J. Membrane Biol. 198, 65–76 (2004)
DOI: 10.1007/s00232-004-0660-4

The Journal of
Membrane
Biology

Effect of Extracellular pH on Presteady-State and Steady-State Current Mediated by the Na^+/K^+ Pump

A. Vasilyev[1], **K. Khater**[1], **R.F. Rakowski**[2]
[1]Department of Physiology and Biophysics, Rosalind Franklin University of Medicine and Science/The Chicago Medical School, North Chicago, IL, 60064, USA
[2]Department of Biological Sciences, Ohio University, Athens, OH 45701, USA

Received: 4 September 2003/Revised: 10 January 2004

Abstract. A ouabain sensitive inward current occurs in *Xenopus* oocytes in Na^+ and K^+ -free solutions. Several laboratories have investigated the properties of this current and suggested that acidic extracellular pH (pH_o) produces a conducting pathway through the Na^+/K^+ pump that is permeable to H^+ and blocked by $[Na^+]_o$. An alternative suggestion is that the current is mediated by an electrogenic H^+-ATPase. Here we investigate the effect of pH_o and $[Na^+]_o$ on both transient and steady-state ouabain-sensitive current. At alkaline or neutral pH_o the relaxation rate of pre-steady-state current is an exponential function of voltage. Its U-shaped voltage dependence becomes apparent at acidic pH_o, as predicted by a model in which protonation of the Na^+/K^+ pump reduces the energy barrier between the internal solution and the Na^+ occluded state. The model also predicts that acidic pH_o increases steady-state current leak through the pump. The apparent pK of the titratable group(s) is ~6, suggesting that histidine is involved in induction of the conductance pathway. ^{22}Na efflux experiments in squid giant axon and current measurements in oocytes at acidic pH_o suggest that both Na^+ and H^+ are permeant. The acid-induced inward current is reduced by high $[Na^+]_o$, consistent with block by Na^+. A least squares analysis predicts that H^+ is four orders of magnitude more permeant than Na^+, and that block occurs when 3 Na^+ ions occupy a low affinity binding site ($K_{0.5}$ = 130 ± 30 mM) with a dielectric coefficient of 0.23 ± 0.03. These data support the conclusion that the ouabain-sensitive conducting pathway is a result of passive leak of both Na^+ and H^+ through the Na^+/K^+ pump.

Correspondence to: R.F.Rakowski; email: rakowski@ohio.edu

Key words: Na^+, K^+-ATPase — Ion well — Access channel — Voltage dependence — ABC transporter — Pre-steady state transient current

Introduction

Evidence for an Extracellular Ion Well for Na^+ Binding from Forward Pumping, Na^+/Na^+ Exchange and Transient Current Measurements

The hypothesis that a high field access channel (ion well) connects the extracellular solution and the extracellular Na^+ binding sites of the Na^+/K^+ pump has been proposed based on the reduction of forward-going (outward) pump current by hyperpolarization (Gadsby & Nakao; 1989, Rakowski et al., 1991). Strong evidence supporting this hypothesis is provided by the observation that the voltage dependence of electroneutral Na^+/Na^+ exchange by the sodium pump across the plasma membrane of the squid giant axon measured as ouabain-sensitive $^{22}Na^+$ efflux is a saturating sigmoid function of membrane voltage whose mid-point is shifted by changes in extracellular $[Na^+]$ (Gadsby, Rakowski, & De Weer, 1993). The existence of a negative slope in the pump current-voltage (*I-V*) relationship of *Xenopus* oocytes (Rakowski et al., 1991) suggested that K^+ also binds to sites within an extracellular ion well. The steady state *I-V* relationship in *Xenopus* oocytes under a variety of conditions can be described by a kinetic model in which both Na^+ and K^+ bind within narrow access channels (Sagar & Rakowski, 1994). Gadsby et al. (1992) showed that in conditions favoring electroneutral Na^+/Na^+ exchange ouabain-sensitive pre-steady-state currents are observed that can be ascribed to the release of extracellular Na^+ within the postulated extracellular

ion well. The current relaxation rate increases with hyperpolarization and is a monoexponential function of membrane potential consistent with the existence of a high field access channel for extracellular Na^+ (Gadsby et. al., 1992; Rakowski, 1993; Holmgren & Rakowski 1994).

A Novel Ouabain-sensitive Current Measured in the Absence of Extracellular Na^+ and K^+

While conducting studies of ouabain-sensitive forward pump current in oocytes, a novel ouabain sensitive current was found by Rakowski et al. (1991). The current was novel because it was observed in external Na^+- and K^+-free solutions and so could not be associated with any known mode of Na^+ or K^+ transport by the sodium pump. The current was inwardly directed and increased in magnitude with hyperpolarization. It was suggested that protons carried the current. This current was further investigated by Efthymiadis, Rettinger & Schwarz (1993) who showed that the inward current increased at low pH_o, depended on the presence of internal ATP and was not affected when internal K^+ was replaced with Na^+. It was also inhibited by external Mg^{2+} and showed a biphasic dependence on $[Na^+]_o$: it increased in magnitude as $[Na^+]_o$ was raised from 0 to about 10 mM and decreased upon further increasing $[Na^+]_o$. The authors postulated the existence of a ouabain-sensitive conducting pore. Wang & Horisberger (1995) provided evidence that protons moved through this pore passively. They showed that a ouabain-sensitive, time-dependent decrease in cytoplasmic pH occurred after extracellular acidification. The reversal potential of the inward current shifted with changes in pH_o, as predicted by the Nernst equation. They also observed biphasic dependence of the current on external Na^+ and suggested that K^+ was also an extracellular inhibitor of the current. Rettinger (1996) observed a similar current in giant patch studies of *Xenopus* oocyte membrane. His conclusion (based on supra-Nernstian shift of the reversal potential) was that the pump operates as a proton transporter at low extracellular pH and uses intracellular ATP to pump protons into the cell. In summary, previous research has described an unusual current presumably mediated by the sodium pump (because it is ouabain-sensitive) but that is not seen when either extracellular Na^+ or K^+ are present at their physiological concentrations. Most studies agree that this current is carried (at least in part) by protons and is inhibited by extracellular cations. On the other hand, the underlying mechanism of the current remains unclear. Proposed interpretations are a passive proton leak or active pumping of protons.

In the present work we investigate the effect of low pH_o on pre-steady-state and steady-state ouabain-sensitive currents in *Xenopus* oocytes and on simultaneous net current and unidirectional $^{22}Na^+$ efflux measurements in squid giant axon. The model we propose to explain these results postulates the existence of a conducting pathway (leak) through the Na^+/K^+ pump that is induced by lowering of the activation energy for ion translocation across an internal occlusion gate (equivalent to an increase in open probability for this postulated gate). The conductance pathway induced by acidic pH_o, is permeable to both H^+ and Na^+ and blocked by high $[Na^+]_o$.

Materials and Methods

Oocytes

Stage V–VI oocytes were obtained from adult African clawed frogs (*Xenopus laevis*, Nasco, LaCrosse, WI). Oocytes were treated for two hours with 2 mg/ml type 1A collagenase (Sigma, St.Louis, MO) dissolved in Ca^{2+} -free oocyte Ringer solution (in mM); 87.5 NaCl, 2.5 KCl, 1.0 $MgCl_2$, 5.0 Tris/HEPES, pH = 7.6. The collagenase-treated oocytes were manually defolliculated and kept at 4°C in normal Barth's saline solution (in mM): 87 NaCl, 3 KCl, 1 $MgCl_2$, 5 Tris/HEPES, pH = 7.6, 50 units/ml Penicillin, 50 mg/ml streptomycin. Oocytes were used within four days after defolliculation.

