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Voltage Dependence of Current through the Na,K-Exchange Pump of *Rana* Oocytes

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Summary. We have studied current (I_{Str}) through the Na,K pump in amphibian oocytes under conditions designed to minimize parallel undesired currents. Specifically, I_{Str} was measured as the strophanthidin-sensitive current in the presence of Ba^{2-}, Cd^{2+} and gluconate (in place of external Cl^-). In addition, I_{Str} was studied only after the difference currents from successive applications and washouts of strophanthidin (Str) were reproducible. The dose-response relationship to Str in four oocytes displayed a mean $K_{0.5}$ of 0.4 μM, with 2–5 μM producing 84–93% pump block. From baseline data with 12 Na^+-preloaded oocytes, voltage clamped in the range [−170, +50 mV] with and without 2–5 μM Str, the average I_{Str} depended directly on V_m up to a plateau at 0 mV with interpolated zero current at −165 mV. In three oocytes, lowering the external [Na^+] markedly decreased the voltage sensitivity of I_p, while producing only a small change in the maximal outward I_{Str}. In contrast, decreasing the external [K^+] from 25 to 2.5 mM reduced I_{Str} at 0 mV without substantially affecting its voltage dependence. At K^+ concentrations of ≤ 1 mM, both the absolute value of I_{Str} at 0 mV and the slope conductance were reduced. In eight oocytes, the activation of the averaged I_{Str} by $[K^+]_o$ over the voltage interval [−30, +30 mV] was well fit by the Hill equation, with $K' = 1.7 \pm 0.4$ mM and n_H (the minimum number of K^+ binding sites) = 1.7 ± 0.4. The results unequivocally establish that the cardiotonic-sensitive current of *Rana* oocytes displays only a positive slope conductance for $[K^+]_o > 1$ mM. There is therefore no need to postulate more than one voltage-sensitive step in the cycling of the Na,K pump under physiologic conditions. The effects of varying external Na^+ and K^+ are consistent with results obtained in other tissues and may reflect an ion-well effect.

Key Words amphibian oocyte · Na,K-exchange pump · strophanthidin · sodium · potassium · voltage sensitivity

Introduction

Na,K-activated ATPase is a nearly ubiquitous enzyme which subserves the function of the Na,K-exchange pump (Skou, 1957). Under physiologic conditions, 3 Na^+ are extruded from the cell, 2 K^+ accumulated, and 1 ATP utilized during each cycle (Albers, Koval & Siegel, 1968; Post et al., 1969). Since one positive charge is ejected per cycle, the pump must be voltage sensitive. Until very recently, efforts to detect such voltage sensitivity under physiologic conditions had been inconclusive (Glynn, 1984; De Weer, Gadsby & Rakowski, 1988). However, work reported over the past five to six years has now documented the voltage dependence of the pump in: cardiac myocytes (Gadsby, Kimura & Noma, 1985; Gadsby & Nakao, 1989), *Xenopus* oocytes (Lafaire & Schwarz, 1986; Eisner, Valdeolmillos & Wray, 1987; Rakowski & Paxson, 1988; Schweigert, Lafaire & Schwarz, 1988), *Rana* oocytes (Wu & Civan, 1988, 1989, 1990), squid giant axon (Rakowski, Gadsby & De Weer, 1989), and reconstituted vesicles (Goldshlegger et al., 1987).

Although the pump is voltage sensitive, the precise nature of the voltage dependence has been unclear. At issue has been the observation by Schwarz and his colleagues (Lafaire & Schwarz, 1986; Schweigert et al., 1988) in the *Xenopus* oocyte, that the current-voltage relationship of the pump displays two regions of voltage sensitivity under physiologic conditions. A positive slope conductance was noted at negative membrane potentials. In this domain, the membrane current was a monotonic, slightly sigmodal function of voltage, consistent with measurements in the cardiac myocyte (Gadsby & Nakao, 1989), squid giant axon (Rakowski et al., 1989), and *Rana* oocyte (Wu & Civan, 1989, 1990). In addition, a negative slope conductance was observed at positive membrane potentials. In the latter domain, the current was monotonically and inversely dependent on voltage. The issue of this second conductance has at least two important implications. First, as pointed out by Lafaire and Schwarz (1986), a region of negative conductance would suggest a second, voltage-dependent step in the pump cycle. Second, in order

to model transport across epithelia (e.g., Civan, 1983) and biological cells in general, more definitive information concerning the voltage dependence of the pump current is necessary.

Rakowski and Paxson (1988) have recently concluded from their study of *Xenopus* oocytes that only one voltage-dependent step need be postulated. However, their study has still not resolved the issue because: (*i*) most of their work was based on measuring current responses to staircase voltage commands, an approach which introduced hysteresis; (*ii*) the instability of the membrane current at positive potentials required that they base their conclusions largely on study of a single preparation free of hysteresis; and (*iii*) Rakowski, Vasilets and Schwarz (1990) have since re-examined the problem and again concluded that, at least under some conditions, amphibian oocytes do display both a negative and a positive conductance.

In addressing this issue, we have modified previous experimental approaches in several respects. First, we have increased the absolute magnitude of the pump current by studying *Rana,* rather than *Xenopus,* oocytes. The former cells have surface areas about 2–4 times larger than those of the latter, while displaying a similar pump density (Weinstein et al., 1982; Richter, Jung & Passow, 1984). Second, we have minimized the contributions of pathways in parallel with the pump current measured as the strophanthidin-sensitive current (I_{Str}). Third, we have chosen the concentration of cardiotonic steroid and experimental protocol to avoid potentially significant contributions to I_{Str} of irreversible effects of pump blockade. With these precautions, we have found that the pump current of *Rana* oocytes displays only a single region of voltage sensitivity, having a positive slope conductance for the negative membrane potentials studied under physiologic conditions.

Materials and Methods

Oocyte Preparation

The mature amphibian oocyte is surrounded by cell and protein layers, which must be removed before voltage-clamp experiments. The diameters of denuded or defolliculated mature oocytes were ≈1.5–2.0 mm, in contrast with those of *Xenopus* stage VI oocytes (≈1 mm).

In the early phases of this study, oocytes were obtained from sacrificed *Rana pipiens*. In later experiments, oocytes were obtained surgically from *Rana* kept on a daily regimen of mealworms. These *Rana* were injected once intraperitoneally with 20–30 IU of human chorionic gonadotropin (CG-2, Sigma Chemical) at least one month prior to use. A portion of the ovarian lobe was extracted from a small incision along the side of the abdomen from frogs anesthetized by 10–15 min immersion in 0.15% MS-222 (A-5040, Sigma) in tap water. Oocytes were denuded or defolliculated by the method of Masui (1967), as modified by Weinstein et al. (1982). In this method, oocytes from the ovarian lobes were preincubated for 20–30 min in a Ca^{2+}-free Ringer's solution containing 2 mM EDTA (ethylene-diamine-tetraacetic acid). The follicle cells were then removed manually with fine-tipped forceps under a dissecting microscope. Denuded cells were incubated at room temperature (20–22°C) either in Ringer's solution without EDTA for ≈1 hr, or in a K^--free Ringer's solution for 3–24 hr prior to study.

