitive transient outward currents, and repolarization elicited inwardly directed transient currents that decayed monoexponentially. The amount of charge translocated (Q), measured as the integral of the transient current, showed a sigmoid voltage dependence that could be described by a Boltzmann equation with a steepness factor appropriate for a single charge moved through the entire membrane field. The relaxation rate (k, the inverse of the exponential time constant) approached a minimum at positive potentials, but increased steeply when the potential was made more negative.

Recently, Rakowski (1993) has studied the voltage dependence of the pre-steady-state charge movement from the endogenous Na/K pump in *Xenopus* oocytes using a two-microelectrode voltage clamp technique. Dihydroouabain (DHO)- and Na_o-sensitive transient currents measured in K^+-free external solutions had the characteristics of membrane charge movement (Chandler et al., 1976): voltage-dependent relaxation rate, saturating sigmoid voltage dependence of the quantity of charge moved, and equality of the charge moved at the on and off of the voltage step. The voltage dependence of charge movement by the Na/K pump in *Xenopus* oocytes was found to be similar to that observed in myocytes by Nakao and Gadsby (1986).

We have studied pre-steady-state charge translocation by the Na/K pump working under Na/Na exchange conditions in *Xenopus* oocytes using the open-oocyte vaseline-gap technique (Taglialatela et al., 1992). With this technique we have been able to explore transient currents 600 μs after the onset

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of the voltage step. Moreover, the technique has allowed us to control the intracellular medium and, therefore, study the intracellular substrate dependence of the charge translocation. In K^+-free solutions, and in the presence of 5 mM ADP, 5 mM ATP, 50 mM Na^+ in the intracellular solution and 100 mM Na^+ in the extracellular solution, a Na_o- or DHO-sensitive component of the transient current could be detected. These currents declined toward zero in the steady-state, and were dependent on the presence of intracellular Na^+ and nucleotides. The transient current was diminished by activation of forward Na/K pumping or addition of 10 μg ml^{-1} oligomycin B. These characteristics strongly suggest that the pre-steady-state currents are associated with the Na/K pump operating in its Na/Na exchange mode. We have found that there is no appreciable difference between the DHO- and Na_o-sensitive transient currents. A two-state model in which only the backward rate coefficient is voltage-dependent fit the experimental data reasonably well. Two findings support the two-state model: (i) transient currents resolved after 600 μs showed no indication of a rising phase, and (ii) the apparent valence of the translocated charge calculated either from the steady-state charge distribution or from the current relaxation rates were in agreement and approximately equal to 1. However, there is a discrepancy between the mid-point voltage V_q measured from the steady-state charge versus voltage relationship and V_k measured from the voltage dependence of the charge relaxation rate that is not predicted by a simple two-state model. The values of V_k are much more negative (~−130 mV) than the corresponding values of V_q (~−60 mV). The two-state model is, therefore, oversimplified.

A preliminary report of these findings has been published (Holmgren and Rakowski, 1993b).

MATERIALS AND METHODS

Preparation of oocytes

Oocyte-positive, adult female African clawed toads (*Xenopus laevis*) were obtained from Xenopus I (Ann Arbor, MI) and were maintained on a high protein diet in fresh water tanks. The animals were anesthetized by immersion in ice for about 30 min until unresponsive to tactile stimulation. Surgery was performed as described by Smith et al. (1991). A small incision is made in the posterolateral side of the ventral part of the animal, and the required amount of ovarian tissue is removed. After suturing, the animals are transferred to a 1-cm deep, cold water container, which is allowed to warm up at room temperature for at least 1 h before returning the animal to its tank. Animals that have undergone surgery are kept in a separate tank. When necessary, animals were killed by decapitation after being anesthetized. Oocytes were isolated by treatment of ovarian tissue for 2–2.5 h with collagenase (type IA; Sigma, St. Louis, MO, 2 mg ml^{-1}) in a modified Ringer solution (Rakowski et al., 1991). Oocytes were incubated at 17°C in either normal Barth or K^+-free Barth solutions (see below) both with 50 μg ml^{-1} of neomycin added. Although the results did not differ depending on the incubation solution, we have found that the oocytes are in better condition and can be used for a longer period after surgery when they are incubated in normal Barth's solution. Oocytes were usually used from 1–4 days after the collagenase treatment.

Solutions for incubation of oocytes

The normal Barth's solution had the following composition (in mM): 88 NaCl, 1 KCl, 2.4 $NaHCO_3$, 0.82 $MgSO_4$, 0.33 $Ca(NO_3)_2$, 0.41 $CaCl_2$, and 5 2-amino-2-hydroxymethylpropane-1,3-diol (TRIS)/4-(2-hydroxymethyl)-1-piperazineethanesulfonic acid (HEPES) (pH 7.1). When needed, KCl was omitted in order to obtain K^+-free Barth solution. Modified Ringer solution contained the following salts (in mM): 87.5 NaCl, 2.5 KCl, 1.0 $MgCl_2$, 5.0 TRIS/HEPES (pH 7.6).

Experimental solutions

100Na 0K external solution had the following composition (in mM): 100 sodium sulfamate, 20 tetraethylammonium sulfamate (TEA), 10 $MgSO_4$, 10 TRIS/HEPES (pH 7.6). 0Na 0K external solution was obtained by equimolar substitution of tetramethylammonium sulfamate for sodium sulfamate. The composition of the internal solution was varied to find optimal conditions. In most of the experiments, the internal solution contained (in mM) 50 sodium sulfamate, 20 TEA sulfamate, 10 $MgSO_4$, 5 MgATP, 5 Tris-ADP, 5 1,2-bis(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid, 30 *N*-methyl-D-glucamine (NMG) sulfamate, 10 TRIS/HEPES (pH 7.3). Na^+-free internal solution was prepared by equimolar substitution of NMG sulfamate for sodium sulfamate. Experiments were performed in K^+- and Cl^--free external and internal solutions to minimize non-pump-mediated currents. Absence of external K^+ also prevents the normal forward mode of operation of the Na/K pump. TEA was present to reduce K-channel-mediated resting and time-dependent currents. ATP and ADP were present to promote electroneutral Na/Na exchange (DeWeer, 1970; Glynn and Hoffman, 1971; Cavieres and Glynn, 1979). Both the internal and external solutions were nominally Ca^{2+}-free to prevent outward Na/Ca exchange current and the activation of Ca^{2+}-dependent anionic current. Finally, the solutions were Cl^--free to reduce chloride current (Miledi and Parker, 1984). Oligomycin B was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 2 mg ml^{-1} and was prepared immediately before each experiment in which it was used. All reagents were purchased from Sigma. The most recent experiments were performed in the presence of 5 mM $Ba(NO_3)_2$, 2 mM $Ni(NO_3)_2$, 10 μM $Gd(NO_3)_3$ in the external solutions. $MgSO_4$ was substituted by magnesium sulfamate. Ba^{2+} and Ni^{2+} were used to block K^+ current and the Na/Ca exchanger (Rakowski, 1993). Gd^{3+} was used to block stretch-activated cation channels (Yang and Sachs, 1989).

Electrophysiological measurements

An open-oocyte, guarded-seal technique (Taglialatela et al., 1992) was used to investigate pre-steady-state charge movement by the endogenous Na/K pump in the vegetal pole of *Xenopus* oocytes. The external compartment is continuously superfused by gravity at a rate of ~2 ml min^{-1} and aspiration by a vacuum pump. Internal perfusion was performed with a cannula connected via polyethylene tubing (0.58 mm I.D.) to a syringe pump (SP100i; World Precision Instruments, Inc., Sarasota, FL) that injects intracellular solution at a rate of 30–50 μl h^{-1}. In some experiments, replacement of intracellular solution was required. In order to minimize the dead space in the perfusion system, a 33G needle ~15-mm long was used in the final section of tubing after a T-junction. At a rate of 50 μl h^{-1}, the new intracellular solution is expected to arrive at the cell ~5 min after the solution up to the T-junction is changed. An additional syringe pump (Sage Instruments; model 355) was used to rapidly clear the solution up to the T-junction and drive the syringe containing the new intracellular solution.

