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Voltage Dependence of Na/K Pump Current in *Xenopus* Oocytes

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Summary. Stage V and VI (Dumont, J.N., 1972, *J. Morphol.* **136:**153–180) oocytes of *Xenopus laevis* were treated with collagenase to remove follicular cells and were placed in K-free solution for 2 to 4 days to elevate internal [Na]. Na/K pump activity was studied by restoring the eggs to normal 3 mM K Barth's solution and measuring membrane current-voltage (*I-V*) relationships before and after the addition of 10 μM dihydroouabain (DHO) using a two-microelectrode voltage clamp. Two pulse protocols were used to measure membrane *I-V* relationships, both allowing membrane currents to be determined twice at each of a series of membrane potentials: (i) a down-up-down sequence of 5 mV, 1-sec stair steps and (ii) a similar sequence of 1-sec voltage pulses but with consecutive pulses separated by 4-sec recovery periods at the holding potential (−40 mV). The resulting membrane *I-V* relationships determined both before and during exposure to DHO showed significant hysteresis between the first and second current measurements at each voltage. DHO difference curves also usually showed hysteresis indicating that DHO caused a change in a component of current that varied with time. Since, by definition, the steady-state Na/K pump *I-V* relationship must be free of hysteresis, the presence of hysteresis in DHO difference *I-V* curves can be used as a criterion for excluding such data from consideration as a valid measure of the Na/K pump *I-V* relationship. DHO difference *I-V* relationships that did not show hysteresis were sigmoid functions of membrane potential when measured in normal (90 mM) external Na solution. The Na/K pump current magnitude saturated near 0 mV at a value of 1.0–1.5 μA cm^{-2}, without evidence of negative slope conductance for potentials up to +55 mV. The Na/K pump current magnitude in Na-free external solution was approximately voltage independent. Since these forward-going Na/K pump *I-V* relationships do not show a region of negative slope over the voltage range −110 to +55 mV, it is not necessary to postulate the existence of more than one voltage-dependent step in the reaction cycle of the forward-going Na/K pump.

Key Words Na/K pump · voltage dependence · membrane current-voltage relationship · dihydroouabain · *Xenopus laevis* · oocyte

Introduction

Early investigations of the voltage dependence of the Na/K pump in squid giant axons (Hodgkin & Keynes, 1954, 1955; Brinley & Mullins, 1974) suggested that over a limited voltage range the voltage dependence of Na/K pump activity was slight. However, recent studies using isolated cardiac myocytes (Gadsby & Noma, 1984; Gadsby, Kimura & Noma, 1985) have demonstrated voltage dependence of ouabain-sensitive current and also addressed the possibility that ouabain-sensitive current might be contaminated by a change in K-channel current resulting from accumulation of extracellular K in a restricted diffusion space following pump inhibition (Isenberg & Trautwein, 1974; Gadsby, 1982, 1984). Using K-channel blocking agents to decrease K conductance, Gadsby et al. (1985) demonstrated that there was only an insignificant residual sensitivity of membrane current to deliberate changes in K concentration in the presence of ouabain. Similar studies in squid giant axons have further shown that steroid-sensitive ^{22}Na efflux is also voltage dependent and that its voltage sensitivity closely parallels that of steroid-sensitive current (De Weer, Rakowski & Gadsby, 1987; Rakowski, Gadsby & De Weer, 1988*b*). Initial voltage-clamp studies of the Na/K pump in developing *Xenopus* blastomeres (Turin, 1982, 1984; Béhé & Turin, 1984) suggested that because of its low passive membrane conductance this preparation was suitable for investigation of the voltage dependence of the pump. Subsequent studies in *Xenopus* oocytes (Eisner, Valdeolimillos & Wray, 1986, 1987) have reported a significant voltage dependence of Na/K pump current at negative membrane potentials under conditions of elevated intracellular Na concentration, but these authors were unable to conduct experiments at positive membrane potentials where Lafaire and Schwarz (1985, 1986) had found that the dihydroouabain-sensitive current exhibited a maximum at about +20 mV and then declined at more positive voltages, giving rise to a region of negative slope.

A region of negative slope in the *I-V* relationship of the forward-going Na/K pump would be of considerable interest since it implies the existence of more than one voltage-dependent step in the overall pump cycle (Chapman et al., 1983; De Weer, 1984, 1986; Läuger & Apell, 1986). We have, therefore, reinvestigated the voltage dependence of Na/K pump current in Na-loaded *Xenopus* oocytes. We find that DHO difference *I-V* relationships that do not show hysteresis (and, hence, are indicative of the steady state expected for Na/K pump current) do not have a region of negative slope. We have also investigated the Na/K pump *I-V* relationship in Na-free solutions and find that under this condition pump current is approximately voltage independent, in agreement with results from cardiac myocytes (Gadsby & Nakao, 1987) and squid giant axon (De Weer et al., 1987; Rakowski et al., 1988*b*).

An abstract of some of these results has been published (Rakowski, 1987). Wu and Civan (1988) have also published an abstract on the voltage dependence of strophanthidin-sensitive current in oocytes of *Rana pipens* and have suggested that extracellular K accumulation on blocking the pump activates a contaminating parallel K-conductance pathway that contributes to the negative slope seen in the difference *I-V* relationship at positive membrane potentials, consistent with the results presented here.