Solutions

The Ca^{2+}-free Ringer's solution used to prepare the oocytes for defolliculation contained (in mM): 110 NaCl, 2 KCl, 1 $MgCl_2$, 2 EDTA, and 5/5 HEPES/NaHEPES (N-2-hydroxyethyl piperazine N'-2-ethane sulfonic acid). The pH was titrated to 7.45 with 1 M NaOH, and the osmolality was 220–225 mOsm. The composition of the OK Ringer's incubation solution consisted of (in mM): 110 NaCl, 1 $CaCl_2$, 1 $MgCl_2$, 5/5 HEPES/NaHEPES, 5 glucose, pH 7.45. Baseline Ringer's solution consisted of (in mM): 110 NaCl, 2 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 5/5 HEPES/NaHEPES, at a pH of 7.5. Gluconate (G) Ringer's solution contained (in mM): 120 NaG, 3 KG, 3 hemi-CaG, 1 hemi-MgG, 2 $CdCl_2$, 1 $BaCl_2$, 5 glucose, and either 5/5 NaHEPES/acid HEPES or 5/5 Tris/HEPES. The pH was titrated to 7.45 with HCl, and the osmolality was 240–245 mOsm. In some experiments, the external Na^+ and K^+ were partially replaced by the impermeant cation N-methyl-D-glucamine (NMDG). When K^+ was varied, the total concentration of NMDG + K^+ was maintained at 25 mM; in those experiments, the [Na^+] in the gluconate Ringer's solution was reduced from 120 to 100 mM. Strophanthidin (S-6626, Sigma) was solubilized in a stock solution of 95% ethanol at 50 mM. Dihydroouabain (D-9267, Sigma) was kept in a stock solution of deionized H_2O at 5 mM. Pump inhibitors were diluted to desired final concentrations just prior to use in the experiments.

Bath Solution Changing Protocol

In all cases, to gauge the effect of a test solution, the holding current was monitored during the application of the solution. Application was considered complete when the holding current level ceased to change during the application of the test solution. This typically occurred well within 40 sec, corresponding to <6 ml of solution. Washout to the control solution was similarly monitored. For ion substitution experiments, and some channel blocker experiments, washout to control levels of holding current took about as long as the application time. Pump inhibitors typically took ≈5 min to wash out, corresponding to ≈40 ml of solution.

Chamber and Impalements

The incubated, denuded oocytes were transferred to a Lucite chamber with a volume of ~1.5 ml, permitting complete volume replacement within 12 sec at a solution flow rate of 8 ml/min. The chamber was mounted securely in the bottom of a binocular dissecting microscope placed on a vibration-isolation table. Solutions were gravity fed from 50-cc polyethylene reservoirs, providing a flow rate through the chamber of ≈10 ml/min. A small

indentation in the soft dental wax in the center of the trough prevented the oocytes from moving during micropipette impalement or during change of solution. The cells were impaled with separate voltage-sensing and current-passing micropipettes. The micropipettes were pulled on a horizontal puller (Flaming-Brown P80/PC, Sutter Instrument, San Rafael, CA), and the tips broken back by touching them momentarily against Kimwipes. The micropipettes had resistances of 2–5 MΩ when filled with 1 M KCL (voltage electrode) or 1 M K acetate (current electrode) solutions.

Shielding and Current Pathways

To minimize capacitance between the voltage and current electrodes, a grounded brass plate was placed between them. A coating of Sylgard was placed around the electrodes starting from ≈0.5 mm from the tip and up to the unpulled section of the electrode to further minimize capacitance and noise due to the meniscus formed by the surface of the bath solution and the electrode shank. The chamber bath solution was connected, via a 1-M KCl agar bridge, to a small pool of saturated KCl grounded through a Ag/AgCl wire.

Electronics

The cells were voltage clamped with a two-electrode system, using an intracellular amplifier (Dagan 8500, Dagan, Minneapolis, MN). The amplifier's current-injection circuitry was modified to pass more current during the first 5 msec of a commanded pulse. Amplified signals were routed to an oscilloscope for continuous monitoring and to a pulse code modulator (Sony PCM-501ES, Sony Co. of America, Park Ridge, NJ) with its frequency response extended down to DC, using a variation of the circuitry published by Bezanilla (1985). The Butterworth low-pass filters in the pulse code modulator (PCM) were replaced with 4-pole Bessel filters for better phase-delay response. The video output of the PCM was stored on a video cassette recorder for later playback and analysis. In some experiments the data were digitized directly and stored on floppy disk. Voltage command signals to the intracellular amplifier were provided by data acquisition software (Notebook, Laboratory Technologies, Wilmington, MA) driving an analog-to-digital/digital-to-analog board (DT2801A A/D-D/A. Data Translation, Framingham, MA) installed in a PC AT-compatible computer. The data acquisition software was used during playback and redigitization of experiments from tape, or during experiments for direct storage on disk. Signals were filtered at 15 kHz with an 8-pole Bessel filter (902, Frequency Devices, Haverhill, MA) and sampled at 200 Hz.

Voltage Pulse Protocol

In the majority of experiments, the oocytes were voltage clamped at a holding potential (V_h) of −50 mV, which was close to the resting potential of the majority of oocytes. Except during the voltage pulse trains and brief moments when the clamp was turned off to record the resting potential, the oocytes were continuously clamped at the holding potential. In a few experiments the cells were held at −60 or −70 mV, but this was rarely done because large hyperpolarization-activated currents (*see* Fig. 1*A*) were very slow to deactivate upon repolarization. The slow deactivation affected the baseline holding current for subsequent pulses. The

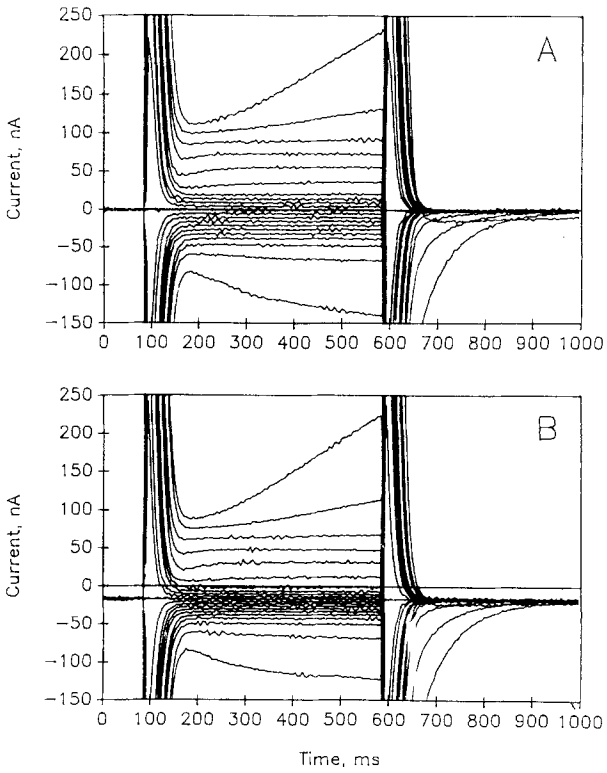

Fig. 1. Effect of strophanthidin on whole-cell currents. Time course of currents measured in the presence of baseline Ringer's solution before (*A*) and after (*B*) adding 50 μM strophanthidin. V_h = −50 mV for both *A* and *B*. Resting potential of the oocyte was −60 mV for *A*, −20 mV for *B*. The lowermost current traces correspond to a clamp potential of −150 mV, and the uppermost current traces to +50 mV. All intervening current traces are separated by 10 mV

voltage pulse train consisted of alternating positive-going and negative-going pulses increasing in 10-mV steps from the holding potential. The pulses were 500 msec long and spaced 3 sec apart to allow for passive redistribution of ions at the holding potential after the voltage stimulus. At V_h = −50 mV, the range of membrane potentials (V_m) tested was −150 to +50 mV. To obtain the difference current between control and test cases, point-by-point subtraction of test currents from control currents was performed at each corresponding clamp potential. To generate data for current-voltage plots, 20 points were averaged, usually over the last 100 msec of each 500-msec test pulse. Over this time period, most of the active and passive conductances were presumed to have reached steady state. This was generally true for experiments done in gluconate Ringer's solution.