The electronic system was designed by Taglialatela et al. (1992) and purchased from DAGAN Corporation (Minneapolis, MN; Model CA-1 High Performance Oocyte Clamp). The holding potential was usually −40 mV. Voltage pulses 40 ms in duration were made from the holding potential to command potentials over the range −160 to + 60 mV in increments of 20 mV. Pulses were applied every 500 ms. Current records shown below usually were the result of averaging 20 repetitions of the pulse protocol. Data were collected using a commercially available analog to digital converter system (TL-1 DMA interface, 100 KHz; Axon Instruments, Inc., Foster City, CA) and software (PCLAMP version 5.5; Axon Instrument, Inc.) running on an IBM compatible computer system (Dell Computer Corp., Austin, TX). The analog signal was filtered at 5 KHz before being digitized, and was sampled every 200 μs. An improvement in signal-to-noise ratio could have been achieved by using a faster sampling rate or by using a 1-KHz filter cut-off for the sampling rate employed.

Transient currents were calculated by subtraction of the current records obtained after halting Na/Na exchange as described below from those records acquired prior to stopping the exchange. Na/Na exchange was stopped by three different means: 1) direct addition of 5 μl of 20 mM DHO to the extracellular compartment (the DHO concentration attained was ~200 μM) (the DHO stock solution was prepared in 100Na 0K external solution); 2) extracellular superfusion with solution containing 20 μM DHO; 3) extracellular superfusion with Na^+-free solution. DHO is a specific and reversible inhibitor of the Na/K pump in *Xenopus* oocytes (Schweigert et al., 1988; LaTona, 1990). A period of 4–5 min was allowed after DHO addition before data sampling. Only 1–2 min is required to obtain complete block. The charge moved (Q) was determined by direct numerical integration of the subtracted current records. Usually, the integration was performed over 35 ms starting 400–800 μs after the start of the voltage step. The relaxation rate was determined by fitting the transient component of the subtracted current records to a single exponential decay equation as follows: $I_t = I_t(0)\exp(-kt)$, where I_t is transient current, t is the time, $I_t(0)$ is the magnitude of I_t extrapolated to time 0, and k is the exponential relaxation rate. The integrals and the relaxation rates of the transient currents for each voltage step were calculated using the CLAMPAN and CLAMPFIT software modules of PCLAMP. Further analysis, least-square curve fitting, and preparation of figures were done with SIGMAPLOT software version 5.0 (Jandel Scientific, Corte Madera, CA). Curve fit parameters are reported ± SD of the value obtained from the least-squares fitting procedure. Experiments were performed at room temperature (~22°C).

RESULTS

Extracellular Na-sensitive transient current

Fig. 1 *B* shows current records obtained from an oocyte in 100Na 0K external solution and internally perfused with 50 mM Na^+, 5 mM ATP, 5 mM ADP, K^+-free solution: conditions that favor the operation of the electroneutral Na/Na exchange mode of the Na/K pump (Glynn, 1985). The records show the average current elicited by voltage clamp pulses to −160, −120, −80, −40, 0 and +40 mV from a holding potential of −40 mV as diagrammed in Fig. 1 *A*. Fig. 1 *C* shows average current records produced with the same pulse protocol but during superfusion with 0Na 0K solution. The transient component of these records is smaller than the corresponding records in Fig. 1 *B*. Fig. 1 *D* shows the Na_o-sensitive currents obtained by subtraction of current records in the absence of Na^+ (*C*) from those in its presence (*B*). The hyperpolarizing steps resulted in transient inward currents which decayed exponentially to zero. Returning to the holding potential produced transient outward currents with slower relaxation time constants. The depolarizing steps caused transient outward currents and the return steps produced transient inward currents. The absence of steady-state current at any voltage tested confirmed the expectation that there should be no steady-state current generated by the electroneutral Na/Na exchange mode of the Na/K pump.

Voltage-dependence of the Na_o-sensitive transient current

The voltage dependence of the Na_o-sensitive transient current records from Fig. 1 *D* is shown in Fig. 2. The quantity of charge moved during the on (Q_{on}, *open circles*) and off (Q_{off}, *filled circles*) of the pulses are shown in Fig. 2 *A*. The

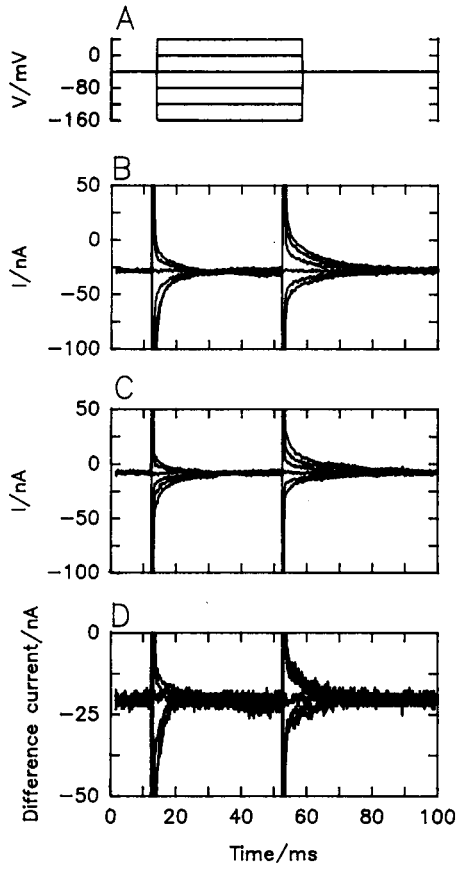

FIGURE 1 Na_o-sensitive transient currents. (*A*) Voltage pulse protocol. The voltage pulses were done in increments of 40 mV from −160 to + 40 mV and are 40 ms in duration. Holding potential −40 mV. (*B*) Current time course in 100Na 0K external solution. (*C*) Current time course in 0Na 0K external solution. Sodium removal reduced the transient current both at the on and off of the pulses. (*D*) Na_o-sensitive current determined by subtraction of records in the absence of sodium (*C*) from those in its presence (*B*). The current declines to zero in the steady-state. Sodium removal typically produced a D.C. offset in the current traces (see *C* and *D*). This probably reflects changes in the liquid junction potential between the 100Na 0K and 0Na 0K external solutions and the agar bridges. Since the electrophoretic mobilities of tetramethylammonium$^+$ and Na^+ are similar, the change in junction potential is expected to be small. The change in bath voltage was measured on changing from 0Na 0K to 100Na 0K solutions and was found to be about 1 mV. Given an oocyte membrane impedance of 50 KΩ of the experimentally controlled area, a 1-mV change in junction potential would be sufficient to explain the ~20 nA current offset.

following equation was fitted to the values of Q_{on}:

$$Q(V) = Q_{min} + Q_{tot}/\{1+ \exp[z_q(V_q - V)F/RT]\}, \quad (1)$$

where Q_{tot} is the total amount of charge moved, z_q represents an apparent valence, V_q is the midpoint potential, V is membrane potential, and F, R, and T have their usual meanings. Fig. 2 *B* shows the voltage dependence of the relaxation rate constants (k) of the transient currents. The value at the holding potential (−40 mV; *filled circle*) is the average of the relaxation rate of the Na_o-sensitive transient current measured at the off of the pulses. The relaxation rate (*solid line*) can be described as the sum of a forward voltage-independent rate constant and a reverse voltage-dependent rate coefficient