Materials and Methods

Oocyte positive, laboratory bred and reared, South African clawed frogs (*Xenopus laevis*, Daudin) were obtained from a commercial supplier (Nasco, Ft. Atkinson, WI). The animals were maintained in recirculating, filtered, dechlorinated fresh water tanks under artificial lighting conditions at room temperature and fed a commercial high-protein diet (Carolina Biological Supply) twice a week. No seasonal variations in oocyte properties were found under these conditions. Animals were immersed in crushed ice prior to decapitation and removal of the ovaries. The ovaries were dissected into small clumps of 10–30 full-grown oocytes and then treated with collagenase (Sigma type IA, 2.5 mg/ml) in normal 3 mM K Barth's medium (*see below*) for 3–5 hr. After collagenase treatment to free the oocytes of enveloping follicular epithelial cells, full-grown, prophase-arrested oocytes of stage V and VI (Dumont, 1972) were selected and transferred to K-free Barth's solution. The eggs were stored in an incubator at 19°C for 2–3 days prior to study. Penicillin (1 U/ml) and streptomycin (1 μg/ml) were usually added to the storage medium, which was changed daily. This procedure yielded oocytes that were Na loaded and had low membrane conductances compared to freshly dissected oocytes. The resting membrane potential of freshly dissected oocytes in 3 mM K Barth's solution averaged -36.8 ± 1.1 mV ($n = 27$) (±SEM) (*cf.* Dascal, Landau & Lass, 1984; Webb & Nuccitelli, 1985) and the membrane conductance measured at a holding potential of −40 mV averaged 202 ± 15 μS cm^{-2}. Membrane potentials of Na-loaded, 2-day-old, collagenase-treated oocytes returned to normal 3 mM K Barth's solution averaged -60.8 ± 3.1 mV ($n = 30$) and the membrane conductance measured under voltage-clamp conditions at a holding potential of −40 mV averaged 54.4 ± 4.6 μS cm^{-2}. After storage for 2 days in K-free, Cl-free solution, oocyte membrane potentials averaged -40.1 ± 5.6 mV ($n = 24$) when returned to 3 mM K chloride-free solution, and the membrane conductance at −40 mV averaged 45.8 ± 6.6 μS cm^{-2}.

The solution used during dissection and collagenase treatment was a modified Barth's solution (Barth & Barth, 1959) of the following composition (in mM): 88 NaCl, 3 KCl, 0.74 $CaCl_2$, 0.66 $NaNO_3$, 0.82 $MgSO_4$, 2.4 $NaHCO_3$; pH was adjusted to 7.6 using 5 mM Tris/HEPES buffer (Tris = tris(hydroxymethyl) aminomethane, HEPES = N-2-hydroxyethyl piperazine-N′-2-ethanesulfonic acid). KCl was omitted from the K-free incubation medium. The chloride-free experimental solution had the following composition (in mM): 88 Na HEPES, 2.4 $NaHCO_3$, 3 K HEPES, 0.82 $MgSO_4$, 0.74 $CaNO_3$, 5 4-aminopyridine (or 3,4-diaminopyridine), 5 Tris/HEPES buffer, at pH 7.6. Na-free, Cl-free solution was prepared by substitution of N-methylglucamine for Na. K HEPES was omitted in the K-free incubation medium. An aqueous stock solution of 10 mM dihydroouabain (DHO) (Sigma) was diluted 1,000-fold in the experimental solution to obtain a final concentration of 10 μM DHO. All experiments were conducted at room temperature (24 ± 2°C).

A conventional two-microelectrode voltage-clamp circuit was used to hold the membrane potential at the desired level. Command voltage steps or pulses were generated using a digital-to-analog converter system under computer program control (Apple IIe). The membrane current and voltage were measured at the end of each voltage pulse or stair step using a 12-bit analog-to-digital converter system. The resting conductance of each oocyte was measured at the initial holding potential using a sequence of 1-sec voltage pulses that were increased by 0.5 mV over a voltage range of ±5 mV from the holding potential. The pulses were separated by 4-sec recovery intervals at the holding potential. The *I-V* data were least-squares fitted by a polynomial to determine the slope conductance at the holding potential. Specific membrane conductance and current density were calculated on the basis of the mean value of the optically measured (80×) oocyte diameter assuming spherical geometry. This method underestimates the actual membrane area since the surface membrane of collagenase-treated oocytes is invaginated (Methfessel et al., 1986).

Two methods were used to determine more extensive *I-V* relationships. The first, used most frequently, was a down-up-down sequence of 1-sec stair steps that began from the holding potential (usually −40 mV), descended to the most negative test voltage (usually −120 mV), ascended from there to the most positive test voltage (usually +60 mV), and then returned in steps to the holding voltage. These staircases incorporated 1-sec, 5-mV decrements or increments at each step and so took approximately 1 min to complete depending on the voltage range examined. The second protocol consisted of a similar down-up-down sequence, but of discrete 1-sec voltage pulses decreased or increased 5 mV and separated by 4-sec intervals at the holding potential. This pulse protocol allowed sufficient time between pulses for the membrane current to return to its steady-state value at the −40 mV holding potential. Both the staircase and pulse protocols generated duplicate *I-V* data that showed significant hysteresis between the first and second current measure-

ments at each potential, indicating that time-dependent ionic currents were activated at extreme positive and negative voltages (*see* Fig. 4). Hysteresis in data obtained with the pulse protocol was less pronounced than with the staircase protocol, but the pulse sequence took five times longer to complete. The presence of hysteresis in *I-V*s obtained using the pulse protocol, despite the return of the holding current to its original value during the interpulse interval, suggests that the return of the holding current is not a sufficient criterion for re-establishment of steady-state conditions. For example, ionic currents that have a reversal potential near the holding potential, or ionic currents that are turned off quickly at the holding potential but recover from inactivation only slowly, will not produce significant current at the holding potential between pulses yet show hysteresis on depolarization. The pulse protocol adopted here was different from that used by Lafaire and Schwarz (1986). They used 20-mV voltage increments and 500-ms pulses applied every 5 sec, but did not repeat measurements at each voltage to determine whether hysteresis might have been evident in the resulting *I-V* relationship.