Pre-Steady-state Transient Pump Current Measurements

The cut-open oocyte technique was used to study transient pump currents (Taglialatela, Toro & Stefani, 1992). The voltage clamp was a CA-1 High Performance Oocyte Clamp (Dagan, Minneapolis, MN) and sampling was done with a 100 kHz TL1 DMA interface AD/DA converter (Axon Instruments, Burlingame, CA). Pclamp6 software was used to collect data and to perform the initial analysis. The composition of the internal solution was (in mM): 5 MgATP, 5 TrisADP, 5 BAPTA, 10 $MgSO_4$, 50 Na sulfamate, 20 TEA sulfamate, 10 Tris/HEPES, 30 N-methyl D-glucamine (NMG) sulfamate. The external solution contained (in mM) 100 Na sulfamate, 20 tetraethylammonium (TEA) sulfamate, 2 $Ni(NO_3)_2$, 5 $Ba(NO3)_2$, 3 Mg sulfamate, 10 μM $Gd(NO_3)_3$. The buffers were 20 MES (pH_o 4.6), 20 HEPES (pH_o 5.6–6.6), 10 mM Tris/HEPES (pH_o 7.6), or 20 mM TRIS (pH_o 8.6).

Steady-State Ouabain-sensitive Current Induced by Acidic pH_o

A standard two-microelectrode voltage-clamp technique (Warner Instruments, OC-725) was used to measure steady-state pump currents (Sagar & Rakowski 1994). The holding potential was −40 mV unless otherwise stated. Steady-state *I–V* relationships were measured over the range −100 to +20 mV in staircase voltage steps of 500 ms duration. The data were filtered at 1 kHz, sampled using a TL-1 AD converter (Axon) and software written in Quick Basic (Microsoft Corp., Redmond, WA). The composition of experimental solution for such experiments was (in mM) 20 or 95 or 120 TMA sulfamate, 20 TEA sulfamate, 100 or 25 or 0 Na sulfamate, 10 Mg sulfamate, 20 HEPES, pH 5.6. To increase the intracellular Na^+ concentration, oocytes were incubated for

30 min before the start of each experiment in solution containing (in mM) 90 Na sulfamate, 5 Tris/HEPES, 2.4 Na citrate (Rakowski et al., 1991).

Simultaneous Current and Flux Measurements in Squid Giant Axon

For simultaneous current and flux measurements we used axons from the hindmost stellar nerves of the squid *Loligo peali*. The experimental chamber was modified from the design of Brinley & Mullins (1967). A cellulose acetate capillary (Fabric Research Corp., Dedham, MA) made porous to small (< 1 kD) molecules by 20 hour soaking in 0.1 N NaOH was introduced inside the axon for internal dialysis. The membrane potential was measured and controlled by means of an internal current-passing wire, a voltage-sensing electrode inserted along the perfusion capillary, and an external reference electrode. The uniformity of the membrane field was ensured by means of two guard chambers that held the end pools at the same potential as the central recording chamber. A voltage-clamp system of special design (Rakowski, 1989; Rakowski, Gadsby & De Weer, 1989) was used to voltage-clamp the axon and to measure the membrane current. As in the microelectrode experiments, current and voltage were sampled at 1 kHz with a TL-1 DMA interface and software written in Quick BASIC. Constant external perfusion was driven by peristaltic pumps (MicroPerpex, LKB Instruments, Gaithersburg, MD). The perfusate was collected over 1.5 min intervals with a fraction collector. The dialysis solution contained (in mM): 25 NMG HEPES, 100 Na HEPES, 50 glycine, 50 phenylpropyltriethylammonium sulfate, 5 dithiothreitol, 2.5 BAPTA NMG, 10 Mg HEPES, 5 MgATP, 5 NMG phosphoenolpyruvate, 5 phosphoarginine and sufficient additional HEPES to titrate the solution to pH_i = 7.4. The external solution contained (in mM): 400 NMG sulfamate, 7.5 $Ca(OH)_2$, 10 Tris/HEPES (pH 7.7) or 20 MES (pH 6.6), 10 EDTA, 1 (3,4)diaminopyridine, adjusted to the indicated pH with sulfamic acid. The final osmolanty of the external solution was adjusted by addition of water to within 1% of the internal solution osmolarity (1012 mosm kg^{-1}). $^{22}Na^+$ efflux was measured as described by Rakowski, et al. (1989). Additional analysis and plotting of data was performed using the Sigma Plot graphic analysis program (SPSS Science, Inc., Chicago, IL).

Results

Measurement of Ouabain-sensitive Current and Its Relaxation Rate

Ouabain-sensitive pre-steady-state transient currents were measured in cut-open oocytes, as shown in Fig. 1*A* to *D*. An exponential slow component of charge relaxation can be separated from the fast initial transient current (not temporally resolved since it has the time course of the change in membrane voltage). This is illustrated in Fig. 1*E* to *G*, in which the relaxation currents are plotted linearly (*E*) and as the logarithm of the absolute value of current (*F*). These example records demonstrate that after 500 μs (the voltage-step rise time) the ouabain-sensitive current behaves as a single exponential. The relaxation time constant of the slow component of transient current is voltage-dependent.

Effect of pH_o on Ouabain-sensitive Difference Current

Ouabain-sensitive difference currents measured for voltage pulses from −160 to +40 mV as described for Fig. 1*A* to *D* are shown in Fig. 2 from four oocytes, one at each indicated pH_o, over the range 8.6 to 4.6. Note that the pulse duration in Fig. 2*C* and *D* is one half that in *A* and *B*. The magnitude of the slow component of current relaxation and its relaxation rate for this and additional data from a total of 20 oocytes over the pH_o, range 8.6 to 5.6 was analyzed as shown in Fig. 1*E* to *G* and the results are plotted in Fig. 3*A* and *B*.

Effect of pH_o on the Slow Component of Pre-Steady-state Pump Current

The normalized integral of the slow component of the ouabain-sensitive relaxation current (Q) is plotted as a function of membrane potential (V) in Fig. 3*A*. The curves at pH_o from 5.6 to 8.6 have been normalized so that they have the same absolute magnitude.

The equation $Q = Q_{min} + Q_{tot}/(1 + \exp(-FZ_q(V - V_q)/RT))$ was fit to the data at each pH to determine Q_{min} and Q_{tot}.

The parameters Z_q and V_q, determined from the fitting procedure, represent the exponential steepness and the mid-point voltage of the curve, and Q_{min} and Q_{tot} are the minimum and total value of the integral of the slow component of charge, respectively. R, T and F have their usual meaning.

There was no consistent effect of pH_o, on the value of Z_q and its value was not significantly different from 1.0. Z_q was, therefore, set equal to 1.0 for all of the fits shown in Fig. 2*A*. There is a statistically significant ($p < 0.01$) and consistent shift of V_q to more positive voltages with acidification. The least-squares values of V_q are −61.1 ± 1.3,−57.5 ± 3.9, −55.0 ± 3.9,−51.7 ± 2.0 mV for pH 8.6, 7.6, 6.6 and 5.6, respectively. However, considering that this effect occurs over 3 pH units (1000-fold change in $[H^+]$) it is a relatively small effect compared to, for example, the effect of external $[Na^+]$ on the steady state Q *vs.* V curve (Rakowski, 1993), Na^+/Na^+ exchange (Gadsby, et al., 1993) or backward pumping (De Weer, Gadsby & Rakowski, 2001) for which there is a shift of ~25 mV per twofold change in concentration (corresponding to an extracellular ion-well depth for Na^+ of about 0.7). We conclude that pH_o changes over the range 8.6 to 5.6 do not alter the apparent valence of the charge moved (Z_q) and have a relatively small effect on the mid-point voltage of the charge distribution (V_q). This suggests that changes in pH_o over this range do not greatly affect the relative energy levels of the stable states of the enzyme.