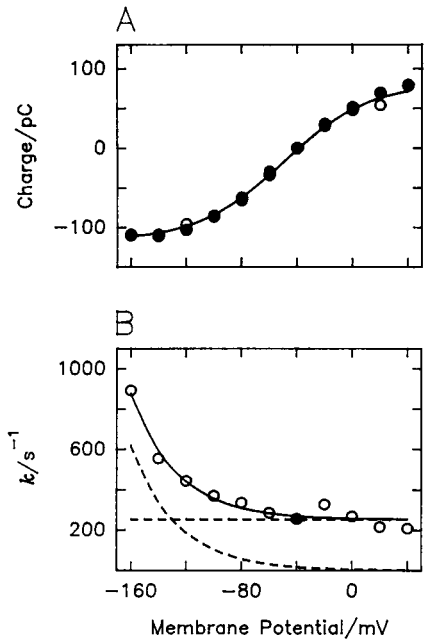

FIGURE 2 Voltage dependence of the Na_o-sensitive transient current. (*A*) Voltage dependence of the charge moved during the on (Q_{on}; *open circles*) and off (Q_{off}; *filled circles*) of the experiment shown in Fig. 1. Filled symbols obscure the open symbols. Q_{on} and Q_{off} were calculated by numerical integration of records in Fig. 1 *D*. The integration started 600 μs after initiating the voltage step, and was terminated at 35 ms. The solid line is a least-square fit of Eq. 1 to Q_{on}. The best-fit parameters are $Q_{min} = -117 \pm 6$ pC, $Q_{tot} = 200 \pm 13$ pC, $z_q = 0.81 \pm 0.10$, and $V_q = -49.9 \pm 3.5$ mV. (*B*) Voltage dependence of the relaxation rates (k). Open circles, calculated from the on transient currents. Filled circle, average obtained from the off transient currents. The SEM is smaller than the radius of the circle. The solid line is a least-square fit of Eq. 2 to the data. The best-fit parameters are $a_k = 252 \pm 2\ s^{-1}$, $z_k = 0.76 \pm 0.11$, and $V_k = -130 \pm 7$ mV. The dashed lines represent the forward and backward rate coefficients from Eq. 2.

(*dashed lines*) according to Eq. 2:

$$k(V) = a_k\{1 + \exp[z_k(V_k - V)F/RT]\}, \quad (2)$$

where a_k is a voltage-independent rate constant, z_k is the apparent valence, and V_k is the value of voltage in which $k = 2a_k$. Equations 1 and 2 are derived from a simple two-state model, which requires that $z_q = z_k$, and that $V_q = V_k$ (Rakowski, 1993). The best-fit values for the parameters are given in the figure legend.

Intracellular substrate dependence of charge translocation

Fig. 3 *A* shows Na_o-sensitive transient currents obtained under normal conditions, i.e., the oocyte was internally perfused with 50 mM Na^+, 5 mM ADP and ATP, and both the internal and external solutions were nominally K^+-free. Removal of intracellular Na^+ diminished the Na_o-sensitive transient currents almost completely (Fig. 3 *B*). Fig. 3 *E* displays the average steady-state charge distribution of two experiments in the presence (*open circles*) and absence (*filled circles*) of intracellular Na^+, respectively. Solid and dashed lines represent the best fit to the data; parameter values are given in the figure legend. The charge moved in the absence of Na^+ is very small making the fit procedure unreliable. Nevertheless, for comparative purposes, we obtained a fit by constraining z_q to be the same as that found under normal conditions. Approximately 86% of the total amount of charge was reduced by intracellular perfusion with Na^+-free solution. Since intracellular Na^+ is a required substrate for Na/Na exchange, its absence is expected to stop the exchange.

Na_o-sensitive transient currents were absent in an experiment that was started without intracellular nucleotides (Fig. 3 *C*). Intracellular perfusion of this same oocyte with a solution containing 5 mM of ATP and 5 mM of ADP resulted in the appearance of Na_o-sensitive transient currents (Fig. 3 *D*). Fig. 3 *F* shows the steady-state charge distribution of Q_{on} in the presence (*open circles*) and absence (*filled circles*) of intracellular nucleotides, respectively. Solid and dashed lines represent the best fit to the data; parameter values are given in the figure legend. The absence of nucleotides abolished ~80% of the charge. Although hydrolysis of ATP is not necessary for Na/Na exchange to occur (Garrahan and Glynn, 1967b), the presence of both ATP and ADP are required (DeWeer, 1970; Glynn and Hoffman, 1971; Cavieres and Glynn, 1979).

Effect of extracellular addition of oligomycin B and K^+

Fig. 4 *A* shows Na_o-sensitive transient currents under normal conditions with the exception that the 100Na 0K and the 0Na 0K external solutions contained 0.5% of DMSO (used to dissolve oligomycin B). Addition of 10 μg ml^{-1} of oligomycin B to the extracellular solutions reduced the Na_o-sensitive transient currents (Fig. 4 *B*). Fig. 4 *E* shows the average steady-state charge distribution under control conditions (*open circles*) and after the addition of oligomycin B (*filled circles*) from this and one additional experiment. Oligomycin B blocked ~80% of the charge translocated. In the absence of K^+, ouabain-sensitive sodium efflux in resealed ghost cells is inhibited 91% by 10 μg ml^{-1} of oligomycin (Garrahan and Glynn, 1967b).

Fig. 4 *C* shows Na_o-sensitive transient currents under normal conditions. Addition of 5 mM of $K^+$to the external solutions resulted in the abolition of the transient currents (Fig. 4 *D*). Fig. 4 *F* displays the average charge distribution from this and two additional experiments in control conditions (*open circles*) and after adding external K^+ (*filled circles*). Addition of external K^+ is expected to activate forward pumping by the Na/K pump, resulting in a reduction of the transient currents and the appearance of steady-state pump current (Bahinski et al., 1988). Averaging the last 15 ms of the traces from Fig. 4 *D* gives a steady-state current versus voltage relationship that saturates at positive potentials at a value of ~5 nA (data not