In addition to determining whether hysteresis was present between the duplicate *I-V* relationships, signalling that the voltage pulses had elicited slowly increasing or decreasing changes in membrane conductance, which persisted after the end of the pulse, we also repeated the *I-V* measurements under constant experimental conditions, at intervals of several minutes, to detect, and hence control for, any systematic trend or drift with time, e.g., a gradual increase in leak conductance (*see* Figs. 2, 3, and 5).

The voltage-measuring electrodes were filled with 3 M KCl and had resistances of 5–15 MΩ. Current-passing electrodes were filled with 2 M K citrate and had resistances of 3–10 MΩ. Current and voltage signals were filtered via 8-pole, low-pass Bessel filters with a 3 db cut-off frequency of 100 Hz. The *I-V* data are presented graphically as a sequence of data points connected by straight line segments. The 5-mV voltage increment used is sufficiently small, however, for the *I-V* relationships to appear as continuous curves.

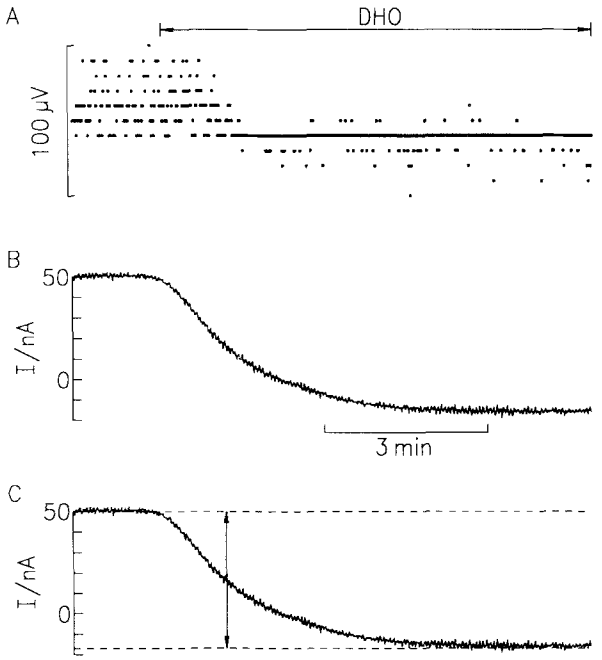

Fig. 1. Voltage-clamped membrane potential record and the change in holding current produced by addition of 10 μM DHO. A collagenase-treated oocyte, stored overnight in K-free Barth's solution to elevate intracellular [Na], was voltage clamped in normal (3 mM K) Barth's solution. (*A*) Membrane voltage. The membrane potential was held near −40 mV and maintained to within ±40 μV during the application of DHO. (*B*) Inward shift in holding current produced by DHO. The time course of the change in current is limited by the time required to completely change the external solution. (*C*) The magnitude of the change in holding current was determined by baseline extrapolation using linear least-squares fits as described in the text. The estimated change in holding current was 67 nA (1.3 μA cm^{-2}). Oocyte diameter was 1.23 mm

Results

The Na/K pump is electrogenic, normally transporting 3 Na outward for every 2 K transported inward (Post & Jolly, 1957; Garrahan & Glynn, 1967), and so produces a net outward current which, in the steady state, largely balances the discrepancy between passive inward Na and outward K currents. If this dynamic steady-state condition is disturbed by stopping the Na/K pump cycle with a cardiotonic steroid, such as ouabain or DHO, that acts specifically on the Na/K pump and has no other site of action, the abolition of outward pump current should cause a net inward shift of the current necessary to clamp the membrane at a constant voltage. The steroid-induced change in holding current, however, it not necessarily a direct measure of the magnitude of Na/K pump current. There are several possible sources of error: for example, steroid-induced, but nonpump-mediated, current changes can arise as a secondary consequence of the accumulation or depletion of ions in restricted diffusion spaces that will occur on stopping the Na/K pump (*e.g.*, Gadsby, 1982, 1984). In addition, it is essential to exclude from the estimation of cardiotonic steroid-sensitive current those systematic drifts or changes in recorded current that occur with time irrespective of the presence or absence of the steroid.

The records illustrated in Fig. 1 provide a means for estimating the maximum contribution from a further source of error, which arises from the fact that it is theoretically impossible to clamp the membrane potential at an absolutely constant voltage when the membrane current is made to vary. A small difference between the command potential and the measured membrane voltage is required as