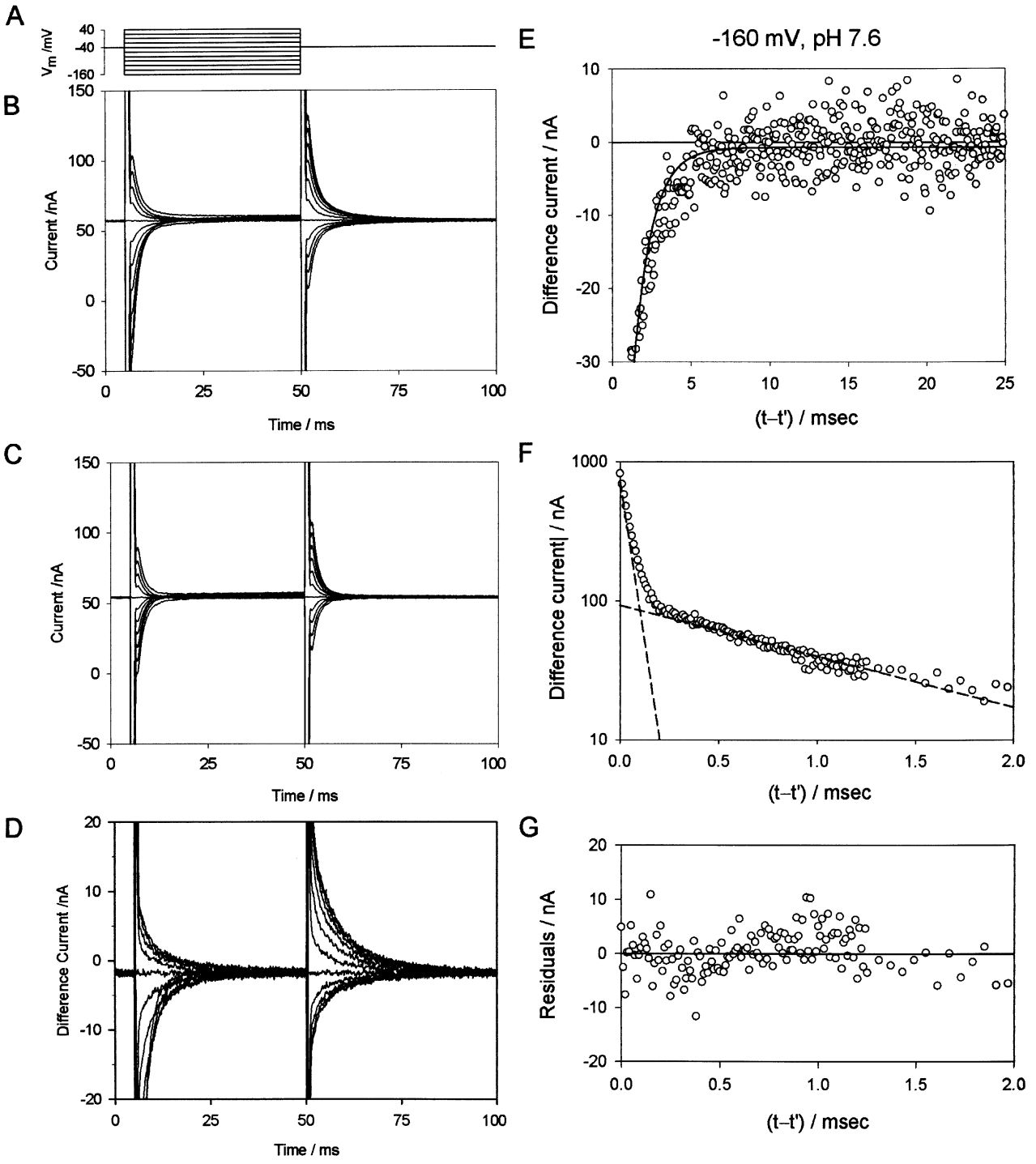

Fig. 1. Procedure for measurement of ouabain-sensitive pre-steady-state transient current (pH_o = 7.6). The series of voltage steps shown in *A* was performed on a cut-open oocyte held at −40 mV. Panel *B* shows the current response before the addition of ouabain. The series was repeated at 5 min intervals until there was no difference between successive current records. Near the start of the next 5 min interval, 20 μM ouabain was added to the solution in the extracellular chamber and at the end of the interval the current response to the voltage steps was recorded as shown in *C*. Panel *D* shows the ouabain-sensitive current obtained by subtraction of the current records shown in Panel *C* from those in Panel *B*. Panel *E* shows the subtracted trace under the same conditions after a −160 mV voltage pulse. The value $t' = 0.08$ ms is the time between the beginning of the voltage pulse and the starting point of analysis. The currents are not temporally resolved in the initial time interval. Panel *F* shows the data during the 2 ms after $t' = 0.08$ ms on a logarithmic current scale. It can be seen clearly that the relaxation of the current is essentially monoexponential for times after 0.5 msec. The dashed straight lines are the slow and fast components of current relaxation obtained by a least-squares fit of the equation $I = I_a e^{-At} + I_b e^{-Bt}$ to the data. Resolution of the time course of the fast component ($I_b e^{-Bt}$) is limited by the rise time of the voltage step and it was, therefore, not further analyzed. Panel *G* plots the residual differences between the fit and the data in *F*.

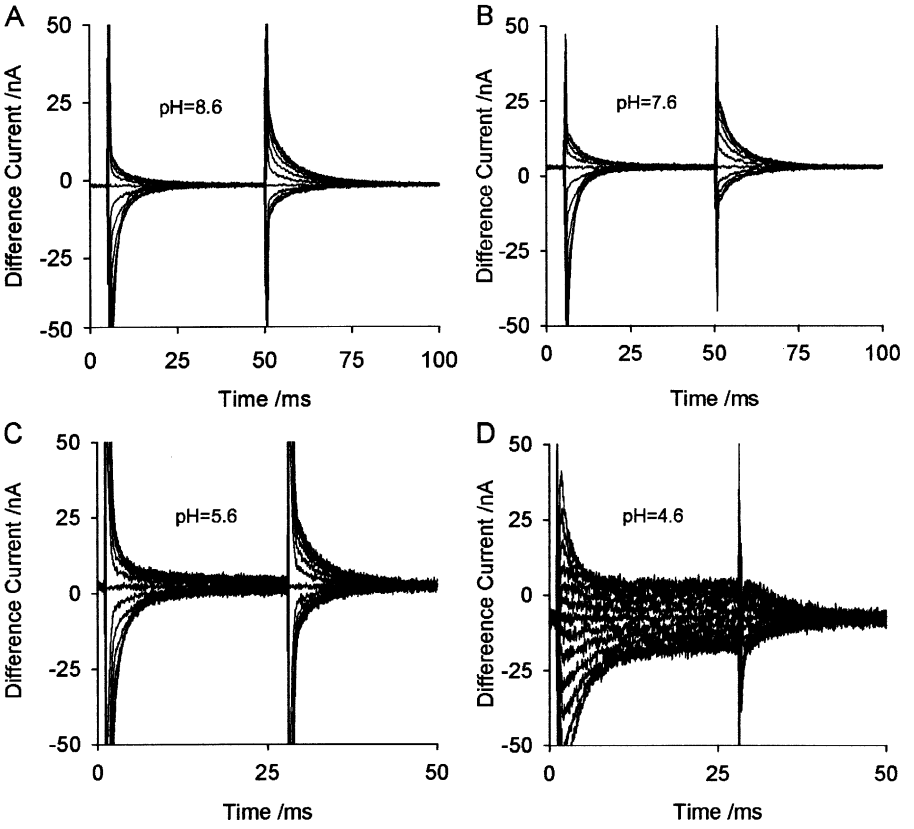

Fig. 2. Ouabain-sensitive difference current records obtained at various pH_o. Difference currents were calculated by subtraction of current records obtained in the presence of 20 μM ouabain from those obtained prior to ouabain addition, as explained in Fig. 1*B* to D. The data at each indicated pH_o was obtained from different oocytes since complete recovery from ouabain addition was not routinely possible. The data in panel 2*B* is the same as that shown in Fig. 1*D* but is shown at a different vertical scale here to permit direct comparison of the current magnitude in all of the panels in Fig. 2. Shorter pulse durations were used in *C* and *D* since the current relaxation rate increases at low pH_o and shortening the pulse duration resulted in greater success at obtaining a complete experiment.