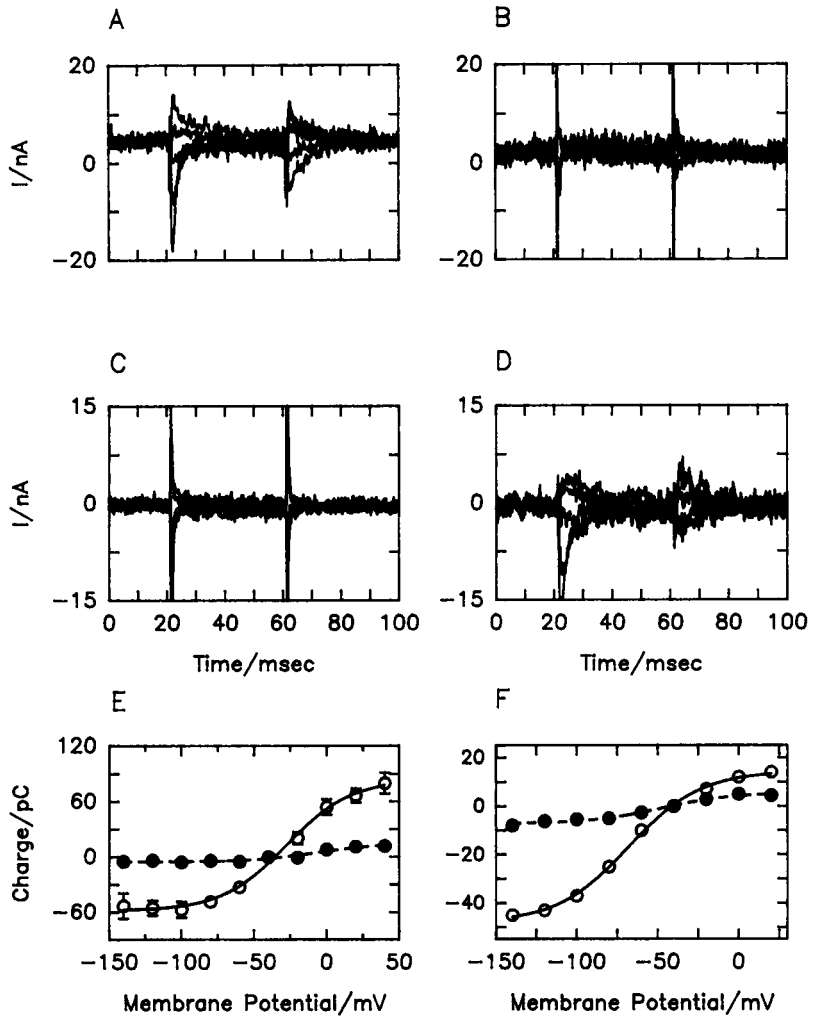

FIGURE 3 Effect of intracellular substrates on charge translocation. (*A*) Na_o-sensitive transient currents in response to voltage pulses in increments of 40 mV from −140 mV to + 20 mV under normal conditions, i.e.: the internal solution contained 50 mM Na^+, 5 mM ATP, and 5 mM ADP, and was K^+-free. (*B*) Na_o-sensitive transient currents after 15 min of internal perfusion with a solution in which sodium sulfamate was replaced by NMG sulfamate. *A* and *B* are from the same oocyte. (*C*) Na_o-sensitive transient currents obtained in the absence of both ATP and ADP. (*D*) Na_o-sensitive transient currents after 30 min of intracellular perfusion with a solution containing 5 mM of ATP and 5 mM of ADP. *C* and *D* are from the same oocyte. All current records shown in this figure are the average of eight runs of the experimental protocol. The data was digitally filtered with a time constant of 0.3 ms after averaging. (*E*) Average steady-state charge distribution from current records of two experiments like the one described in Fig. 3, *A* and *B*. Open circles, charge distribution in the presence of intracellular Na^+. The solid line is a least-square fit of Eq. 1 to the data. The best-fit parameters are Q_{min} = −58.4 ± 5.8 pC, Q_{tot} = 143 ± 15 pC, z_q = 1.14 ± 0.26, and V_q = −27.5 ± 5.4 mV. Filled circles, charge distribution in the absence of intracellular Na^+. The dashed line is a least-square fit of Eq. 1 to the data with the constraint that z_q = 1.14. The best-fit parameters are Q_{min} = −5.3 ± 2.3 pC, Q_{tot} = 20.2 ± 10.3 pC, and V_q = −7.5 ± 26.8 mV. (*F*) Steady-state charge distribution of Q_{on} from current records shown in 3, *C* and *D*. Open circles, charge distribution in the presence of intracellular nucleotides. The solid line is a least-square fit of Eq. 1 to Q_{on}. The best-fit parameters are Q_{min} = −48.1 ± 1.3 pC, Q_{tot} = 62.6 ± 2.0 pC, z_q = 1.14 ± 0.08 and V_q = −67.8 ± 1.5 mV. Filled circles, charge distribution in the absence of intracellular Na^+. The dashed line is a least-square fit of Eq. 1 to Q_{on} in the absence of nucleotides with the constraint that z_q = 1.14. The best-fit parameters are Q_{min} = −7.3 ± 0.7 pC, Q_{tot} = 12.8 ± 1.6 pC, and V_q = −48.0 ± 5.8 mV.

shown). Between 30 and 40 nA of maximal Na/K pump current per oocyte has been previously reported under similar ionic conditions (Rakowski et al., 1991). Since the area of membrane from which we were recording was ~1/5 of the total area of an oocyte, we should expect steady-state Na/K pump currents of the order of 6–8 nA, which is close to the value obtained experimentally. The transient component of the Na/K pump current was diminished by approximately 88% after activation of forward pumping.

Comparison between Na_o-sensitive and DHO-sensitive transient currents

Na_o-sensitive and DHO-sensitive transient currents, measured in the same oocyte, are shown in Figs. 5, *A* and *B*, respectively. At the beginning of the experiment, the cell was superfused with 100Na 0K solution and 20 repetitions of a pulse protocol consisting of voltage clamp pulses, every 20 mV, from −160 to 0 mV from a holding potential of −40 mV, was applied. Subsequently, the cell was superfused with 0Na