Results

Baseline Measurements

Figure 1*A* shows the typical currents elicited in the mature *Rana* oocyte by polarizing pulses at a holding potential (V_h) of −50 mV. The membrane conduc-

tance increased at both hyperpolarized and depolarized voltages. This outward and inward rectification is typical of egg membranes (Hagiwara & Jaffe, 1979). The large capacitative transients were also typical of the *Rana* oocyte. In later experiments, the voltage-clamp amplifier was modified to reduce the charging time. It should be noted, that the current used for steady-state pump current measurements (the 100-msec interval starting 400 msec after initiating the voltage pulse) was unaffected by the initial capacitative transient. It is clear from Fig. 1*A* that the transient decayed to steady state well within 200 msec after beginning the pulse.

Strophanthidin-Sensitive Current Without Channel Blockers

Initial efforts at obtaining the strophanthidin-sensitive currents (I_{Str}) in the *Rana* oocyte were done without ion channel blockers. As illustrated by Fig. 1*A* and 1*B*, adding 50 μM strophanthidin produced a small but noticeable decrease in membrane conductance. The small inward shift of the holding current in Fig. 1*B* reflects pump inhibition by the aglycone. Difference currents (I_{Str}) were obtained by the point-by-point subtraction of the currents in Fig. 1*B* from those of Fig. 1*A* at the corresponding voltages. These currents are presented as functions of test voltage in Fig. 2*A*. The absolute currents of Fig. 1*A* and *B* (and corresponding difference currents) display clear time dependences at both -150 and $+50$ mV.

The qualitative shape of the I_{Str}-V_m relationship of Fig. 2*A* was seen while studying 18 of an initial series of 27 oocytes, untreated with channel blockers. However, at some point in their study, seven of the oocytes displayed the relationship of Fig. 2*B*. Finally, 10 oocytes of the series even displayed a positive conductance over the range of positive membrane potentials. Six of the oocytes, like that of Fig. 2, displayed qualitatively different voltage dependence at different times during the course of study.

One can reasonably explain the coincidence of the region of variability of the I_{Str}-V_m curves with the onset of time-dependent currents by assuming that the background conductances change in the presence of strophanthidin. Because the pump contributes only a fraction of the total membrane current, any change in ion channel conductance can produce large current artifacts in the derived I_{Str}-V_m relations. On the basis of this reasoning, experiments were conducted involving ionic substitutions and channel inhibitors in an effort to minimize contributions to I_{Str} from sources other than the pump.

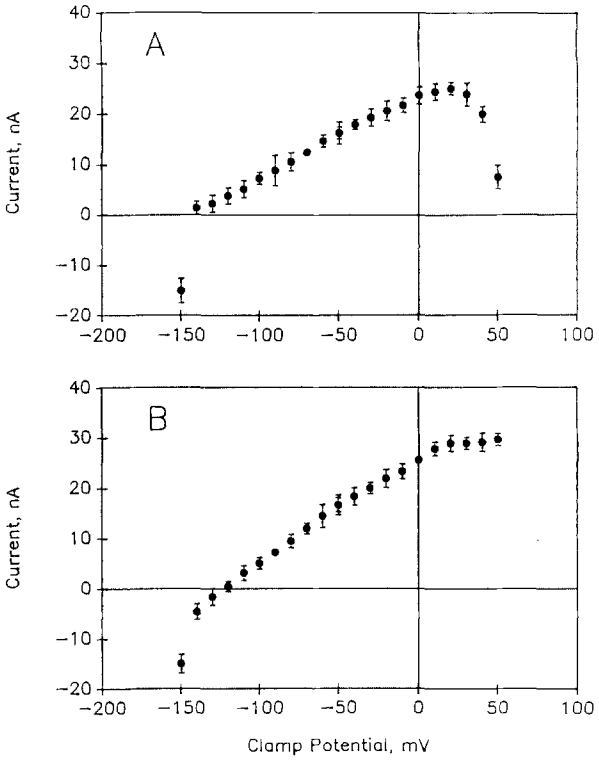

Fig. 2. Strophanthidin-sensitive current as a function of voltage. (*A*) Average of the difference currents obtained by point-by-point subtraction of currents in Fig. 1*B* from corresponding currents in Fig. 1*A*, plotted against stimulus potential. (*B*) Average strophanthidin-sensitive currents in the same oocyte as *A* at a later time. Average difference currents at each stimulating potential were taken from 20 points, starting 400 msec after the start of the stimulus voltage pulse, to the end of the pulse 100 msec later. Error bars present ±1 SD

Contributions of K^+ Channels to I_{Str}

Several considerations suggested that current through voltage-activated K^+ channels was a likely basis for the variable response of I_{Str} to positive membrane potentials (Wu & Civan, 1988): (*i*) the oocyte membrane has been reported to display both delayed and inward rectifiers (Hagiwara, Miyazaki & Rosenthal, 1976); (*ii*) in a preliminary study of four oocytes, we observed a hyperpolarization-activated, Ba^{2+}-blockable current, similar to the inward rectifier reported for *Mediaster* oocytes (Hagiwara et al., 1976); and (*iii*) the putative changes in background conductance produced by blocking the pump were likely to arise from the accumulation of either cytosolic Na^+ or extracellular K^+ in diffusion-limited layers. Figure 3 presents representative results obtained by alternately clamping the external $[K^+]$ ($[K^+]_o$) at 2 and at 0 mM in a series of 11 oocytes bathed with strophanthidin. It is clear from the plot

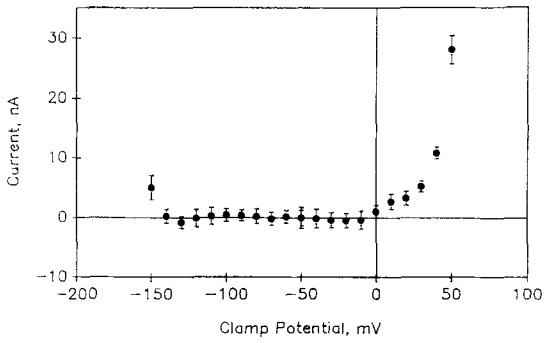

Fig. 3. K^+-sensitive currents in strophanthidin (0, 2 mM K^+). Average of the difference currents, obtained by point-by-point subtraction of currents in the presence of strophanthidin with 0 mM K^+ from currents obtained in the presence of strophanthidin with 2 mM K^+, plotted against stimulus potential. $V_h = -50$ mV. Error bars present ±1 SD

of average difference currents *vs.* clamp potential that the outward current across the depolarized membrane is larger with $[K^+]_o$ fixed at 2 mM than at 0 mM. This observation is consistent with the operation of the K^+ inward rectifier (Hagiwara et al., 1976). The datum suggests that pump inhibition, leading to an increase in the K^+ activity at the extracellular surface of the membrane, could activate an extra component of outward current during membrane depolarization. An apparent negative conductance at depolarized potentials could thereby be generated by subtracting these currents from the control strophanthidin-untreated currents. This reasoning leads to the expectation that raising the external K^+ concentration should suppress the apparent negative conductance.