Experiments at pH_o = 4.6

Four successful experiments like that shown in Fig. 2*D* were conducted at pH_o = 4.6. The results from these experiments differed from those at more alkaline pH_o in that the steepness of the *Q vs. V* curve was reduced substantially (Z_q = 0.51 ± 0.02), however the value of V_q (−50.7 ± 4.9 mV) followed the same trend towards more negative values as the data over the pH range 8.6 to 5.6 discussed above. This large change in Z_q is not predicted by the model presented here and presumably reflects an effect of pH at additional sites. Further analysis of the data at pH_o = 4.6 has been deferred to a future set of more complete experiments at this and more acid pH_o.

Effect of pH_o on the Relaxation Rate of the Transient Current

Previous work has shown that the relaxation rate of the slow component of pre-steady-state pump current can be described by an equation of the form: $K = K_0 + K_1 \exp(-\gamma FV/RT)$ (Gadsby et al., 1992; Holmgren & Rakowski, 1994). In our experiments at pH = 7.6 we find that the relaxation rate declines with depolarization as described in previous work, but there is a measurable increase of the current relaxation rate at the most positive membrane potentials (Fig. 3*B*). As the pH is made more acidic the right-hand limb steepens and the U-shaped voltage dependence of the relaxation rate is much more apparent. This is a significant change in the voltage dependence of the relaxation rate in contrast to the relatively minor effect on the mid-point voltage (V_q) suggesting that lowering pH_o changes one or more of the rate coefficients governing charge movement (one such rate coefficient (k_2) is plotted in Fig. 3*C*, *see* Discussion).

Steady-state Component of the Ouabain-sensitive Current Measured in Na^+- and K^+-free Solutions in Oocytes

As pH is lowered to 5.6 and 4.6 the steady-state component of ouabain-sensitive current is increased (Fig. 2*C* and *D*). This steady-state current is expected based on previous reports of acid-induced steady-state ouabain-sensitive current (Rakowski et al., 1991; Efthymiadis et al., 1993; Wang & Horisberger, 1995). These authors are in agreement that the current is carried by protons and is inhibited by other monovalent cations. We propose that the channels opened by acidic pH_o also permit Na^+ to carry current (*see* Discussion). The properties of the steady-state current are examined in Figs. 4 and 5 below.

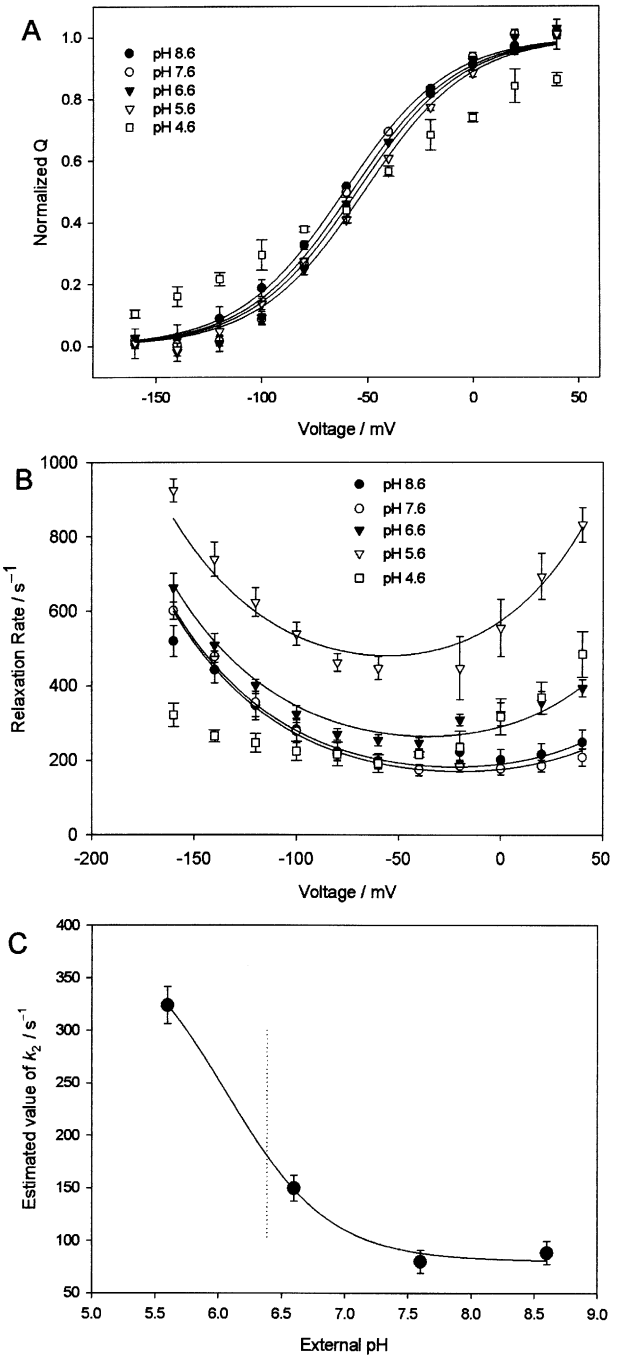

Fig. 3. Characteristics of the slow component of transient current at different pH_o (*A*) The magnitude of the slow component of the transient charge movement at each pH_o and during each voltage pulse was integrated by simple addition of the difference current from time $t' = 0.08$ ms. The integrated charge was normalized to the value $Q_{total} = (Q_{max} - Q_{min})$ where Q_{max} was measured at +40 mV and Q_{min} was measured at −160 mV. The regression lines were calculated from the least-squares fit of the equation $Q = Q_{min} + Q_{tot}/(1 + \exp(-FZ_q(V - V_q)/RT))$. (*B*) Relaxation rate of the slow component of pre-steady-state current. The solid lines are calculated from a least-squares fit to Appendix Eq. A3. (C) The least-squares values of k_2 from the fits in *B* are plotted here as a function of pH_o. The best fit of $k_2 = k_2^0 \exp(\text{const}/10^{(pH - pK_a)} + 1)$ (derived by assuming the "titratable" transitional energy level $\varepsilon = \varepsilon o + \Delta\varepsilon/(K_a/[H^+]_o + 1)$) to these data gives an estimate for pK_a = 6.38 ± 0.15 (dotted vertical line). The mean values and standard error in *A* to *C* were calculated from data obtained in the following number of oocytes (*n*) at each pH_o: 8.6 ($n = 5$), 7.6 ($n = 6$), 6.6 ($n = 5$), 5.6 ($n = 4$) and 4.6 ($n = 4$).

^{22}Na Efflux through the Na^+ Pump in Squid Giant Axons in Na^+- and K^+-free Solutions is pH_o Sensitive

To test whether there is an acid pH_o -sensitive increase in steady-state Na^+ permeability through the Na^+/K^+ pump, we performed an experiment that simultaneously measured unidirectional sodium efflux and net current across the membrane of squid giant axon (Fig. 4). The experiment was conducted at a holding potential of −40 mV so that the electrochemical gradient for protons is inward at both pH_o = 7.7 and 6.1, while that for Na^+ is outward (external Na^+-free solution). Under this zero-trans condition, Na^+ translocation is directly measured as ^{22}Na efflux and protons are presumably the only charged species available to carry inward current (internal and external anions presumed to be impermeant and no other permeant cation species present in the external solution.)