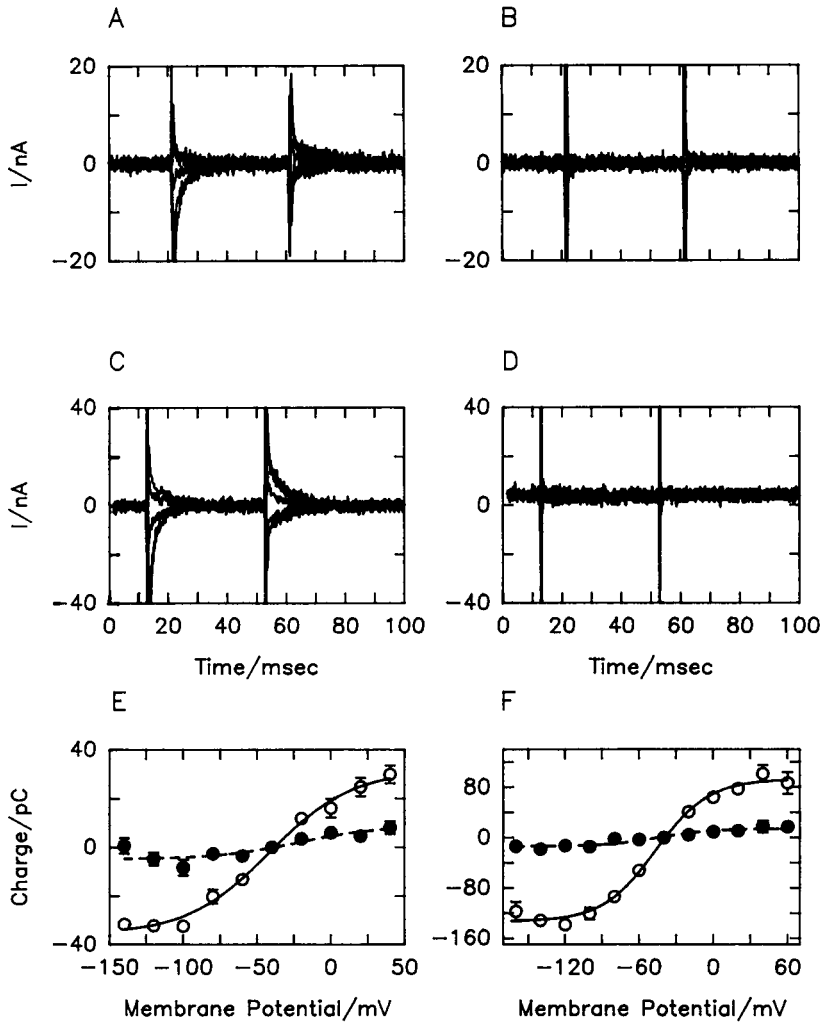

FIGURE 4 Inhibitory effect of oligomycin B and external K^+ on pre-steady-state charge translocation. (*A*) Na_o-sensitive transient currents obtained under normal conditions, but the external solutions contained 0.5% of DMSO. The pulse protocol was the same as described in Fig. 3 *A*. (*B*) Na_o-sensitive transient currents obtained in the presence of 10 μg ml^{-1} of oligomycin B in the extracellular solutions. *A* and *B* are from the same oocyte. (*C*) Normal Na_o-sensitive transient currents in response to the same pulse protocol as described in Fig. 3 *A*. (*D*) DHO-sensitive transient currents in the presence of 5 mM of K^+ in the external solution. *C* and *D* are from the same oocyte. (*E*) Average steady-state charge distribution from current records of two experiments like the one described in Fig. 4, *A* and *B*. Open circles, charge distribution under control conditions. The solid line is a least-square fit of Eq. 1 to the data. The best-fit parameters are Q_{min} = −35.7 ± 2.9 pC, Q_{tot} = 67.4 ± 5.9 pC, z_q = 0.91 ± 0.16, and V_q = −41.5 ± 4.5 mV. Filled circles, charge distribution in the presence of 10 μg ml^{-1} of oligomycin B in the external solutions. The dashed line is a least-square fit of Eq. 1 to the data with the constraint that z_q = 0.91. The best-fit parameters are Q_{min} = −4.9 ± 2.3 pC, Q_{tot} = 13.8 ± 6.7 pC, and V_q = −22.8 ± 25.8 mV. (*F*) Average steady-state charge distribution from current records of three experiments like the one described in Fig. 4, *C* and *D*. Open circles, charge distribution in normal conditions. The solid line is a least-square fit of Eq. 1 to Q_{on}. The best-fit parameters are Q_{min} = −133 ± 6 pC, Q_{tot} = 226 ± 10 pC, z_q = 1.19 ± 0.15, and V_q = −46.5 ± 2.9 mV. Filled circles, charge distribution in the presence of 5 mM of K^+ in the external solutions. The dashed line is a least-square fit of Eq. 1 to the data with the constraint that z_q = 1.19. The best-fit parameters are Q_{min} = −14.0 ± 3.6 pC, Q_{tot} = 28.6 ± 6.3 pC, and V_q = −44.0 ± 14.4 mV.

0K solution and 20 more repetitions of the same pulse protocol were done. The cell was returned to 100Na 0K in order to reactivate the Na/Na exchange mode of the Na/K pump. After 20 additional repetitions of the protocol were performed in this solution, the oocyte was superfused with the same external solution but now containing 20 μM DHO and the protocol was repeated for a final 20 times. Transient current records were obtained by subtraction of average current records in 100Na 0K external solution from: 1) those obtained in the absence of extracellular Na (Fig. 5 *A*), and 2) those obtained after the addition of 20 μM DHO (Fig. 5 *B*). Fig. 5 *C* shows the charge movement associated with the Na_o-sensitive and DHO-sensitive current. Solid and dashed lines represent the best fit to Q_{on} calculated from the Na_o-sensitive and DHO-sensitive transient currents, respectively. The total amount of charge moved as measured from the DHO-sensitive transient current was approximately 20% larger than that determined from the Na_o-sensitive current. This difference is not unreasonable. At the high concentration of DHO used in this experiment (20 μM) we expect a 98% block of the Na/K pump given a K_i of 0.4 μM for inhibition of the Na/K pump by DHO in *Xenopus* oocytes