This prediction was tested by alternately clamping the $[K^+]_o$ at 2 and 10 mM in studying a series of eight oocytes bathed with strophanthidin. Contrary to expectation, the difference currents still displayed variable responses to depolarizing test pulses. These results indicate that all of the variability in voltage sensitivity displayed by I_{Str} (for positive values of V_m) could not be ascribed solely to voltage-activated K^+ channels. These channels do likely contribute to that variability (Fig. 3), so that 1–2 mM Ba^{2+} was included in the bathing medium later chosen to rigorously define the I_{Str}-V_m relationship. However, one or more additional voltage-activated parallel conductances seemed to be playing a role.

Voltage-Activated Cl^- Conductance

Calcium-activated Cl^- conductances have been seen in *Xenopus* oocytes by Miledi and Parker (1984) and in *Rana* oocytes by Cross (1981). Co^{2+} is known to block the Ca^{2+} channel (Hagiwara & Takahashi, 1967) and is effective in abolishing the Ca^{2+}-dependent current in *Xenopus* oocytes (Barish, 1983; Miledi & Parker, 1984). With this background, eight preliminary experiments were conducted in which 10 mM concentrations of Co^{2+} or Cd^{2+} were added to the media bathing the *Rana* oocytes. In the absence of other inhibitors, the Co^{2+} or Cd^{2+} markedly inhibited the slowly activating outward current produced by depolarization and reduced the total membrane conductance of the oocyte membrane. For this reason, 1 mM Cd^{2+} or Co^{2+} was included in the bathing medium used in the later studies of I_{Str}.

Although Cd^{2+} and Co^{2+} likely suppressed some Cl^- channels, additional Cl^- channels remained operative. In a series of five oocytes exposed to 1 mM Co^{2+} and 0.5 mM Ba^{2+}, extracellular Cl^- was replaced by 112 mM gluconate. The depolarization-activated outward currents were thereby reduced. A voltage-activated Cl^- conductance has also been described in *Xenopus* oocytes (Barish, 1983). To suppress current through the Cl^- channels, the medium finally used for studying the I_{Str}-V_m relationship also contained gluconate in place of Cl^-.

Figure 4 presents whole-cell currents measured with an oocyte bathed with the final gluconate Ringer's medium chosen for the major phase of the study. Displayed are the records obtained before addition of strophanthidin (Fig. 4*A*), during exposure to 2 μM strophanthidin (Fig. 4*B*), and after washoff of the aglycone (Fig. 4*C*). The withdrawal of external Cl^- and the addition of 1 mM Ba^{2+} and Cd^{2+} have suppressed the large time- and voltage-dependent currents illustrated in Fig. 1.

Dose-Response Experiments with Cardiotonic Steroids

It was valuable to determine the dose-response relationship of strophanthidin in order to strike a balance between two conflicting technical aims. First, we wished to block as many pump units as possible in order to maximize the strophanthidin difference currents. On the other hand, we also wished to limit the dosage of the pump blocker in order to minimize the washout time. Otherwise, the experiment becomes so long as to lead to run-down problems (*cf.* Gadsby & Nakao, 1989).

Figure 5*A* shows strophanthidin-sensitive currents for a single oocyte exposed to varying concentrations of the aglycone, plotted against stimulus potential. Note that all the curves cross through zero current at ≃ −130 mV and that all are simply scaled versions of each other. This implies that the partially inhibited pump yields an I_{Str} that does not differ in

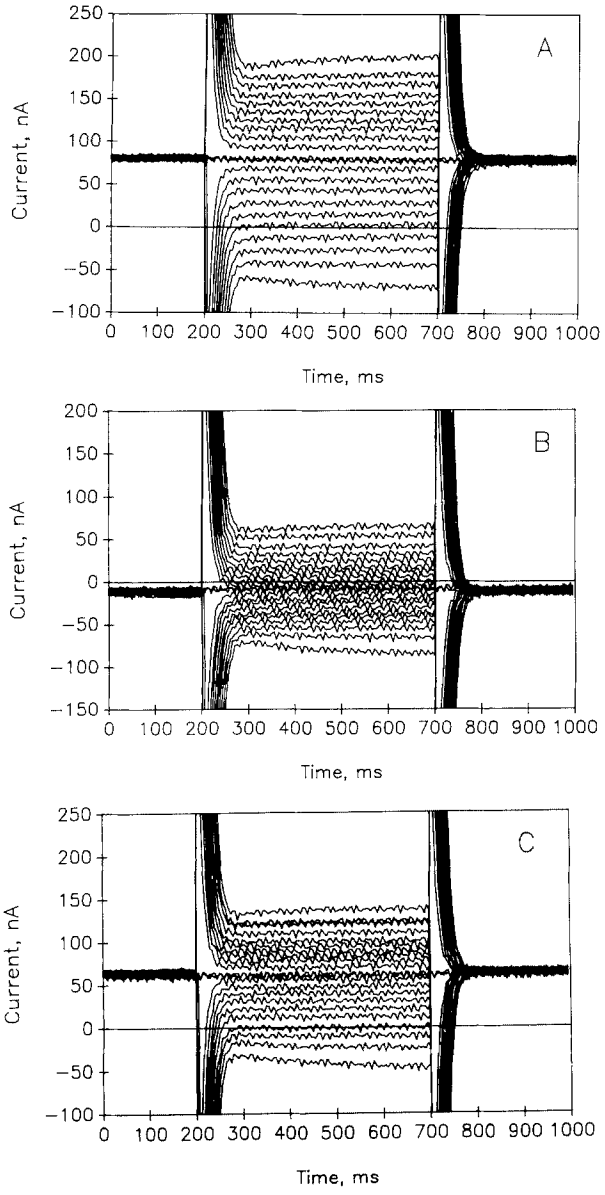

Fig. 4. Effect of strophanthidin on whole-cell currents of an oocyte bathed with gluconate-Ringer's solution containing 1 mM Ba^{2+} and 1 mM Cd^{2+}. Records (*A*), (*B*) and (*C*) were obtained before, during and after perfusion with 2 μM strophanthidin, respectively. The oocyte had been previously exposed to one cycle of washing the aglycone on and off.

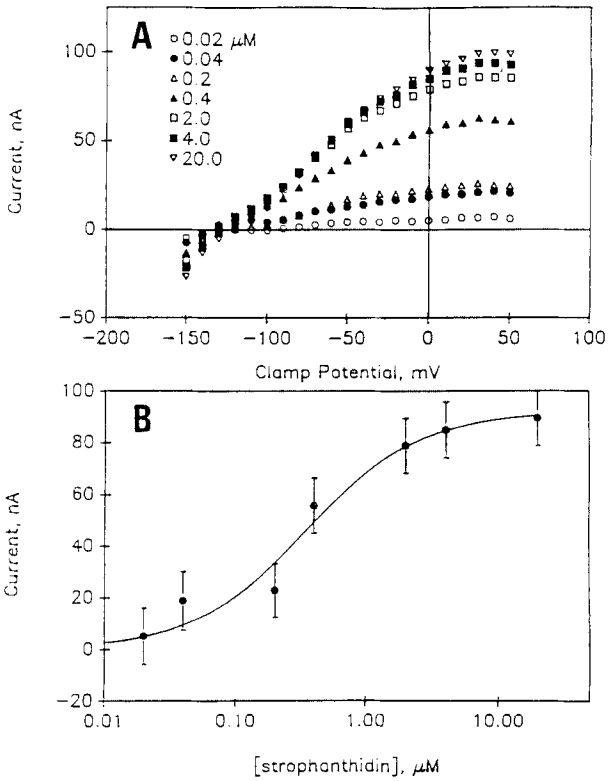

Fig. 5. Strophanthidin dose response. Increasing concentrations of strophanthidin were applied at 2-min intervals to a single oocyte continuously exposed to 1 mM Cd^{2+} and 1 mM Ba^{2+}. The measurements were obtained 45 sec after each addition of strophanthidin, 15–20 sec after the holding potential had reached a stable level. (*A*) Strophanthidin-sensitive currents, obtained at a range of concentrations, plotted against stimulus potential. Order of symbol legends represents order of application of doses of strophanthidin. (*B*) Strophanthidin current at 0 mV plotted against applied dose. Solid line represents a Michaelis-Menten fit with V_{max} = 92.2 nA, $K_{0.5}$ = 0.35 μM. Error bars represent ± 1 SD