As seen in Fig. 4*A*, at pH 7.7 in the absence of external Na^+ or K^+, there is a dihydrodigitoxigenin (H_2DTG)-sensitive component of Na^+ efflux (−3.8 pmol cm^{-2} s^{-1} at *Aa*). Simultaneous current measurements (Fig. 4*B*) show that at pH 7.7 there was a very small component of outward current blocked by H_2 DTG (−0.14 μA cm^{-2} at *Ba*). This small H_2 DTG-sensitive outward current and $^{22}Na^+$ efflux can be explained either by forward pumping, with protons acting as K^+ congeners ($3Na^+/2H^+$ exchange) (Polvani & Blostein, 1988), or by $3Na^+$/ATP (uncoupled) electrogenic transport (Garrahan & Glynn, 1967; Glynn & Karlish, 1976; Cornelius, 1989; 1990). The measured ^{22}Na efflux of 3.8 pmol cm^{-2} s^{-1} would result in an outward current of 0.37 μA cm^{-2} if it were generated by uncoupled $3Na^+$/ATP transport. The observed reduction in outward current produced by addition of H_2 DTG was only 0.14 μA cm^{-2} . On the other hand, the net current change expected for $3Na^+/2H^+$ exchange is one-third of 0.37 μA cm^{-2} (0.12 μA cm^{-2}), which is quite close to the observed magnitude of the measured change in current. We, therefore, favor $3Na^+/2H^+$ exchange as the explanation of the measured change in current and flux. The magnitude of the Na^+ efflux increased significantly when the external pH was lowered to 6.1 (+10 pmol cm^{-2} s^{-1} at *Ab*) and there was an inward shift of holding current (≥−1 μA cm^{-2} at *Bb*). The increase in Na^+ efflux upon changing to pH 6.1 cannot be explained either by $3Na^+/2H^+$ exchange or by uncou-

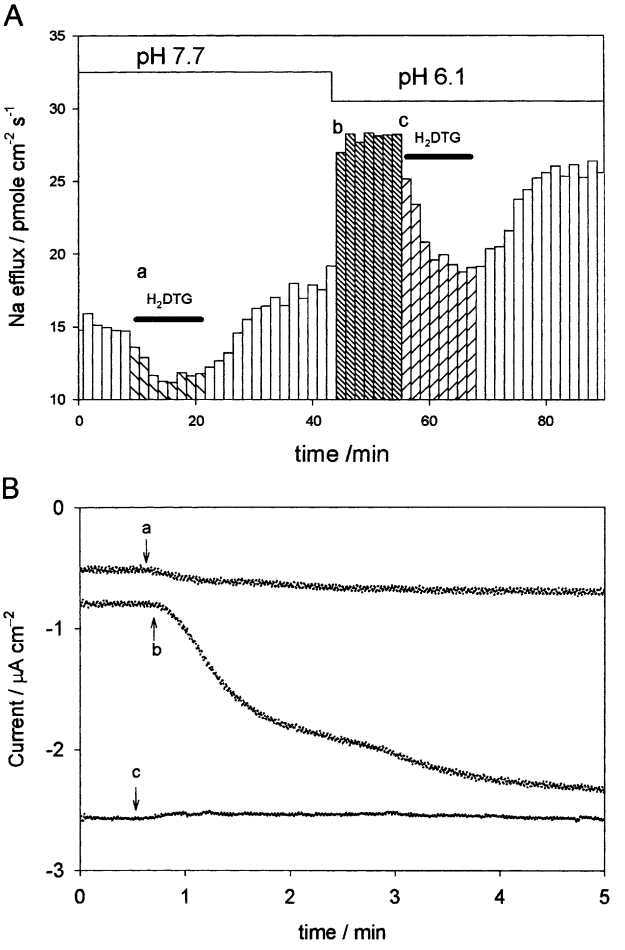

Fig. 4. Simultaneous flux and current measurements in squid giant axon in Na^+-and K^+-free external solutions. (*A*) Addition of H_2 DTG at pH 7.7 produced a block of 3.8 pmol cm^{-2} s^{-1} of $^{22}Na^+$ efflux (at *a*). After a 20 min washout of H_2DTG, pH_o was changed to 6. 1 (at *b*), resulting in an increase of $^{22}Na^+$ efflux of 10 pmol cm^{-2} s^{-1} of which 6.8 pmol cm^{-2} s^{-1} was blocked by a second addition of H_2DTG (at *c*). (*B*) Changes in holding current upon addition of H_2DTG. Holding potential = −40 mV. In contrast to part *A*, which shows only the efflux carried by Na^+, these records represent net current changes (the sum of current carried by Na^+ and protons). The addition of H_2DTG (10 μM) at pH 7.7 (*a*) resulted in net decrease of outward current (0.14 μA cm^{-2}) possibly mediated by $3Na^+/2H^+$ exchange. (*b*) Changing pH to 6.1 after washing out H_2DTG resulted in a large inwardly directed current (> 1 μA cm^{-2}). This net inward current occurred despite an increased in $^{22}Na^+$ efflux (10 pmol cm^{-2} s^{-1}). An inward proton flux of about 20 pmol cm^{-2} s^{-1} is required to explain the observed change in current. (*c*) The addition of H_2DTG at pH 6.1 did not produce a larger block of outwardly directed current, which would be expected if lowering pH_o increased $3Na^+/2H^+$ exchange. Instead, H_2DTG blocked a small inward current (0.1 μA cm^{-2}) that would result if the inward current carried by H^+ were slightly larger than the outward current carried by Na^+. The results support the hypothesis that low pH_o induces a Na^+ and H^+ conducting pathway through the Na/K pump.

pled Na^+ efflux, since the current change recorded simultaneously is in the inward direction. The data suggest that external acidification produces a state of the Na^+/K^+ pump that is permeable to both Na^+ and H^+. This is supported by the data shown at the second application of H_2 DTG (pH_o of 6.1). The H_2 DTG-sensitive drop in ^{22}Na efflux was −6.8 pmol cm^{-2} s^{-1} (at *Ac*) and this was accompanied by block of only a small net inward current (0.1 μ*A* cm^{-2} at *Bc*). This directly demonstrates that acid pH_o increased H_2DTG-sensitive (pump-mediated) ^{22}Na efflux and this efflux of positive charge must have been accompanied by an inwardly directed current (presumably carried by H^+ since only impermeant anions were present) in order to produce the small net inward current change that was observed at *Bc*. The results support the suggestion that both H^+ and Na^+ are permeant through a conducting pathway activated by low pH_o.

Ouabain-sensitive Steady-state Current Measured in Oocytes at Acid pH_o

We studied the voltage dependence of acid pH_o-induced ouabain-sensitive steady-state current further, using the two-electrode oocyte clamp technique. The results of a typical experiment at pH_o 5.6 are shown in Fig. 5. In Na^+ -free solution (*circles* in *E*) there is a voltage-dependent ouabain sensitive inward current. This is consistent with protons being the primary current carrier in the absence of external Na^+ or K^+. The addition of 25 mM Na^+ to the external solution (*squares*) results in a significant increase of the magnitude of the ouabain-sensitive inward current. This result, taken together with the results of the Na^+ efflux experiment shown in Fig. 4, strongly suggests that Na^+ and H^+ are both carriers of passive (channel-mediated) current induced by low pH_o. A further increase of the Na^+ concentration to 100 mM (*triangles*) results in a reduction of the inward current seen at very negative voltages. This [Na^+] and voltage-dependent inward current and its inhibition at high [Na^+] suggests the existence of a regulatory Na^+-binding site within the membrane field that inhibits the ouabain-sensitive current through the pump. We propose that Na^+ acts both as a carrier of the acid pH-induced current leak through the pump and as an inhibitor of that current.