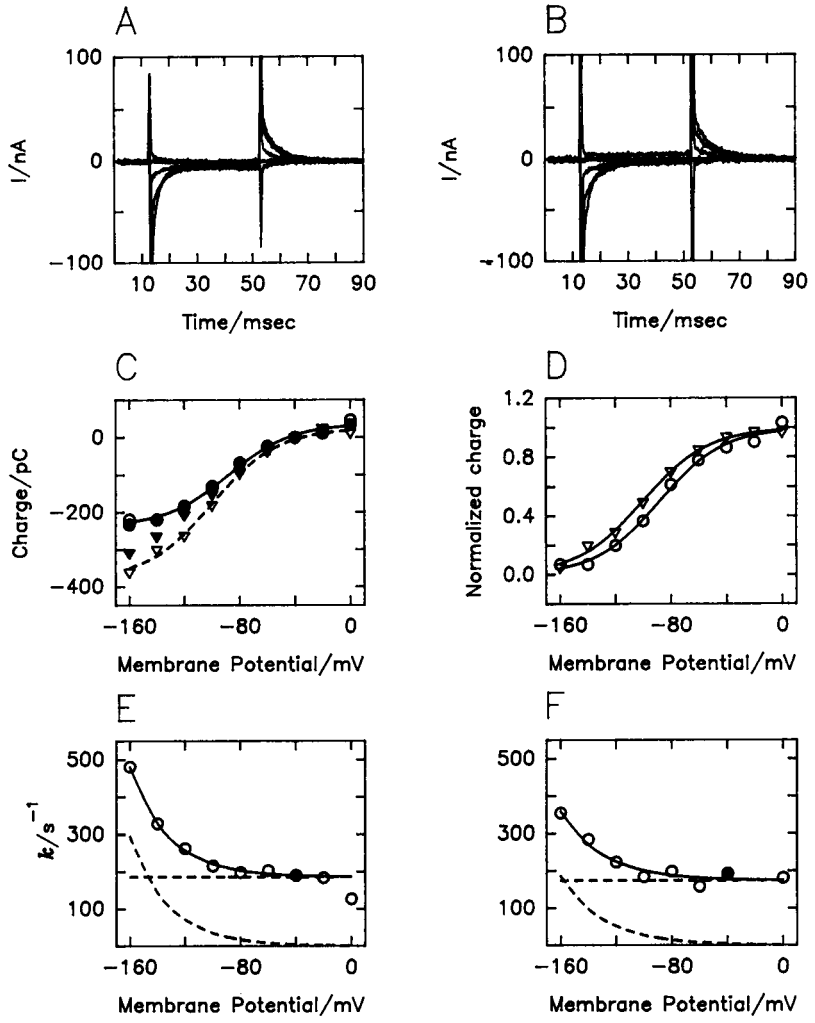

FIGURE 5 Comparison of Na_o-sensitive and DHO-sensitive transient currents. The pulse protocol was similar to that in Fig. 1 except that the range of voltages explored was from −160 to 0 mV. (*A*) Na_o-sensitive transient currents obtained by subtraction of current records in the 0Na 0K solution from those obtained in 100Na 0K solution. (*B*) DHO-sensitive transient currents obtained by subtraction of current records in the presence of DHO from those in its absence. The records in *A* and *B* were obtained from the same oocyte. The Na_o-sensitive transient currents are quite similar to the DHO-sensitive transient currents. (*C*) Voltage dependence of Q. Q_{on} and Q_{off} for the Na_o-sensitive transient currents are given by open and filled circles, respectively. The solid line is a least square fit of Eq. 1 to the data. The best fit parameters are $Q_{min} = -238 \pm 17$ pC, $Q_{tot} = 275 \pm 24$ pC, $z_q = 1.1 \pm 0.2$, and $V_q = -88 \pm 4$ mV. Q_{on} and Q_{off} for the DHO-sensitive transient currents are given by open and filled triangles, respectively. The dashed line is a least square fit of Eq. 1 to the data. The best fit parameters are $Q_{min} = -381 \pm 20$ pC, $Q_{tot} = 406 \pm 26$ pC, $z_q = 1.07 \pm 0.13$, and $V_q = -100 \pm 3$ mV. (*D*) Normalized charge at the on of the pulses versus membrane potential. Open circles, Na_o-sensitive transient data; open triangles, DHO-sensitive transient data. The solid lines through these data represent the best fit to the equation: $\{Q(V) - Q_{min}\}/Q_{tot} = 1/\{1 + \exp[z_q(V_q - V)F/RT]\}$. (*E*) Voltage dependence of k for the Na_o-sensitive transient currents. Open circles, values of k from the on of the pulse. Filled circle, average k of the transient currents from the off. The SEM is smaller than the radius of the circle. The solid line is the best fit of Eq. 2 to the data. The best fit parameters are $a_k = 186 \pm 4\ s^{-1}$, $z_k = 0.90 \pm 0.05$, and $V_k = -147 \pm 1.5$ mV. The dashed lines represent the forward and backward rate coefficients from Eq. 2. (*F*) Voltage dependence of k for the DHO-sensitive transient currents. Open circles, values of k from the on of the pulse. Filled circle, average k of the transient currents from the off. The SEM is smaller than the radius of the circle. The solid line is the best fit of Eq. 2 to the data. The best fit parameters are $a_k = 174 \pm 11\ s^{-1}$, $z_k = 0.82 \pm 0.2$, and $V_k = -158 \pm 5$ mV. The dashed lines represent the forward and backward rate coefficients from Eq. 2. Measurements of the relaxation rate of current records at positive potentials are subject to large errors because the currents are small and the time constant long. For these reasons the value of k at 0 mV in *E* and the value of k at −20 mV in *F* were not included in the curve fitting procedure.

(Schweigert et al., 1988). On the other hand some sodium probably remained in the chamber after a change to Na-free solution due to incomplete Na removal. Normalized values of Q_{on} from Na_o- and DHO-sensitive transient currents are shown in Fig. 5 *D*. Fig. 5, *E* and *F*, show the voltage dependence of k from the Na_o- and DHO-sensitive transient currents, respectively. Fig. 6 is a summary of the voltage dependence of the charge moved and the relaxation rates from Na_o-sensitive ($n = 6$, *A* and *C*) and DHO-sensitive ($n = 5$, *B* and *D*) transient currents.

Table 1 summarizes the mean ± SE of the parameters obtained from the data from individual oocytes to Eqs. 1 and 2. The parameters obtained from Na_o-sensitive and DHO-sensitive transient currents were not statistically significantly different (right-most column). Therefore, it is likely that both signals report the same event. A single factor analysis of

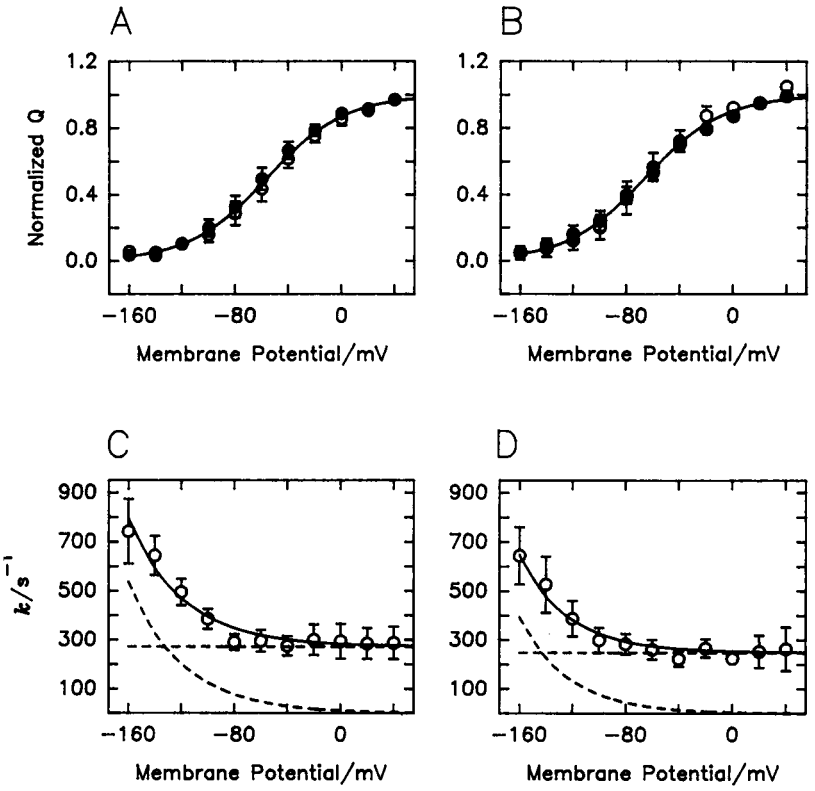

FIGURE 6 Summary of the voltage dependence of pre-steady-state charge translocation. (*A*) Voltage dependence of the normalized charge from Na_o-sensitive transient currents ($n = 6$). (*B*) Membrane potential dependence of the normalized charge from DHO-sensitive transient currents ($n = 5$). Open circles in *A* and *B* are the normalized charge moved at the on and filled circles represent the normalized charge moved at the off of the pulses. Solid lines are the best fit to the data using the normalized version of Eq. 1. The best fit parameters are: (*A*) $z_q = 0.87 \pm 0.05$, $V_q = -56.2 \pm 1.7$ mV; (*B*) $z_q = 0.86 \pm 0.05$, $V_q = -66.6 \pm 2.0$ mV. (*C*) k from Na_o-sensitive currents and (*D*) k from DHO-sensitive currents. Solid lines are the result of the best fit to the data using Eq. 2. The best fit parameters are: (*C*) $a_k = 272 \pm 34\ s^{-1}$, $z_k = 0.62 \pm 0.17$, and $V_k = -132 \pm 13$ mV; (*D*) $a_k = 249 \pm 39\ s^{-1}$, $z_k = 0.69 \pm 0.29$, and $V_k = -143 \pm 14$ mV. Boltzmann fits of averaged data tend to give an understimate of the exponential steepness factor z_k. The mean values obtained from the best fit parameters of the individual experiments are shown in Table 1. The dashed lines in *C* and *D* represent the forward and backward rate coefficients from Eq. 2.

TABLE 1 Comparison of charge movement parameters from Na_o-sensitive and DHO-sensitive experiments

Parameter	Na_o-sensitive ($n = 6$)	DHO-sensitive ($n = 5$)	t test*
$z_{q\text{-on}}$	0.96 ± 0.05	1.10 ± 0.07	$0.1 < p < 0.2$
$z_{q\text{-off}}$	0.95 ± 0.05	0.85 ± 0.06	$0.2 < p < 0.5$
Q_{tot} (pC)	230 ± 15	268 ± 34	$0.2 < p < 0.5$
V_q (mV)	−56.2 ± 5.1	−67.0 ± 7.6	$0.2 < p < 0.5$
a_k (s^{-1})	266 ± 39	228 ± 31	$0.2 < p < 0.5$
z_k	0.80 ± 0.05	0.86 ± 0.10	$p > 0.5$
V_k (mV)	−129.3 ± 10.0	−135.1 ± 9.0	$p > 0.5$

* A two-tailed t test was performed for each parameter. At a significance level of 0.05, all the comparable means between the Na_o-sensitive and DHO-sensitive transient currents are not statistically significantly different.

variance test was performed on z_q(on), z_q(off) and z_k for Na_o-sensitive transient current with the result that the mean of these three parameters were not statistically different ($0.05 < p < 0.10$). Similar results were obtained when the test was performed for DHO-sensitive transient currents.