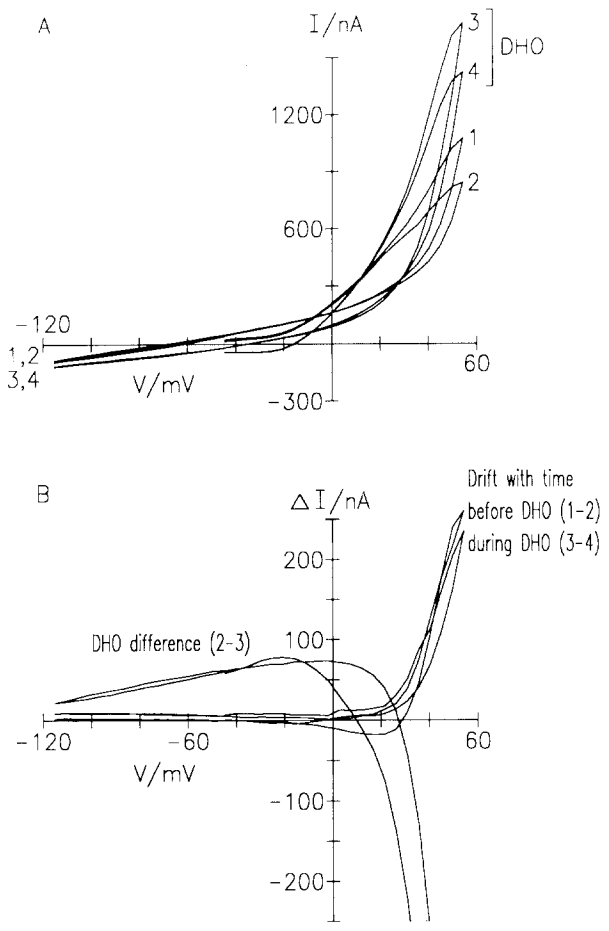

Fig. 2. DHO difference current-voltage (*I-V*) relationships and slow drift with time. (*A*) A 1-sec down-up-down voltage staircase protocol from a holding potential of −40 mV was used to measure 4 successive membrane *I-V* relationships. *I-Vs 1* and *2* were measured before the application of 10 μM DHO, and *3* and *4* were measured in the presence of DHO. Approximately 4 min elapsed between each successive *I-V*. (*B*) Difference *I-Vs* were determined by subtraction of each successive pair of *I-Vs*. The difference *1–2* shows slow changes that occurred during the 4-min interval before application of DHO. The difference *3–4* shows the slow current changes that occurred during a 4-min interval after DHO was applied. The difference *2–3* represents a combination of the current changes caused by action of DHO and drift with time. The presence of hysteresis in the DHO difference *I-V* relationship at voltages more positive than about −30 mV but less than +20 mV is an indication of contamination of the steady-state Na/K pump *I-V* by time-dependent ionic currents. Oocyte was the same as in Fig. 1

an input signal for the voltage-clamp amplifier to generate output current.

Collagenase-treated oocytes were stored for 2 days in K-free Barth's solution to inhibit the pump and elevate the internal Na concentration. The eggs were then returned to normal 3 mM K Barth's solution. In the experiment illustrated in Fig. 1, the membrane potential of the egg was held near −40 mV and the change in holding current upon application of 10 μM DHO was recorded. The current change produced by DHO is due primarily to the block of outwardly directed Na/K pump current and secondarily to any changes in ionic channel currents that result from ion accumulation or depletion. The measured DHO-induced shift will be underestimated by an amount we can calculate from the small change in the voltage signal (Fig. 1*A*, ca. 40 μV) and from the membrane slope conductance measured just before addition of DHO (104 μS cm^{-2} for this oocyte). The estimated error is 4.2 nA cm^{-2}, or 0.21 nA for this 1.28-mm diameter egg. This technically necessary current error is ~0.3% of the magnitude of the observed DHO-sensitive current of 67 nA.

The magnitude of the DHO-induced current change shown in Fig. 1*B* can be measured as shown in Fig. 1*C*. The baseline holding current data recorded before adding DHO are fitted with a straight line (sloping if necessary) using a linear least-squares technique, and the current data recorded in the period approximately 8 to 10 min after addition of DHO are similarly fitted. The magnitude of the DHO-sensitive current is then estimated as the distance between the two extrapolated baselines at the time when the current change reaches half the vertical distance between the extrapolated baselines (arrows in Fig. 1*C*). In this oocyte the DHO-induced current change was 67 nA, equivalent to a current density of 1.3 μA cm^{-2}. This current change is considerably greater than the value of 15 nA per oocyte reported by Lafaire and Schwarz (1986) at saturating concentrations of internal Na, and is also greater than the value of about 10 nA per oocyte reported by Eisner et al. (1987), also for Na-loaded oocytes at −40 mV. The reason for the almost fivefold greater DHO-sensitive current observed in our experiments is not clear. It is apparently not due to greater Na loading because the measurements of both other groups were made at levels of internal Na that were presumably close to saturating. Our currents, however, are smaller than the value of 90.5 nA per oocyte reported by Marx, Ruppersberg and Rüdel, (1987), who did not use collagenase to remove follicular cells. It seems possible, therefore, that some difference attributable to the collagenase, such as contaminating protease activity, might have resulted in a severe decrease in pump activity in the studies of Lafaire and Schwarz (1986), and a smaller decrease in our studies.

Figure 2 shows typical results of an experiment designed to measure the degree of hysteresis and to show systematic changes in membrane current over periods of several minutes. Both phenomena might be expected to make a DHO difference *I-V* relation-

ship, obtained by subtracting *I-V* data recorded in the presence of DHO from those recorded before its addition, an incorrect measure of the Na/K pump *I-V* relationship. *I-V* relationships were determined by a down-up-down 1-sec voltage staircase of 5-mV steps. The curves labeled *1* and *2* in Fig. 2*A* were obtained 4 min apart, 1 min before addition of 10 μM DHO. Those labeled *3* and *4* were also obtained 4 min apart, the former 3 min after adding DHO. That is, all four *I-V*s were determined at 4-min intervals in succession. All four *I-V* curves in Fig. 2*A* show significant hysteresis at positive voltages. The pattern is the same in all cases. During the ascending voltage staircase, an outward current seems to be activated, which persists during the subsequent descending voltage staircase from the most positive potential, +55 mV, but decreases in magnitude with hyperpolarization. Eventually the current crosses the *I-V* curve obtained during the ascending limb near 0 mV, and finally becomes somewhat more inward than the current recorded initially. This complicated "figure-eight" pattern of hysteresis suggests that both inward and outward components of ionic current are activated during the 1-sec staircases used. Indeed, both a voltage-dependent inward Na current activated during prolonged depolarizations (Baud, Kado & Marcher, 1982) and an outward, Ca-activated, chloride current (Miledi, 1982; Barish, 1983; Miledi & Parker, 1984) have been described and characterized in *Xenopus* oocytes.