Discussion

The experimental conditions in the experiments conducted in cut-open oocytes were designed to promote the phosphorylated Na^+-bound states of enzyme shown within the box in Fig. 6. As suggested by previous work, the binding/release of Na^+ to and from the outside-facing (E_2) states of the pump results in pre-steady-state transient current (Figs. 1*D* and 2). The behavior of the pre-steady-state current-relaxation rate at normal pH_o is well accounted for

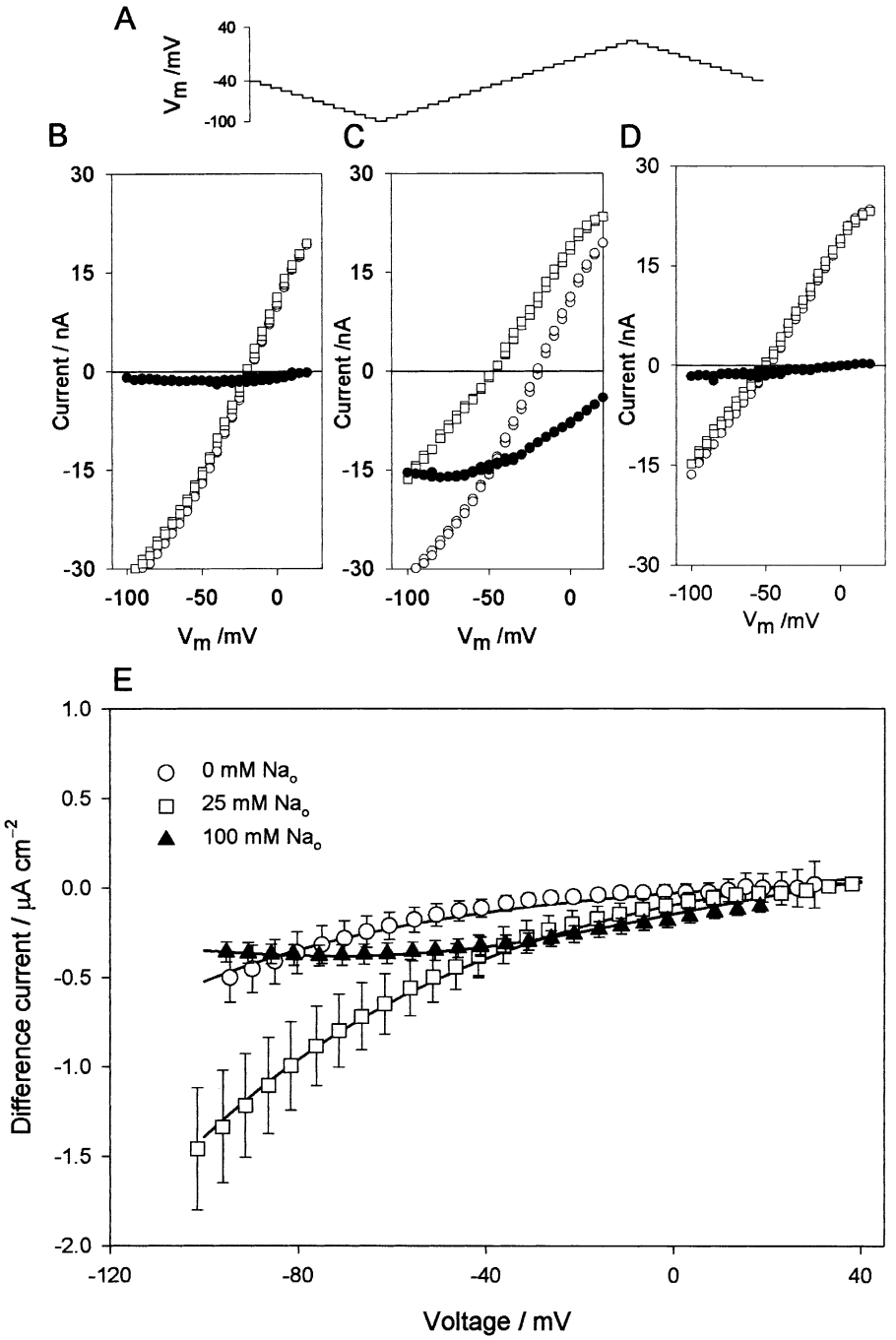

Fig. 5. Steady-state ouabain-sensitive current induced by low pH_o. (A) Staircase voltage protocol. The duration of each step is 500 ms. (B). *Empty symbols*: Current measured at the end of each voltage step for two successive *I–V* curves obtained 5 min apart in 100 mM $[Na^+]_o$. *Filled circles*: The time-only control subtraction of these two successive *I–V* records is nearly flat. (C) *Empty circles*: *I–V* obtained before the addition of ouabain; *Empty squares*: *I–V* obtained after addition of ouabain; *Filled circles*: ouabain-sensitive difference current. (*D*) *Empty symbols*: two successive *I–V* records obtained 5 min apart in the presence of ouabain. *Filled circles*: time-only control subtraction of these two successive records obtained in the presence of ouabain. (*E*) Steady-state ouabain-sensitive difference current obtained at 0, 25, and 100 mM $[Na^+]_o$ (mean value ± SEM from 4 oocytes at each $[Na^+]_o$). The lines are least-squares fits of Appendix Eq. A2 to the data (*see* Discussion).

by the existence of an extracellular access channel (ion well) that signals the occupancy and release of Na^+ ions at the external face of the enzyme (Holmgren & Rakowski, 1994). At low pH_o, however, we postulate that the activation energy required for the Na_3E_1 ATP $\rightarrow$ P-E_1Na_3 transition is reduced, opening a conducting pathway for Na^+ and H^+ to the cytoplasm. This is equivalent to

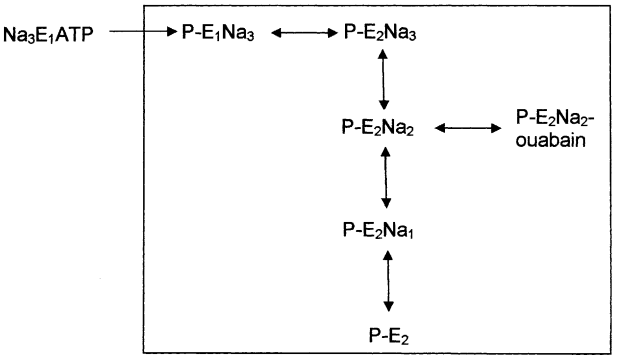

Fig. 6. Partial Post-Albers model of the pump cycle (after Wuddel & Apell, 1995). The enzyme states within the box are assumed to be isolated by the experimental design of the experiments conducted in cut-open oocytes. The overall pump cycle is restricted to the Na^+ half of the pump cycle, owing to the absence of intracellular and extracellular K^+. Phosphorylated, Na^+-bound states are favored by operating at saturating intracellular [Na^+] and [ATP] and reversal of the ATP-E_1 Na_3 $\rightarrow$ P-E_1 Na_3 step is avoided by omission of ADP and provision of substrate for the regeneration of ATP. Reversal of this step is required to allow isotopic Na^+/Na^+ exchange to occur. We postulate that low pH_o reduces the transition-state energy for this step, resulting in a Na^+- and H^+ -conducting pathway through the Na/K pump (*see* Fig. 7).

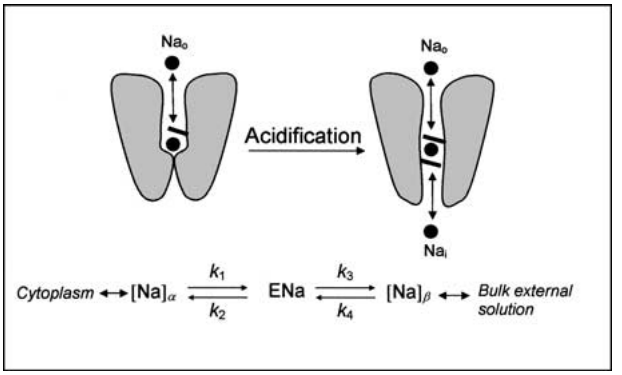

Fig. 7. Leak through the Na/K pump induced by low pH_o. We propose that in the absence of extracellular K^+, Na^+ occluded within the enzyme (*ENa*) can move either to the external or internal solutions when the extracellular solution is acidified. Na^+ in the cytoplasm and bulk external solution is in rapid equilibrium across internal and external ion wells. The effective concentrations of Na^+ at its internal and external binding loci are given by $[Na]_\alpha$ and $[Na]_\beta$, respectively. We postulate that acidification of the extracellular solution results in protonation of certain amino acid(s) within the pump molecule that produces a parallel reduction of the rate coefficients k_1 and k_2, as expected from a reduction in the energy required for the transitions between the internal binding locus and ENa. The pathway is also permeable to H^+ with f representing the ratio of each rate coefficient for H^+ compared to the corresponding rate coefficient for Na^+. The rate coefficients to k_1 to k_4 are assumed to be voltage independent. The model may be regarded as one in which access to the occlusion site may be achieved either *via* an internal route over the fractional electrical distance α or an external route over dielectric distance β. For a monovalent ion, $\alpha+\beta=1$.

increasing the open probability of the innermost of two "gates" responsible for Na^+ occlusion within the enzyme.