Comparison of Q_{on} and Q_{off} and calculation of Na/K pump density

Fig. 7 shows Q_{on} vs. Q_{off} measurements for pulses between −160 and + 60 mV for 16 experiments in which the Na/K pump was stopped by any of the three different experimental methods. Within experimental error, equality of Q_{on} and Q_{off} is found for all voltages explored. The mean value of the total amount of charge moved per oocyte for the DHO-sensitive transient currents was 270 ± 30 pC. Given a linear capacity

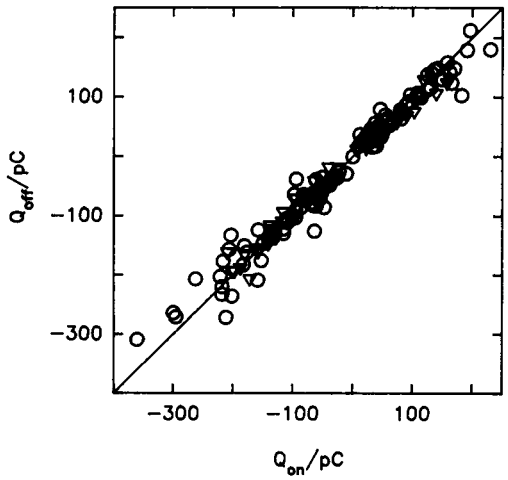

FIGURE 7 Equality of on and off charge movement. Values of Q_{on} versus Q_{off} obtained from pulses between −160 and +60 mV are plotted. Results were obtained from 16 experiments in which Na/Na exchange was stopped by any of the three methods described under Materials and Methods. Open circles, experiments in which the intracellular [Na] was 50 mM (12 experiments). Open triangles, experiments in which the intracellular [Na] was 25 mM (four experiments). The solid line is the theoretical relationship $Q_{on} = Q_{off}$.

of 0.18 μF per oocyte (Vasilets et al., 1990), a specific membrane capacitance of 1 $\mu F/cm^2$, and further assuming that one net charge is moved per pump site and that the area of membrane under experimental control is ~20% of the total membrane area, we find that the Na/K pump site density in the vegetal hemisphere is 460 ± 60 μm^{-2}.

Absence of rising phase in the transient currents

Fig. 8 *A* shows DHO-sensitive transient currents obtained with the on of voltage pulses of 10 ms in duration from −40 mV to command potentials of −100, −80, −60, −20, 0, and 20 mV. Fig. 8 *B* displays the same current records expanded in both the *x* and *y* directions, starting the *x* axis at the time of the onset of the voltage steps, i.e., 1.68 ms. A rising phase was not observed even in transient currents resolved from ~500 μs after the beginning of the voltage step. A fast current component was not temporally resolved (see Discussion).

DISCUSSION

Charge translocation and Na/Na exchange.

Five modes of operation of the Na/K pump are well established (Glynn, 1985): 1) Forward and reverse ATP-driven Na/K exchange, 2) K/K exchange, 3) ATP-driven Na-efflux, 4) ATP-driven Na/Na exchange, and 5) Na/Na exchange without net hydrolysis of ATP. The absence of K^+ in both the internal and external solutions should prevent both modes 1 and 2 of pump operation. Under the conditions used to obtain Na_o-sensitive transient currents, i.e., absence of extracellular Na^+ and K^+, the Na/K pump can catalyze an ATP-driven Na^+ extrusion (Garrahan and Glynn, 1967a; Glynn, 1985). This mode occurs by normal Na^+ efflux, followed by a slow hydrolysis of the phosphoenzyme. The stoichiometry and the turnover rate have been estimated to be ~3 Na^+/1ATP and 3 s^{-1}, respectively (Cornelius, 1989). With 460 pumps μm^{-2} and a total area of ~3.6×10^6 μm^2 (area of the dome under experimental control) we expect a steady-state current mediated by ATP-driven uncoupled sodium efflux between 1.8 and 3.6 nA. The possible presence of uncoupled sodium efflux of this magnitude should not produce a significant difference between Na_o- and DHO-sensitive transient currents.

In most of the experiments, we did not observe steady-state current in either the Na_o- or DHO-sensitive currents (e.g., Figs. 1, 3, 4, and 5). In the absence of K^+ and ADP, but the presence of high Na^+ concentrations, the pump is known to operate in a ATP-driven electrogenic Na/Na exchange mode (probably 3 Na/2 Na) (Lee and Blostein, 1980). Since the oocytes were internally perfused with 5 mM of ADP, we can exclude this mode of operation as a possible contaminant of our results. The conditions of these experiments, i.e., the absence of K^+in the internal and external solutions, the presence of intra- and extracellular Na^+, and the presence of intracellular ATP and ADP, strongly favor the operation of the Na/K pump in its classical 3 Na/3 Na exchange mode without net hydrolysis of ATP (Glynn, 1985) (Fig. 9). As expected for this mode of pump operation, charge translocation is abolished by the lack of ATP and ADP (Fig. 3), intracellular (Fig. 3) or extracellular Na^+ (Fig. 1), and is blocked by DHO (Fig. 5), oligomycin B (Fig. 4) and the activation of forward Na/K pumping (Fig. 4). We conclude, therefore, that the charge translocation studied here originates from the Na/K pump operating in its electroneutral Na/Na exchange mode.

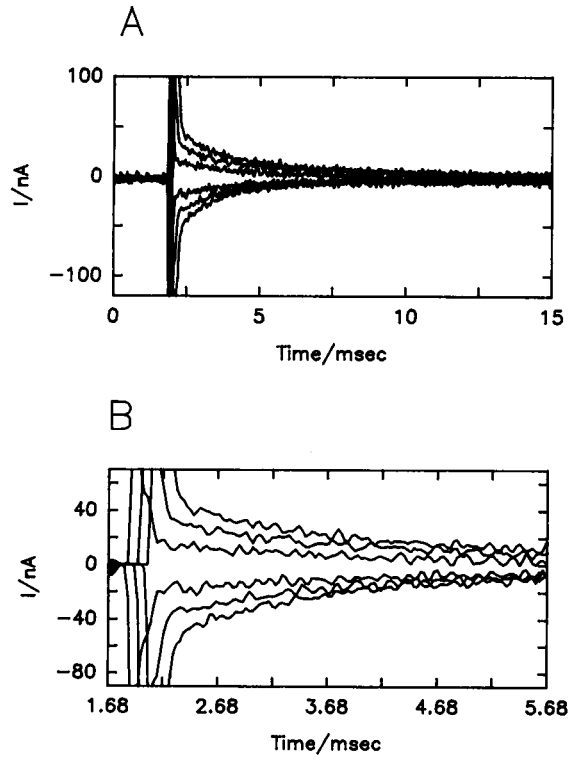

FIGURE 8 Absence of rising phase in DHO-sensitive transient currents. (*A*) DHO-sensitive currents obtained from a holding potential of −40 mV to comand potentials of −100, −80, −60, −20, 0, and 20 mV. The pulses were 10 ms in duration, and the data was sample every 30 μs. (*B*) Same current traces as shown in *A* but expanded in both *x* and *y* axes. The *x* axis was displaced in order to start at the onset of the pulse.

Voltage dependence of the transient current relaxation rate.