This figure-eight pattern of hysteresis is quite reproducible over periods of several minutes, at least within the voltage range from −110 to 0 mV, as can be seen in Fig. 2*A*, and as demonstrated by the difference *I-V* relationships plotted in Fig. 2*B*. There is a significant systematic drift in ionic current with time only for voltages more positive than about +20 mV. Since systematic drift with time is small at negative potentials, such changes should not contribute greatly to the DHO difference *I-V* relationship in that voltage range, but are likely to have a major effect on the ability to estimate the DHO-sensitive current at potentials positive to +20 mV. The portion of the control records for ionic current drift with time that deviates significantly from the zero-current axis and that shows marked hysteresis is unpredictably variable in magnitude and direction. In Fig. 2, the portion of the time-drift control that shows hysteresis is positive and similar in magnitude both before and during exposure to DHO; in Fig. 3*B*, both controls are negative and differ by a factor of about 2 in magnitude; in Fig. 5*B* the control before DHO is negative while that during exposure to DHO is positive. This highly variable hysteresis at positive voltages may either add to or attenuate hysteresis in DHO difference *I-V*s in an unpredictable way. We, therefore, exclude from consideration as a valid measure of Na/K pump current any portion of a DHO difference *I-V* measured at voltages more positive than the voltage at which the time-drift control begins to deviate significantly from the zero-current axis and exhibit hysteresis.

The DHO difference *I-V* plot (*2–3*) shown in Fig. 2*B* was obtained by subtracting the *I-V* relationship recorded shortly after adding DHO (*3*) from that recorded just before DHO addition (*2*). The greater hysteresis from −20 to +20 mV between the difference currents originating from ascending and descending limbs of the voltage staircase in this *I-V* relationship (*2–3*) than in the "time-drift" difference curves (*1–2* and *3–4*) suggests that addition of DHO causes a change either in the magnitude of the persisting conductance activated during the ascending staircase or a change in its reversal potential. Since Na/K pump current is known to reach its steady-state magnitude within a few milliseconds following voltage steps (Gadsby et al., 1985; Bahinski, Nakao & Gadsby, 1988), the presence of hysteresis in DHO difference *I-V* curves at voltages where minimal drift with time occurs indicates that they include components other than Na/K pump current, and so provides a stringent criterion for excluding them from further consideration. By this criterion, the only region of the DHO difference *I-V* relationship in Fig. 2*B* that can be considered to represent Na/K pump current is that part negative to about −40 mV. The data indicate that Na/K pump current declines on hyperpolarization from −40 mV, and that pump current reversal does not take place until well beyond −120 mV. The down-turn in the DHO difference current at positive voltages is clearly due to contaminating ionic current, since there is marked hysteresis and since a second reversal potential at positive voltages is not expected for a fixed-stoichiometry 3Na/2K pump.

Figure 3 illustrates data obtained from an oocyte treated similarly to that in Fig. 2, but which fortuitously showed no significant hysteresis in the DHO difference *I-V* plot until about +30 mV. As in the previous experiment, a 1-sec down-up-down voltage staircase was used to determine *I-V* relationships between about −110 mV and +55 mV. Two sets of *I-V* data were measured before, and two after, adding DHO (Fig. 3*A*). In this oocyte, there was virtually no systematic drift with time of membrane current over the voltage range from −110 mV to +30 mV, as shown by the difference *I-V* plots in Fig. 3*B*. The DHO difference current is free of hysteresis over this wide voltage range and appears to be a saturating function of membrane