In the present work we demonstrated that the dependence of the current relaxation rate on voltage changes significantly as pH is decreased to 5.6. It becomes U-shaped rather than mono-exponential. In attempting to extend the simple ion-well model to explain this behavior, we find that the observed behavior can be predicted by assuming that low pH_o decreases the energy barrier between bound Na^+ (ENa) and the intracellular medium such that the Na^+ -bound state can communicate with both the intracellular solution as well as the extracellular medium. This is illustrated in Fig. 7.

This model predicts that the pre-steady-state current-relaxation rate (A) can be described by Eq. 3 of the Appendix:

$$A = k_1[\mathrm{Na}]_i \exp(\alpha FV/RT) + (k_1k_3/k_4) + k_3 + k_4[\mathrm{Na}]_o \exp(-\beta FV/RT)$$

This expression predicts that the voltage dependence of the relaxation rate is U-shaped with the magnitude of the left and right branches of the curve dependent on the values of k_4 and k_1 respectively. We postulate that lowering pH_o decreases the energy barrier between the Na^+ -bound state and the state that releases Na^+ to the inside; therefore, the rate constants k_1 and k_2 will be proportionately affected. This will not result in a net change of steady-state charge distribution but will affect its relaxation rate.

Fig. 3*B* shows the results of fitting Appendix Eq. 3 to the relaxation rate data at various pH_o. The best-fit parameters are given in Table 1.

We plotted the least-squares values of k_2 *vs.* pH in Fig. 3*C*. This allows us to obtain an estimate of the pK_a of the titratable group(s) that are involved in inducing the current by means of the equation

$$k_2 = k_2^0 \exp\left(\mathrm{const}/(10^{(\mathrm{pH_o}-\mathrm{p}K_a)}+1)\right).$$

Assuming a single binding site, the best fit predicts the pK_a to be 6.38 ± 0.15. This is consistent with a histidine contributing the titratable group, although owing to alterations of the local environment within the enzyme other acidic side chains may be involved.

As is evident from Eq. A2 of the Appendix, lowering the energy barrier between the Na^+-occluded state and the internal solution (k_1/k_2 step) should also increase the magnitude of the steady-state component of the current (limited by $E_{tot}Fk_2$ at the most negative potentials). However, the steady-state component of Eq. A1 cannot fully explain the behavior of the currents in Fig. 5, since those data suggest that external Na^+ also acts as an

Table 1. Best fit parameters for Fig. 3*B*

	α	k_1/k_2	k_2	k_4
pH 8.6			88 ± 11 s^{-1}	
pH 7.6	0.53 ± 0.03	0.010 ± 0.001 mM^{-1}	80 ± 11 s^{-1}	0.26 ± 0.05 s^{-1} mM^{-1}
pH 6.6			149 ± 12 s^{-1}	
pH 5.6			324 ± 18 s^{-1}	

See text and Appendix for details, $\beta = 1 - \alpha$; $k_3 = k_2k_4/k_1$.

inhibitor of the acid-induced steady-state current. The inhibition occurs at high external Na^+ and the most negative membrane potentials. To accommodate this observation we made the additional assumption that Na^+ binds to a locus within the membrane field that causes the current to be blocked according to the reduction of E_{total} to E^*_{total} given by:

$$E^*_{total} = E_{total}/\{1 + (([Na]_o \exp(-\gamma FV/RT))/K_{0.5})^n\}$$

where γ is the dielectric distance at which the blocking Na^+ ions bind, n is the Hill coefficient for the binding, and $K_{0.5}$ is the $[Na^+]_o$ concentration that results in half-maximal binding. We also assumed that H^+ ions permeate through the same pathway as Na^+ without interaction (that is, the Na^+ and H^+ currents obey the independence principle) and that the rate coefficients for H^+ transitions are related to those for Na^+ as $k^*_i = fk_i$, where f is the ratio of the rate coefficient for H^+ to that for Na^+. After making these two assumptions, the expression for the total steady-state current becomes

$$I_{ss} = FE^*_{total}(I_{ss}(Na) + I_{ss}(H))$$

where $I_{ss}(Na)$ is derived in the Appendix (Eq. A2) and $I_{ss}(H)$ is produced by replacing $k^*_i = k_i f$ and sodium ion concentration with hydrogen ion concentration. To fit the data in Fig. 5*E*, the rate constants k_1 to k_4 and the dielectric coefficients α and β were fixed at the values obtained from fitting the rate constant data at pH 5.6 (Table 1). Fig. 5*E* shows the results of this fit. The best fit values are $\gamma = 0.23 \pm 0.03$, $n = 3.02 \pm 0.27$, $K_{0.5} = 130 \pm 30$ mM, and $f = 2.8 \pm 0.7 \times 10^4$. The goodness of the fit supports the hypothesis that the acid-induced current is mediated by a conducting pathway through the Na^+/K^+ pump, blockable by ouabain and permeable to both Na^+ and H^+, with H^+ being more than 4 orders of magnitude more permeant than Na^+. Note that the high value of f predicts a very high rate of H^+-current relaxation—well beyond the resolution limit for the cut-open oocyte experiments. In K^+-free solutions, block by Na^+ occurs when 3 Na^+ ions occupy a low-affinity, shallow binding site at a dielectric distance similar to that found for the activation of forward pumping by K^+ (Rakowski et al., 1991).

Comparison with Previous Work

After the initial discovery of the inwardly-directed acid-induced ouabain-sensitive current in oocytes in Na^+- and K^+-free solution (Rakowski, et al., 1991), Efthymiadis, et al. (1993) further characterized the phenomenon and showed that the current was increased by acidic pH_o, and blocked by a variety of external cations including Na^+. Wang and Horisberger (1995) provided strong support for the conclusion that the conductance pathway opened by acidic pH is permeable to protons since the reversal potential of the current closely follows the equilibrium potential for H^+ in Na^+-free external solutions. They also reported that the current was initially enhanced and then blocked by Na^+_o. Our results are in agreement with the above studies and in addition we suggest that Na^+ is permeant at concentrations below those that produce block. We also have shown that the relaxation rate of ouabain-sensitive pre-steady-state current is slowed by acidification and we propose a simple model that explains the link between the increased steady-state current and slowing of pre-steady state current. Rettinger (1996), on the other hand, has suggested that the inward current induced by acid pH is a consequence of the operation of the electrogenic H^+-ATPase. The evidence cited to support this conjecture is his observation that under certain conditions the slope of the relationship between the reversal potential of the current and extracellular $[H^+]$ has a slope that is greater than RT/F (supra-Nernstian) and, therefore, it is inferred that it cannot arise from a passive conductance pathway. This conclusion, however, is not warranted. The equation for the reversal potential (V_{rev}) written in the form of the Goldman-Hodgkin-Katz equation taking into account that both H^+ and Na^+ are permeant is:

$$V_{rev} = (RT/F)\ln\left\{\frac{[H^+]_o + \alpha[Na^+]_o}{[H^+]_i + \alpha[Na^+]_i}\right\}$$

where $\alpha = P_H/P_{Na}$, the permeability ratio of H^+ and Na^+. (Note that the parameter α in this equation is not the same as the dielectric coefficient α in Table 1.) The expectation by analogy with classical work on the resting potential of skeletal muscle is that the presence of the $[Na^+]$ terms will reduce the slope of

the V_{rev} *vs.* $[H^+]_o$ relationship at low $[H^+]_o$ and that the slope will approach RT/F as $[H^+]_o$ is increased. However, since P_{Na} is a function of $[H^+]_o$ and P_H is a function of $[Na^+]_o$, the value of α is not constant and the reversal potential may behave in either super-or sub-Nenstian fashion depending on the values of the equilibrium potentials for H^+ and Na^+ and the dependence of α on $[Na^+]_o$ and $[H^+]_o$.