Charge translocation was analyzed by a simple model in which the charge is distributed between two states and only the backward rate coefficient is voltage-dependent

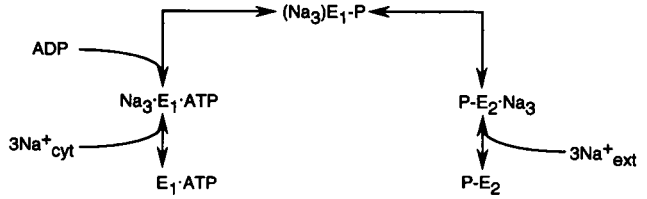

FIGURE 9 Simplified kinetic scheme of Na/Na exchange. In the absence of K^+ and the presence of ADP, ATP, and Na^+, the Na/K pump operates mainly as a three for three Na/Na exchange. The enzyme can assume two major conformational states designated E_1 and E_2. E_1 has ion-binding sites facing the cytoplasm and E_2 has ion-binding sites facing the extracellular medium. When the pump is phosphorylated by ATP in the E_1 state, Na^+ ions become occluded (i.e., trapped inside the protein). After the conformational change to E_2, Na^+ ions are release to the extracellular medium. All reactions are reversible under the conditions required for Na/Na exchange. (Figure modified from Läuger, 1991, p. 178.)

(Rakowski, 1993). This asymmetrical voltage dependence is expected for access channel models of external Na^+ binding (at least at low levels of access channel occupancy) (Gadsby et al., 1993). The data shown in Figs. 2, 5, and 6 support this access channel model in that the relaxation rate appears to become voltage-insensitive as the membrane is depolarized.

Adequacy of a pseudo-two state model

The charge distribution data reported by Nakao and Gadsby (1986) obey a Boltzmann distribution in which approximately a single positive charge is translocated across the entire membrane field. On the other hand, as noted by De Weer (1990), the voltage dependence of k behaves as if the charge traverses only 40% of the entire membrane field. Similarly, Rakowski (1993) found that the apparent valence calculated from the charge distribution (z_q) is close to 1, whereas the apparent valence derived from the current relaxation rate (z_k) was ~0.5. We find that z_q and z_k are not significantly different either for Na_o- or DHO-sensitive transient currents (see Table 1).

The presence of a rising phase in the transient currents would be strong evidence that the charge translocation occurs between more than two states. In *Xenopus* oocytes, previous studies of charge movement associated with the Na/Na exchange were done with a two-microelectrode voltage clamp (Rakowski, 1993). With this technique, the time constants of the voltage steps are usually between 1 and 1.5 ms, limiting the study to events that have relaxation rates slower than about 2 ms. The open-oocyte vaseline-gap technique has allowed us to make voltage steps with time constants as fast as 200 μs. The capacity transient associated with these steps ended at most in 600 μs. No evidence of a rising phase has been observed for times longer than this temporal resolution (Fig. 8). The fast component of the DHO-sensitive transient current detected in Fig. 8 could reflect equilibration of ions within an external access channel, however, this current component was not temporally resolved.

There is one major inconsistency between our data and the predictions of a simple pseudo two-state model. The value of V_q, which corresponds to the voltage at which the forward and backward rate coefficients are equal, should be the same as V_k. This discrepancy indicates that the two-state model is oversimplified. Our values of V_k (~−130 mV) are usually much more negative than the corresponding values of V_q (−60 mV). With this exception a simple two-state model describes the pre-steady-state current reasonably well between membrane potentials of −140 and +60 mV. Multistate models predict that k should become a saturating sigmoid function of membrane potential. In order to expand the experimentally accessible range of voltage, solutions containing niflumic acid (300 μM) to block chloride channels (Kubo et al., 1993) and gadolinium (10 μM) to block stretch-activated cation channels (Yang and Sachs, 1989) are presently being used. Hilgemann (personal communication), using a giant patch technique, has reported that k saturates at extreme negative voltages.

Comparison of Na_o-sensitive and DHO-sensitive transient currents

Rakowski (1993) found no difference between Na_o-sensitive and DHO-sensitive transient currents. Our results support those findings (see Table 1). This similarity is not unexpected since both subtractions should have similar characteristics if the same charge translocating steps have been isolated by the experimental design.

Comparison of parameter values with previous measurements

At an extracellular [Na^+] of 90 mM, Rakowski (1993) found that z_q was close to 1, V_q was ~−40 mV, z_k was ~0.5, a_k was ~100 s^{-1}, and V_k was ~−50 mV. We found that z_q and z_k were both close to 1, V_q was ~−60 mV, a_k was ~250 s^{-1}, and V_k was ~−130 mV.

The fact that we performed our experiments in the nominal absence of internal and external K^+ may have contributed to have faster a_k. Rakowski (1993) did not have access to the intracellular medium, so his experiments were done in the presence of intracellular K^+. Intracellular K^+ may displace some of the enzyme from the E_1·ATP state to preceding potassium-associated states. If those states relax to the E_1·ATP state slowly, then the value of the overall charge translocation rate would be slower in the presence of internal K^+. Our values of a_k are closer to those estimated from guinea pig ventricular myocytes (Nakao and Gadsby, 1986) but, those experiments were performed at 37°C.

The value of V_q we obtained is ~20 mV and ~40 mV more negative than that reported by Rakowski (1993) in *Xenopus* oocytes and Nakao and Gadsby (1986) in cardiac myocytes, respectively. Since mid-point voltages may be affected by such factors as surface charge density, it is probably not worthwhile to speculate about differences that may occur between species. However, it may be worthwhile to compare data obtained from *Xenopus* oocytes further. The Q vs. V relationship is known to shift toward positive potentials with increasing $[Na]_o$ (Rakowski, 1993). If the Q vs. V relationship is similarly affected by internal [Na], this may explain the quantitative difference between the results. Our values of V_k are also more negative than the values obtained by Rakowski (1993).

Comparison of Q_{on} and Q_{off} and calculation of Na/K pump density

Within experimental error, equality of Q_{on} and Q_{off} was found for all voltages explored. This is in good agreement with our results in *Xenopus* oocytes using the two-microelectrode voltage clamp technique (Holmgren and Rakowski, 1993a). Approximate equality of Q_{on} and Q_{off} was found for all voltages and was independent of holding potential in the range −100 to 0 mV. The previous discrepancies between Q_{on} and Q_{off} (Rakowski, 1993) were eliminated when the current integral was calculated by direct integration rather than the extrapolation method originally used.

The Na/K pump site density in the vegetal hemisphere was found in Results to be 460 ± 60 μm^{-2}. If *Xenopus* oocytes have ~2/3 of their Na/K pumps in the vegetal pole (W. Schwarz, personal communication) and assuming that the area of the vegetal and animal poles are equal, our estimation of pump density corresponds to ~340 pumps μm^{-2} if the pumps were distributed homogeneously over the cell surface. This result is in good agreement with values of 270–420 pumps μm^{-2} obtained from measurements of transient currents of the whole oocyte with the two-microelectrode voltage clamp technique (Rakowski, 1993) and values of 330–360 ouabain binding sites μm^{-2} (Vasilets et al., 1991).

Substrate dependence and origin of the charge translocation

The charge translocation studied here appears to result from the functioning of the Na/K pump in its electroneutral Na/Na exchange mode, since it depends on the presence of intracellular Na^+ and nucleotides, and extracellular Na^+. Oligomycin B is known to block the spontaneous change from $(Na)_3E_1$-P to P-$E_2 \cdot Na_3$ (Glynn, 1993) (see Fig. 9). Oligomycin B also blocked Na_o-sensitive transient currents, suggesting that charge translocation requires that the deocclusion/occlusion reaction occur. Nakao and Gadsby (1986) found that charge translocation in myocytes can occur in the nominal absence of internal ADP, which would force the pump to operate in the right-hand half of Fig. 9. Measurement of oligomycin B-sensitive transient currents could be a useful experimental tool. If oligomycin-B sensitive transient currents have the same characteristics as Na_o- and DHO-sensitive transient currents, this would confirm the suggestion of Nakao and Gadsby (1986) that only the deocclusion/occlusion reaction makes a kinetic contribution to the current relaxation rate.

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