voltage dependence from the fully inhibited pump-derived I_{Str}. Figure 5*B* shows the current data at 0 mV of Fig. 5*A* plotted against strophanthidin concentration. The solid line is a Michaelis-Menten fit with $K_{0.5}$ = 0.35 μM and V_{max} = 92.2 nA. The average $K_{0.5}$ for the oocyte of Fig. 5, in the interval [−50, +50 mV] was 0.34 μM. The average $K_{0.5}$ of four oocytes was 0.38 ± 0.07 μM. For the majority of experiments, either 2 or 5 μM strophanthidin was used, corresponding to either 84 or 93% pump inhibition, respectively. Application of the aglycone at these concentrations produced a relatively rapid and complete block of the pump current,usually within 40 sec. Washout at these doses of strophanthidin required an ~5-min wash at 8–10 ml solution/min for maximum recovery of pump activity.

Another commonly used pump inhibitor, dihydroouabain (DHO), was also tested. A nonlinear Michaelis-Menten fit of two DHO dose-response curves (*not shown*) at 0 mV gave an average $K_{0.5}$ = 2.0 μM in 1 mM $BaCl_2$. For comparison, the values of 0.40 ± 0.04 μM without $BaCl_2$, and 3.3 ± 0.55 μM in 5 mM $BaCl_2$ at V_m = +20 mV have been reported in *Xenopus* oocytes by Schweigert et al. (1988). This is consistent with the lower $K_{0.5}$ observed in the present study, where the external [Ba^{2+}] was 1 mM. DHO has been used to inhibit the pump in *Xenopus*

oocytes (Lafaire & Schwarz, 1986; Rakowski & Paxson, 1988; Schweigert et al., 1988).

Time Dependence of Channel Block

In all experiments conducted with 50 μM strophanthidin, the difference currents derived from the control and initial application currents were quantitatively distinct from those derived from the washout control and initial application currents. This may reflect the existence of two populations of the Na,K exchange pump in *Rana* oocytes. The observed effect could be due to a large difference in binding affinity for the aglycone in the two populations. Due to the large unstirred layer at the surface of the oocyte, it may be very difficult to wash off strophanthidin from the high affinity population of the pump. It is, however, not possible to distinguish the two-population, two-affinity explanation from one in which there is only a single pump population, but in which some fraction of the pumps is in diffusion-limited spaces obstructing washout.

During long experiments ($\geq$1 hr) with a particular oocyte, the total membrane conductance of the voltage-clamped oocyte clearly decreased with time. This was possibly due to run-down of the parallel conductances or to the time required for the Ba^{2+}, Co^{2+} and Cd^{2+} to have access to and block their target channels. It was also clear that the I_{Str} became more reproducible while the conductance decreased. To monitor this effect of decreasing conductance of I_{Str}, the holding current just before the stimulating voltage pulse was monitored along with I_{Str}. In Fig. 6*A*, the estimates of I_{Str} derived from application of strophanthidin (filled circles) are different from those derived from the washout of strophanthidin (open circles). The two sets of estimates differ in the magnitudes of the peak currents and qualitatively in their voltage-dependences in the depolarized region (above -10 mV). In the depolarized domain, the washout I_{Str} exhibits negative conductance. Note also the variability in the currents at potentials more negative than -130 mV. Figure 6*B* displays the corresponding differences in holding current measured just before initiating the voltage pulse. The filled circles represent data points generated by subtracting the holding currents in the presence of strophanthidin from the corresponding holding currents prior to addition of inhibitor. The open circles symbolize the values calculated by subtracting the holding currents in the presence of strophanthidin from those after washoff. Both sets of holding difference currents exhibit pronounced "wing" effects. This presumably reflects instability in the holding current after large hyperpolarizing and

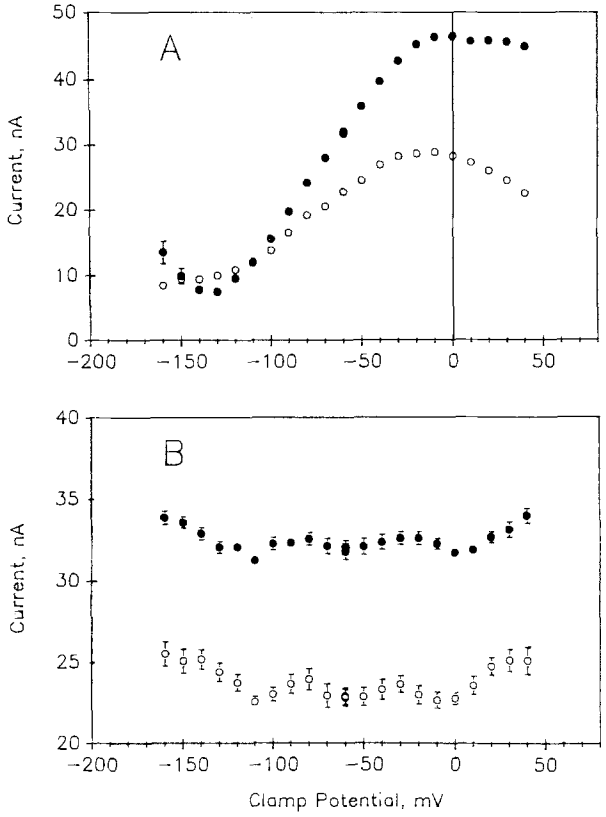

Fig. 6. Early pump currents and holding current. (*A*) Pump current obtained by subtracting the currents from the first application of strophanthidin from those in control solution (filled circles) and from those in control solution after washing out the aglycone (open circles), plotted against stimulus potential. (*B*) Difference holding currents (at $V_h = -60$ mV) representing the 20 time points just before the voltage stimulus, plotted against stimulus potential. Data points in *B* correspond to data with matching symbols in *A*. Error bars present $\pm$ 1 SD

depolarizing voltage pulses open up ion channels. In contrast to I_{Str} in Fig. 6*A*, in the same oocyte ~40 min later, after two complete cycles of applying and washing out strophanthidin, the application I_{Str} (open circles) and washout I_p (filled circles) in Fig. 7*A* are nearly identical, save for a slight difference in peak current magnitude. The "wing" effect is also much less apparent in the plot of the corresponding holding difference currents in Fig. 7*B*, indicating that the holding currents had reached steady states in the intervals between successive test pulses.