Supported by NIH grant NS 22979.

Appendix

Consider the kinetic relationship:

$$E + Na_i \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ENa \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E + Na_o$$

where *ENa* represents an "occluded" state of the enzyme reached either from the inside (*i*) or outside (*o*) of the membrane. The rate constants k_1, k_2, k_3, and k_4 are assumed to be independent of the membrane potential. The local concentrations of Na^+ are described according to the Boltzmann equations:

$$[Na]_\alpha = [Na]_i e^{\alpha FV/RT}$$

$$[Na]_\beta = [Na]_o e^{-\beta FV/RT}$$

where α and β represent the fraction of the membrane field crossed by Na^+ to reach its occluding site from the inside and outside, respectively. Now, if we write the reaction rates as follows:

$$K_1 = k_1[Na]_i e^{\alpha FV/RT}$$

$$K_4 = k_4[Na]_o e^{-\beta FV/RT}$$

and consider that: $E_{total} = E + ENa$, we can write an equation describing the behavior of the *ENa* state of the pump:

$$d(ENa)/dt = E_{total}(K_1 + K_4) - ENa(K_1 + k_2 + k_3 + K_4).$$

This differential equation can be solved for a voltage step where the initial state is described by a set of constants: K_1^0, k_2^0, k_3^0, K_4^0 and the final state described by the set of constants: K_1, k_2, k_3, K_4, of which only K_1 and K_4, differ from the initial ones due to their voltage dependence. If we solve this equation with appropriate initial conditions we arrive at the following expression for *ENa*:

$$ENa = (B/A) + \{(B_o/A_o) - (B/A)\}e^{-AT}$$

where $A = K_1 + k_2 + k_3 + K_4$, $A_o = K_1^0 + k_2^0 + k_3^0 + K_4^0$, $B = E_{total}(K_1 + K_4)$ and $B_o = E_{total}(K_1^0 + K_4^0)$. Having written this expression for *ENa* as a function of time (t) after the start of the voltage pulse, we can now write an explicit expression for the net current:

$$I = F[E(K_1\alpha - K_4\beta) + ENa(k_3\beta - k_2\alpha)].$$

Replacing E and *ENa* with the expressions derived above and simplifying, we may write the following equation for current as a function of time:

$$I = FE_{total}(K_1k_3 - K_4k_2)/(K_1 + k_2 + k_3 + K_4) + F[(B_o/A_o) - (B/A)][(k_3 + K_4)\beta - (k_2 + K_1)\alpha]e^{-AT}. \quad (A1)$$

The left part of this equation represents the steady-state component of the total current:

$$I_{ss} = FE_{total}(K_1k_3 - K_4k_2)/(K_1 + k_2 + k_3 + K_4). \quad (A2)$$

The requirement for microscopic reversibility imposes the following constraint on the values of the rate constants:

$$(k_2k_4)/(k_1k_3) = 1.$$

The expression for the relaxation rate of the transient component of the current (A) is, therefore:

$$A = k_1[Na]_i e^{\alpha FV/RT} + (k_1k_3/k_4) + k_3 + k_4[Na]_o e^{-\beta FV/RT}. \quad (A3)$$

References

Brinley, F., Mullins, L. 1967. Sodium extrusion by internally dialyzed squid axons. *J. Gen. Physiol.* **50:**2303–2331

Cornelius, F. 1989. Uncoupled Na^+ efflux of reconstituted shark Na, K-ATPase is electrogenic. *Biochem. Biophys. Res. Commun.* **160:**801–807

Cornelius, F. 1990. Variable stoichiometry in reconstituted shark Na, K-ATPase engaged in uncoupled efflux. *Biochim. Biophys. Acta.* **1026:**147–152

De Weer, P., Gadsby, D.C., Rakowski, R.F. 2001. Voltage dependence of the apparent affinity for external Na^+ of the backward-running sodium pump. *J. Gen. Physiol.* **117:**315–328

Efthymiadis, A., Rettinger, J., Schwarz, W. 1993. Inward-directed current generated by the Na^+, K^+ pump in Na^+ - and K^+-free medium. *Cell Biol. Int.* **17:**1107–1116

Gadsby, D.C., Nako, M. 1989. Steady-state current-voltage relationship of the Na/K pump in guinea pig ventricular myocytes. *J. Gen. Physiol.* **94:**511–537

Gadsby, D.C., Nakao, M., Bahinski, A., Nagel, G., Suenson, M. 1992. Charge movements via the cardiac Na, K-ATPase. *Acta Physiol. Scand. Suppl.* **146:**11–23

Gadsby, D.C., Rakowski, R.F., DeWeer, P. 1993. Extracellular access to the Na, K pump: pathway similar to ion channel. *Science* **260:**100–103

Garrahan, P.J., Glynn, I.M. 1967. The behavior of the sodium pump in red cells in the absence of external potassium. *J. Physiol.* **192:**159–174

Glynn, I.M., Karlish, S.J.D. 1976. ATP hydrolysis associated with an uncoupled sodium flux through the sodium pump: Evidence

for allosteric effects of intracellular ATP and extracellular sodium. *J. Physiol.* **256:**456–496

Holmgren, M., Rakowski, R.F. 1994. Pre-steady-state transient currents mediated by the Na/K pump in internally perfused Xenopus oocytes. *Biophys. J.* **66:**912–922

Polvani, C., Blostein, R. 1988. Protons as substitutes for sodium and potassium in the sodium pump reaction. *J. Biol. Chem.* **263:**16757–16763

Rakowski, R.F. 1989. Simultaneous measurement of changes in current and radiotracer flux in voltage-clamped squid giant axon. *Biophys. J.* **55:**663–671

Rakowski, R.F. 1993. Charge movement by the Na/K pump in *Xenopus* oocytes. *J. Gen. Physiol.* **101:**117–144

Rakowski, RF., Gadsby, D.C., De Weer, P. 1989. Stoichiometry and voltage dependence of the sodium pump in voltage-clamped, internally-dialyzed squid giant axon. *J. Gen. Physiol.* **93:**903–941

Rakowski, RF., Vasilets, L.A., LaTona, J., Schwarz, W. 1991. A negative slope in the current-voltage relationship of the Na^+/K^+ pump in *Xenopus* oocytes produced by reduction of external $[K^+]$. *J. Membrane Biol.* **121:**177–187

Rettinger, J. 1996. Characteristics of Na^+/K^+ -ATPase mediated proton current in Na^+- and K^+-free extracellular solutions. Indications for kinetic similarities between H^+/K^+-ATPase and Na^+/K^+-ATPase. *Biochim. Biophys. Acta* **1282:** 207–215

Sagar, A., Rakowski, R.F. 1994. Access channel model for the voltage dependence of the forward-running Na^+/K^+ pump. *J. Gen. Physiol.* **103:**869–893

Taglialetala, M., Toro, L., Stefani, E. 1992. Novel voltage clamp to record small, fast currents from ion channels expressed in *Xenopus* oocytes. *Biophys. J.* **61:**78–82

Wang, X., Horisberger, J.-D. 1995. A conformation of Na^+-K^+ pump is permeable to proton. *Am. J. Physiol.* **268:**C590–C595

Wuddel, I., Apell, H.J. 1995. Electrogenicity of the sodium transport pathway in the Na, K-ATPase probed by charge-pulse experiments. *Biophys. J.* **69:**909–921