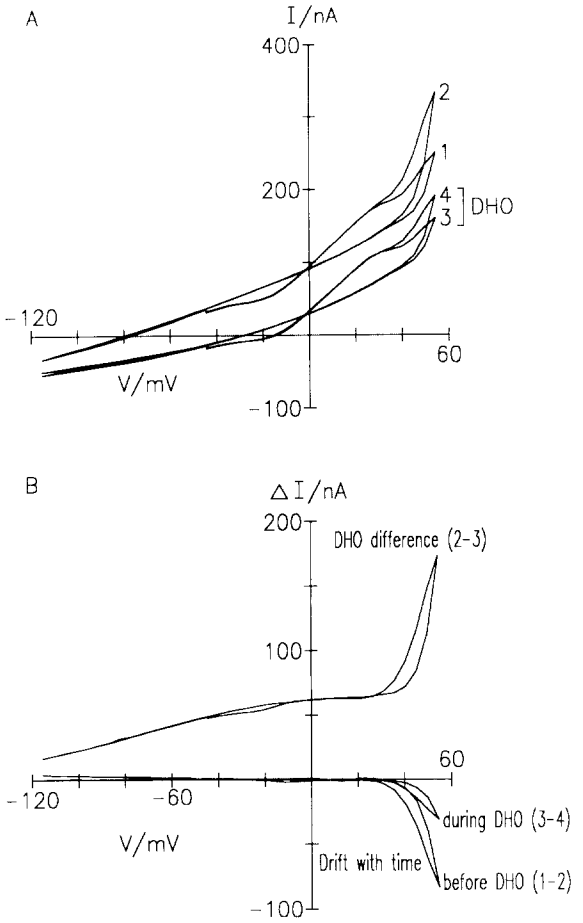

Fig. 3. Currents that give rise to hysteresis are not necessarily DHO-sensitive. (*A*) Four successive *I-V* relationships were determined in an oocyte using a 1-sec down-up-down voltage staircase as in Fig. 2. *I-Vs 1* and *2* were determined prior to the application of DHO, and *3* and *4* were determined in the presence of 10 μM DHO. (*B*) Slow changes in the *I-V* relationships with time were quite small as shown by the time-drift difference currents determined before (*1–2*) and after (*3–4*) the application of DHO. Holding potential was −40 mV. Oocyte diameter was 1.29 mm

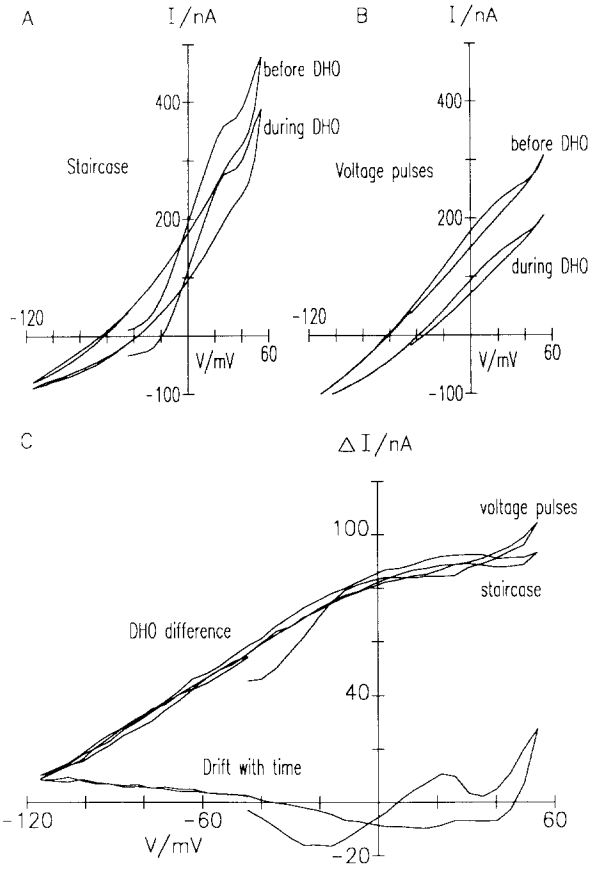

Fig. 4. Comparison of *I-V* relationships determined using both staircase and pulse protocols. (*A*) Membrane *I-V* relationships measured using a 1-sec down-up-down voltage staircase, before and after the application of 10 μM DHO. (*B*) Membrane *I-V* relationships measured using a down-up-down sequence of 1-sec voltage pulses separated by 4-sec intervals at the holding potential of −40 mV, before and after application of 10 μM DHO (same oocyte as in *A*). (*C*) DHO difference *I-V* relationships obtained by appropriate subtraction of the membrane *I-V* data shown in (*A*) and (*B*). Note the absence of significant hysteresis in the subtracted *I-V* curves despite the presence of hysteresis in the original *I-V* curves. The record of current drift with time was obtained by subtracting a final staircase *I-V* from the staircase *I-V* during exposure to DHO shown in (*A*). Oocyte diameter was 1.13 mm

potential. There is no suggestion of a region of negative slope at positive membrane potentials, although some drift of current with time opposite in sign to that observed in the two time-drift controls evidently contaminates the DHO difference *I-V* relationship at voltages more positive than about +30 mV.

The magnitude of the hysteresis in the original *I-V* curves in Fig. 3*A* is considerably greater than that in the DHO difference curve in Fig. 3*B* (note expanded scale), demonstrating that the ionic current responsible for the hysteresis seen without DHO is not necessarily altered by DHO. Conceivably, however, inhibition of the Na/K pump by DHO might cause changes in ion concentration that could, in turn, modify inward Ca current and/or outward Cl current (Miledi, 1982; Barish, 1983; Miledi & Parker, 1984), as well as K current, and thereby contribute to the estimated DHO difference current.

Steady-state Na/K pump *I-V* relationships are expected not only to be free of hysteresis, but also to be independent of the pulse protocol used to determine them. To verify this requirement, we carried out a series of experiments like that represented in Fig. 4. First, in the absence of DHO,