Recognizing that it was possible to get reproducible I_{Str} by cycling an oocyte through several applications and washouts of strophanthidin, the I_{Str} of four oocytes from the same frog were obtained by this method. The pump current of the four oocytes was averaged, with the I_{Str} of each oocyte itself a mean of four different current measurements. For an oocyte diameter of 1.5 mm, linear-regression analysis

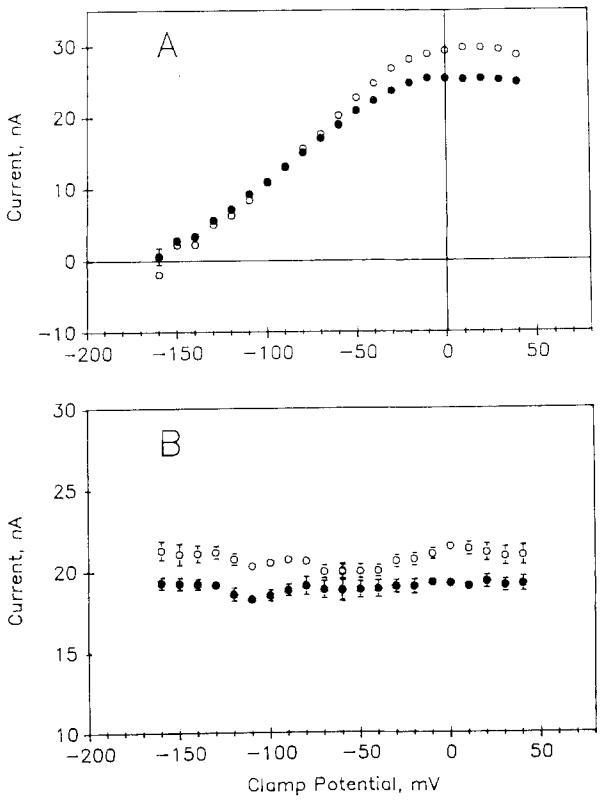

Fig. 7. Late pump current and holding current. Same oocyte as that of Fig. 5, ≈40 min later. (*A*) Pump current obtained by subtracting the currents from the first application of strophanthidin from those in control solution (open circles) and from those in control solution after washing out the aglycone (filled circles), plotted against stimulus potential. (*B*) Difference holding currents (at $V_h = -60$ mV) representing the 20 time points just before the voltage stimulus, plotted against stimulus potential. Data points in *B* correspond to data with matching symbols in *A*. Error bars present ± 1 SD

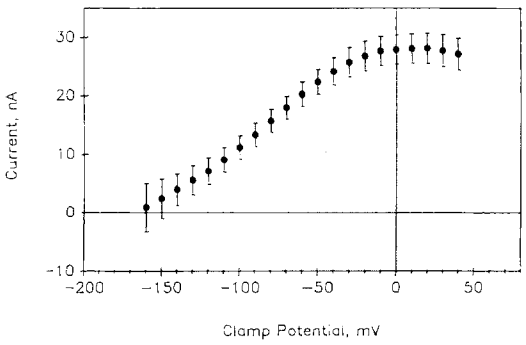

Fig. 8. Average of scaled I_{Str}. Mean pump current from 12 oocytes that yielded bracketed, reproducible I_{Str}-V_m relations (i.e., like the curves of Fig. 7) were scaled to peak currents at 0 mV and then averaged together. Error bars represent ± 1 SD. The external Na^+ and K^+ concentrations were 120 and 3.0 mM, respectively

yielded a conductance of 3.2 $\mu S \cdot cm^{-2}$. Assuming a density of 1000 pumps · μm^{-2} (Weinstein et al., 1982; Richter et al., 1984), the measured total conductance is equivalent to a single pump conductance of ~32 attoS (10^{-18} S). This estimate is comparable to the values of ~60 attoS in cardiac myocytes (calculated from Fig. 6*C* of Gadsby & Nakao, 1989) and ~60 attoS in squid giant axon (Rakowski et al., 1989).

I_{Str} in Standard Gluconate Ringer's Solution

Having established the proper dosage of strophanthidin and the optimum number of application and washout cycles, the average I_{Str} of 12 oocytes was obtained. This average I_{Str} is plotted in Fig. 8. Since each oocyte had a different peak current magnitude, it was necessary to scale all the individual I_{Str} to the peak current value at 0 mV before averaging. The curve is remarkably smooth with relatively small, similarly sized standard deviations, suggesting that the parallel conductances (which vary considerably from oocyte to oocyte) in the 12 oocytes were effectively blocked. It is very similar in shape to the I_{Str} seen by Gadsby and Nakao (1989) in cardiac myocytes, displaying a monotonically rising phase with increasing membrane depolarization and saturating at a plateau region at large depolarizations. There is a hint of a sag in the plateau at extreme depolarizations, as seen also in Gadsby and Nakao (1989), but it is not statistically significant. The plateau was reached at ≃ 0 mV. The zero current potential was estimated to be ≃ −165 mV, from the intercept of a linear-regression analysis.

Effect on I_{Str} of Changes in External Sodium Concentration

The dependence of I_{Str} on external $[Na^+]$ ($[Na^+]_o$) has been shown in a number of preparations (Béhé & Turin, 1984; Gadsby & Nakao, 1987; Rakowski & Paxson, 1988; Nakao & Gadsby, 1989; Rakowski et al., 1989). These reports have shown that the sensitivity of the pump current to voltage is greater at high than at low $[Na^+]_o$, with little or no sensitivity in Na^+-free medium. To test the effect of varying $[Na^+]_o$ on I_{Str} of the *Rana* oocyte, a series of bracketed experiments were performed. In each case, $[Na^+]_o$ was lowered by replacement with N-methyl-D-glucamine, maintaining $[K^+]_o$ constant at 3 mM. Figure 9*A* shows the results obtained with one oocyte. Because of possible run-down effects and peak-current enhancements at low external $[Na^+]$, all data from a given oocyte were normalized to the

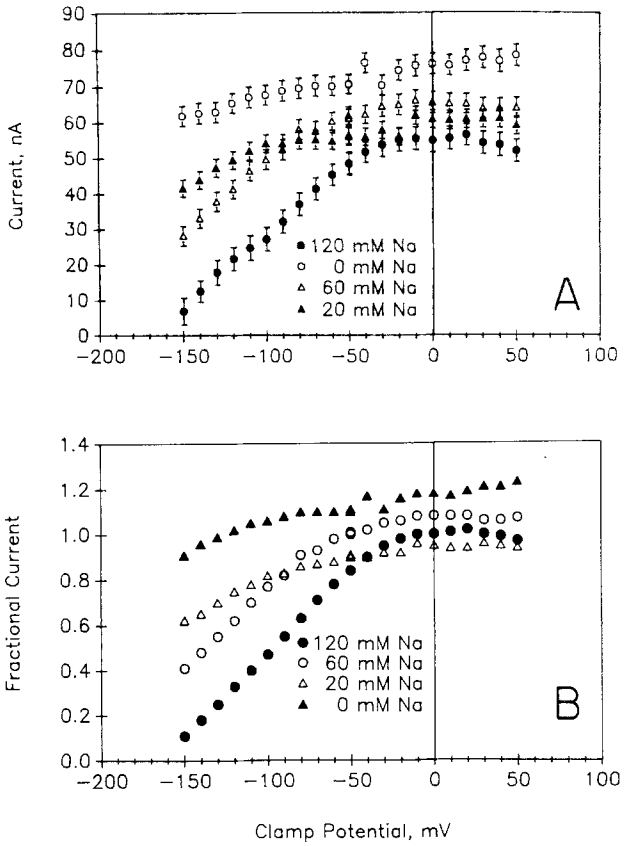

Fig. 9. Effect of external $[Na^+]$ on I_{Str}. Order of symbol legends indicates order of solution application. (*A*) Unscaled current of a single oocyte plotted against stimulus potential at four different values of $[Na^+]_o$. (*B*) Averaged values obtained from measurements with three oocytes. For each oocyte, the values of I_{Str} have been normalized to the currents at 120 mM $[Na^+]_o$ and 0 mV. Solution-change sequences were chosen to reverse the directions of the expected slope changes with each change of $[Na^+]_o$. $V_h = -50$ mV. Indications of the uncertainties have been omitted from *B* for clarity. All three oocytes were studied at 120 and 20 mM $[Na^+]_o$. At 0 mV, the fractional I_{Str} was 0.95 ± 0.16 (mean ± SE for 20 mM Na^+). Two measurements were obtained at the other Na^+ concentrations. The two measurements of the mean (± SE) at 0 mV for the other Na^+ concentrations were: (0 mM), 0.97 ± 0.03 and 1.39 ± 0.02; (60 mM), 0.97 ± 0.02 and 1.194 ± 0.021

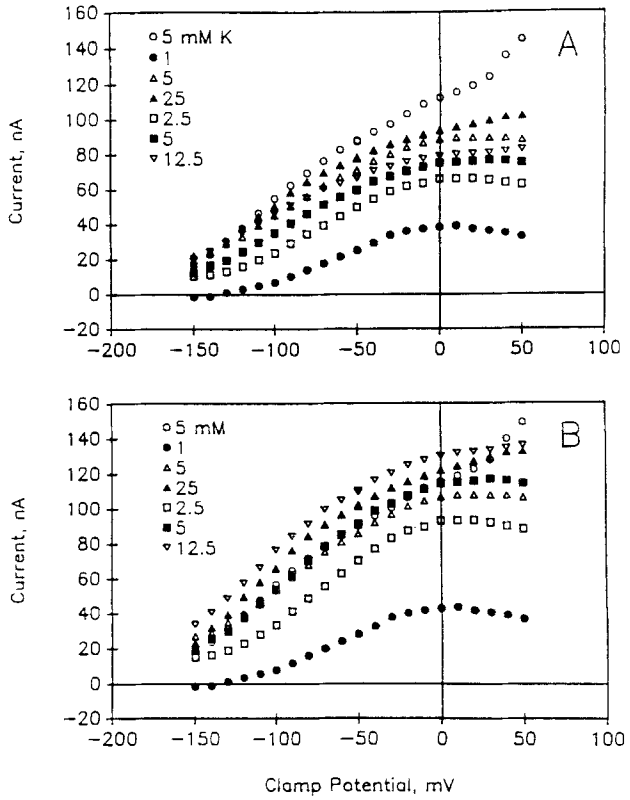

Fig. 10. Effect of external $[K^+]$ on I_{Str}. Order of symbol legends indicates order of solution application. (*A*) Pump current plotted against stimulus potential at five different values of $[K^+]_o$. (*B*) Rundown-corrected pump current of *A* plotted against stimulus potential. Solution-change sequence was chosen to allow correction of run-down of pump current by bracketing solution applications with $[K^+]_o = 5$ mM. $V_h = -50$ mV. $[Na^+]_o = 100$ mM. Error bars left out for clarity

values of I_{Str} at 0 mV. The direction in which the $[Na^+]$ was changed—increasing or decreasing from test solution to test solution—was varied to distinguish responses due to the change in test solution from possible long-term trends or drift in overall current response. The order of the symbol legends, appearing as an inset in Fig. 9*A*, corresponds to the order in which the test solutions were applied.

The voltage sensitivity was correlated only with the change in $[Na^+]_o$. Elevating the external Na^+ concentration markedly increased the voltage sensitivity. In contrast, the effect of $[Na^+]_o$ on the peak difference current was relatively modest. The averaged values obtained from three oocytes (Fig. 9*B*), suggest that I_{Str} at 0 mV was generally inversely dependent on $[Na^+]_o$. However, this was not uniformly observed. For example, the peak value of I_{Str} at 20 mM Na^+ was slightly (but not significantly) less than that at 120 mM. Furthermore, in one of the two oocytes subjected to a step change in $[Na^+]_o$ from 120 to 0 mM, the fractional I_{Str} was insignificantly changed (decreasing by 0.03 ± 0.03).

Effect on I_{Str} of Changes in External K^+ Concentration

At a fixed $[Na^+]_o$ of 100 mM, I_{Str} was measured as a function of $[K^+]_o$. Figure 10 summarizes some of the results of these experiments. The uncorrected I_{Str}-V_m relationships are presented in Fig. 10*A*. To take into account the run-down of pump current due to the extended length of each experiment (4–6 hr), the current data were run-down-corrected relative

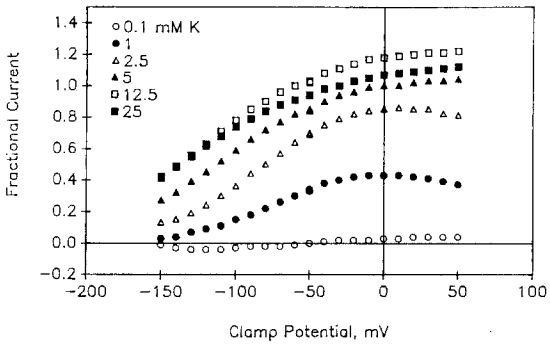

Fig. 11. Influence of external $[K^+]$ on I_{Str}. Order of symbol legends indicates order of solution application. Combined data from eight oocytes. Data from each oocyte were first corrected for run-down, then scaled to the average of the 0-mV current values from the bracketing 5-mM $[K^+]_o$ runs. The scaled values were then averaged at each external $[K^+]_o$. For clarity, the error bars display ± 1 SE, rather than 1 SD. Most of the values of SE are sufficiently small to be included within the data symbols

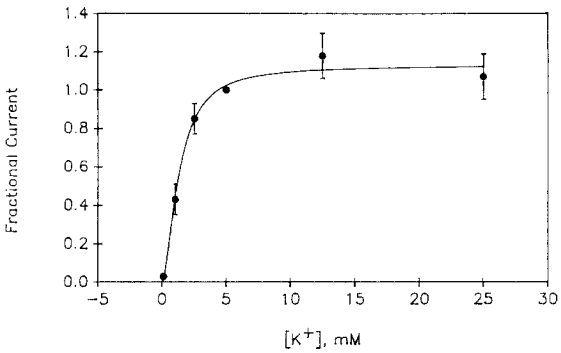

Fig. 12. Saturating activation of I_{Str} by external $[K^+]$. Current values at 0 mV from the six curves of Fig. 11 plotted against $[K^-]_o$. The solid line is a least-squares, nonlinear fit of the Hill equation:

$$\frac{v}{V_{max}} = \frac{\{[K^+]_o\}^{n_H}}{\{[K^+]_o\}^{n_H} + K'}$$

where v is taken to be the fractional current, the relative V_{max} = 1.13, K' = 1.62 mM, and n_H = 1.70

to currents obtained at 5 mM K^+. This was done by obtaining the 0-mV current values at each of the 5 mM K^+ runs for a given oocyte. Together with the times of the 5 mM K^+ runs (with the first 5 mM K^+ run set to time = 0), an exponential was fit through these 0-mV current values. Using the fitted exponential as a model of pump run-down, the time (t) at each K^+ run was used to calculate a number, $f_r(t)$, representing the fraction of pump still operating. Dividing the pump current at each $[K^+]_o$ run by its corresponding $f_r(t)$ then yielded the back-corrected current values. The validity of an exponential run-down of I_p is supported by similar run-down findings in cardiac myocytes by Gadsby and Nakao (1989). The run-down-corrected data are presented in Figure 10*B*. Using the run-down-corrected data, the curves for a given oocyte were normalized to the average 0-mV current value at 5 mM external $[K^+]$, and the resulting 5 mM K^+—normalized data were averaged across eight oocytes. This result is presented in Fig. 11.