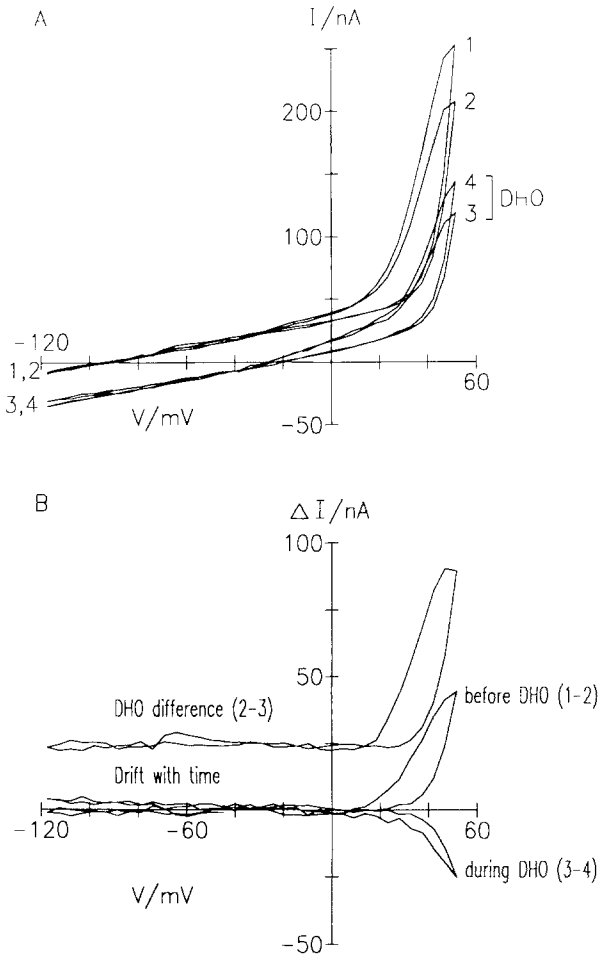

Fig. 5. DHO difference *I-V* relationship in Na-free (and Cl-free) external solution. Collagenase-treated oocytes were Na loaded by incubation for 2 days in K-free, Cl-free, Na-containing medium (*see* Materials and Methods). The experiment was then conducted in Na-free, Cl-free, 3 mM K solution using a 1-sec staircase protocol. (*A*) Four successive membrane *I-V* relationships measured in the same oocyte; *1* and *2* were obtained before the application of 10 μM DHO, and *3* and *4* were measured in the presence of DHO. (*B*) The four membrane *I-V* relationship in (*A*) have been subtracted to give difference *I-V* curves as indicated. Slow drifts in the *I-V* relationships are shown for the pair of *I-V* curves before DHO (*1–2*) and after DHO (*3–4*). Holding potential was −40 mV. Oocyte diameter was 1.21 mm

membrane *I-V* curves were obtained both by the down-up-down, 1-sec step, staircase protocol used for the previous figures, and by a down-up-down, 1-sec pulse protocol with pulses separated by 4-sec periods of recovery at the holding voltage, −40 mV. Current measurements at the various voltages were made in the same sequence with both protocols. These staircase and pulse protocols were then repeated after application of DHO. The resulting membrane *I-V* relationships are illustrated in Fig. 4*A* and 4*B*. They show clearly that the hysteresis elicited by the staircase protocol (Fig. 4*A*) is considerably greater than that produced by the pulse protocol (Fig. 4*B*), presumably because of the lack of recovery periods at −40 mV. However, despite the very different shapes of the membrane *I-V* relationships obtained with the two protocols, the DHO difference *I-V* relationship (Fig. 4*C*), obtained by appropriate subtraction, are nearly identical and show only slight hysteresis. An additional staircase *I-V* relationship was obtained in this oocyte at the conclusion of the experiment in the presence of DHO, to determine whether any systematic current drift with time occurred in the 12-min interval between this final staircase *I-V* and the previous staircase *I-V* measured during exposure to DHO. Since the time interval between staircase *I-V*s in DHO was 3 times longer than the 4-min interval required to measure the DHO difference *I-V*, and since an intervening voltage pulse protocol was done, the current drift with time shown in Fig. 4*C* represents a worst-case estimate of the time-drift error. The slight residual hysteresis present in the DHO difference traces can probably be attributed to currents associated with the drift with time.

The DHO difference *I-V* relationships shown in Fig. 4*C* constitute the best result obtained from experiments on a total of 39 oocytes using both staircase and pulse protocols, as judged by the virtual absence of hysteresis and the agreement of the curves obtained with the two alternative protocols. The DHO difference *I-V* curve shown in Fig. 4*C* is, consequently, most likely to be a reliable representation of the Na/K pump *I-V* relationship in *Xenopus* oocytes under these conditions. It is a simple saturating function of membrane potential with a sufficient hint of sigmoidicity at negative membrane potentials (*see also* Fig. 3*B*) to discourage the use of extrapolation to estimate the reversal potential for Na/K pump current. The estimate of the Na/K pump reversal potential is also complicated by the need to correct for current drift with time of about one-third of that shown in Fig. 4*C*. Again, there is no evidence to suggest the existence of a region of negative slope at positive membrane potentials.

Gadsby and Nakao (1987) working with cardiac myocytes and Rakowski et al. (1988*b*) with squid giant axons have shown that the Na/K pump current in Na-free external solution is relatively voltage independent. In Fig. 5, we show that this same result is obtained in *Xenopus* oocytes. Membrane *I-V* relationships obtained using a staircase protocol both before and during exposure to DHO show significant hysteresis in solutions free of both Na and Cl, as previously seen in normal Barth's solution. However, the hysteresis that occurs between about −20 and +20 mV is not altered by DHO as revealed

by the difference data shown in Fig. 5*B*. At voltages more positive than +20 mV, the DHO difference current appears to be contaminated by current drifts as suggested by the general shape of the hysteresis in the control *I-V* subtractions (*1–2* and *3–4*). Since the magnitude and direction of the hysteresis in the drift control records is different before and during DHO, no prediction of the effect of such drift on the DHO difference current can be made for voltages more positive than about +20 mV. In Na-free solution, the steady-state Na/K pump current appears to be practically voltage independent over the range −120 to +20 mV. The amplitude of the pump current in Na-free solution is lower than that measured in Na-loaded oocytes bathed in normal, 80 mM, external Na, but this might reflect loss of internal Na in Na-free solutions rather than being a direct consequence of low external [Na].

Discussion

We have reinvestigated the dihydroouabain-sensitive *I-V* relationship in Na-loaded *Xenopus* oocytes to determine if there is a region of negative slope that can be ascribed to the Na/K pump at positive membrane potentials. The stringent criterion that steady-state Na/K pump *I-V* relationships must be free of hysteresis has been applied in evaluating the data, and we have found that dihydroouabain-sensitive *I-V* relationships that do not show hysteresis are simple saturating sigmoid functions of membrane potential without any region of negative slope (Figs. 3*B* and 4*C*). In the absence of external Na, when the Na pump operates in a downhill mode, the Na/K pump current seems approximately voltage independent (Fig. 5). These results are similar to recent data obtained in cardiac myocytes (Gadsby & Noma, 1984; Gadsby et al., 1985; Gadsby & Nakao, 1987) and squid giant axons (Gadsby, Rakowski & De Weer, 1986; De Weer, Rakowski & Gadsby, 1987; Rakowski et al., 1988*b*).

When a region of negative slope does occur in DHO difference *I-V* relationships, it is accompanied by hysteresis (Fig. 2*B*). Two sources could contribute to such hysteresis: (i) slow drifts in the membrane *I-V* relationship that occur during the time required for DHO to act and, hence, for *I-V* measurements in the absence and presence of DHO to be recorded, and (ii) changes in ionic current that are a secondary consequence of stopping the pump. The presence of slow changes in *I-V* shape can be revealed by performing successive *I-V* measurements, under identical conditions, separated by the time interval required for DHO application. In most cases, these slow drifts become significant and show hysteresis only at voltages more positive than about +30 mV (*cf.* Figs. 2*B*, 3*B*, and 5*B*). The DHO difference *I-V* relationship usually exhibits additional hysteresis in the voltage range −30 to +30 mV. This suggests that DHO indirectly alters some time-dependent ionic current.

The DHO-sensitive "inward" current seen at positive potentials in Fig. 2*B* probably does not, in fact, reflect reduction of inward current in the presence of DHO, but rather an increase in an outward current, possibly carried by K ions and activated by external K as suggested by Fig. 12 of Lafaire and Schwarz (1986). Because of the order of subtraction of data in determining the DHO difference current (*2–3*), an augmented K-dependent outward current in DHO would give rise to a negative deflection in the difference *I-V* relationship. Since this K-dependent current is not blocked by tetraethylammonium (Lafaire & Schwarz, 1986), it represents a significant technical problem. We do not imply that DHO has any direct action on K conductance, but suggest an indirect action as a consequence of the extracellular K accumulation expected to occur upon stopping the Na/K pump. DHO-sensitive currents might also arise as a consequence of local changes in [Na] which might, in turn, affect Na/Ca exchange and, subsequently, Ca-dependent Cl current, or Ca-channel current. The time dependence that can produce hysteresis in the DHO difference *I-V* relationship, and thereby signal the presence of contaminating ionic currents, could reflect the time courses either of voltage-dependent gating processes or of changes in ion concentrations near the cell membrane, or both.

We wish to emphasize that it is not absolutely necessary for the "raw" *I-V* relationships to be free of hysteresis. In fact, as illustrated in Fig. 4*C*, identical DHO difference *I-V* curves, relatively free of hysteresis, can result from *I-V* measurements made with different protocols (staircase or pulse) that produce varying degrees of hysteresis in the *I-V* curves obtained before and after DHO addition. In that case, it can be further argued that the derived DHO-sensitive current is independent of the magnitude of the total membrane current recorded. At a voltage of +20 mV in Fig. 4*C*, for example, four different pairs of current subtractions yield essentially the same DHO difference current, enhancing confidence in that result.

The experiments do not rule out the possibility of contamination of the DHO difference current by *steady-state* nonpump currents; however, such currents are inferred to be small (or absent) by comparison of the present results with similar results from

cardiac myocytes (Gadsby et al., 1985) and squid giant axons (De Weer et al., 1987) in which such contaminating currents have been demonstrated to be small.

We have evaluated here the possible contribution of the technically necessary voltage-error signal to errors in the estimated Na/K pump current magnitude (Fig. 1) and find that this unavoidable error represents only 0.3% of the pump current magnitude under the conditions of our experiments.

De Weer and Rakowski (1984) reported evidence obtained on internally dialyzed squid giant axons that the *I-V* relationship of the Na/K pump made to operate in the reverse mode, by lowering internal ATP and removing internal Na and external K, appeared to include a region of negative slope. More recent data, collected using an additional end-pool clamp system to eliminate current flow from the cannulated ends of the axon and in the presence of 200 mM internal tetraethylammonium and 20 mM internal phenylpropyltriethylammonium to diminish K conductance (Rakowski, De Weer & Gadsby, 1988*a*), have shown this not to be the case, in agreement with results obtained on the reverse Na/K pump in isolated cardiac myocytes (Bahinski et al., 1988). Therefore, there is no compelling evidence for a region of negative slope in either the forward or reverse Na/K pump *I-V* relationship. Given the absence of a region of negative slope, there is no necessity to postulate the existence of more than one voltage-dependent step in the overall Na/K pump cycle. Nakao and Gadsby (1986) have described strophanthidin-sensitive membrane charge movements in cardiac myocytes under experimental conditions expected to support pump-mediated Na/Na exchange, and Bahinski et al. (1988) have demonstrated the absence of pump-mediated charge movements under conditions of K/K exchange. Taken together, these results suggest that the principal or only voltage-dependent step in the Na/K pump cycle is a Na-dependent step (De Weer, Gadsby & Rakowski, 1988*a*,*b*).

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