Over the range from 0.1 to 12.5 mM, lowering the $[K^+]_o$ progressively reduced I_{Str} measured at 0 mV. The slightly anomalous shape and low peak current of I_p at 25 mM $[K_o]$ may be due to the competition of K^+ with strophanthidin for the binding site (Forbush, 1983). The voltage-insensitive current ($V_m \geq 0$ mV) was clearly K^+ dependent, but reducing $[K^+]_o$ had very little effect on the voltage sensitivity of I_{Str} over the concentration range 2.5 to 25.0 mM. This qualitative impression was quantified by calculating the maximum value of the slope conductance, using the method of least squares. For each $[K^+]_o$, the value of the slope (± SE) and the voltage range, over which the calculation was performed, were: (*i*) 25.0 mM, 6.65 ± 0.04 V^{-1}, [−150, −120] mV; (*ii*) 12.5 mM, 7.2 ± 0.1 V^{-1}, [−130, −90] mV; (*iii*) 5.0 mM, 6.85 ± 0.08 V^{-1}, [−120, −90] mV; and (*iv*) 2.5 mM, 6.8 ± 0.1 V^{-1}, [−100, −60] mV. At 1.0 mM K^+, the maximum slope {over the range [−80, −40] mV} was reduced significantly to 3.83 ± 0.06 V^{-1}. At a still lower $[K^+]_o$ of 0.1 mM, the voltage dependence was of doubtful significance; the maximum slope conductance calculated over the range [−80, −40] mV was 0.88 ± 0.05 V^{-1}. We should emphasize that, in the presence of strophanthidin, the $[K^+]_o$ did not significantly affect the voltage dependence of current from oocytes bathed with Cl^--free medium containing Ba^{2+} and Cd^{2+}. With strophanthidin present, the difference current obtained by subtracting the currents measured at 1 mM from those at 25 mM $[K^+]_o$ generated a flat line parallel to the V_m axis. The maximum deviation from this line was less than a single SE of the I_{Str}-V_m relationships at these $[K^+]_o$ presented in Fig. 11.

The currents at 0 mV (from Fig. 11) are plotted against external K^+ concentration in Fig. 12. The solid line through the data represents a nonlinear Hill fit with K' = 1.62 mM, relative V_{max} = 1.13 and n_H = 1.70. The average of the seven Hill fits across the interval [−30, +30 mV] of the averaged I_p in Fig. 11 gives a K' of 1.67 ± 0.35 mM, relative maximal current of 1.12 ± 0.05, and n_H of 1.71 ± 0.36 (at a $[Na^+]_o$ of 100 mM), in comparison to $K_{0.5}$ of 1.50 ± 0.32 mM and relative maximal current of 1.30 ± 0.10 at $[Na_o]$ = 150 mM and 0 mV (Nakao & Gadsby, 1989), and $K_{0.5}$ of 1.9 mM, n_H of 1.7 at −70 mV (Stimers, Shigeto & Lieberman, 1990) for cardiac

myocytes, and $K' = 2.3$, $n_H = 1.68$ determined at -28 mV in *Xenopus* oocytes (Marx, Ruppersberg & Rodel, 1987).

Discussion

Baseline Measurements of Pump Current

The true current through the Na,K-exchange pump (I_p) is estimated as the current inhibitable by cardiotonic steroids (I_{Str}) more reliably than as the change in current produced by removing Na^+, K^+ or ATP (De Weer et al., 1988). Nevertheless, I_{Str} is only $\simeq$20% of the total transmembrane current of amphibian oocytes, so that several factors can distort the apparent voltage sensitivity of I_p. Our data indicate that voltage-produced changes in K^+, Cl^- and Ca^{2+} fluxes (through pathways parallel to the pump) can substantially alter the voltage sensitivity of I_{Str}. To minimize contaminations from these active components, it has been essential to preincubate *Rana* oocytes with Ba^{2+} and Cd^{2+} or Co^{2+} in Cl^--free media for $\geq$ 30 min. A second source of contamination is hysteresis. To reduce this contribution, we have applied voltage pulses of alternating sign, rather than of staircase format (Rakowski & Paxson, 1988). Finally, the data can be potentially distorted by time-dependent spontaneous events or by secondary changes induced by cardiotonic steroids. We have addressed this problem by using low concentrations of strophanthidin (the aglycone of ouabain), facilitating rapid washoff of the inhibitor. With these precautions, we have been able to impose a stringent criterion for data acceptability. Results have been retained for analysis only from those oocytes displaying reversibility of pump inhibition. Under our conditions, $\simeq$7–16% of the pump current was not blocked. However, as documented by Fig. 5*A*, the voltage dependence of I_p (when normalized) is identical whether the pump is only partly or fully inhibited.

Figure 8 presents the results obtained with 12 *Rana* oocytes under our baseline conditions, with an external K^+ concentration ($[K^+]_o$) of 3 mM. Over negative membrane potentials, the normalized I_{Str} displays the positive slope conductance described in other tissues. At positive membrane potentials, I_{Str} is independent of voltage. We believe that Fig. 8 unequivocally documents that the negative slope conductance, sometimes observed in *Xenopus* and *Rana* oocytes at similar values of $[K^+]_o$ (Fig. 2*A*; Lafaire & Schwarz, 1986), does not reflect the true voltage sensitivity of pump current during physiologic cycling of the pump. This point is important since [as emphasized by Gadsby and Nakao (1989)] the putative negative slope conductance of I_{Str} in amphibian oocytes has constituted the sole datum requiring postulation of more than one voltage-sensitive step in the pump cycle.

Effects of External Na^+ and K^+ on the Voltage Sensitivity of I_{Str}

Lowering the external Na^+ concentration ($[Na^+]_o$) is expected to increase the effective affinity of the external binding site for K^+ and thereby speed forward cycling of the pump (e.g., Civan & Bookman, 1982). Contrary to this expectation, the magnitude and even sign of the response to removing external Na^+ has been reported to vary among the preparations studied. At a membrane potential of 0 mV, I_{Str} is increased by $\simeq$20% in squid axon (Rakowski et al., 1989), increased by only $\simeq$5% in cardiac myocytes (Nakao & Gadsby, 1989), and actually lowered in *Xenopus* oocytes (Béhé & Turin, 1984; Rakowski & Paxson, 1988). From the mean of two measurements (Fig. 9*B*), eliminating external Na^+ entirely increased I_{Str} at 0 mV by approximately 18%. However, the effect of reducing $[Na^+]_o$ was inconstant, and (as illustrated by the data of Fig. 9*B* at 20 mM external $[Na^+]_o$) a consistent inverse dependence was not observed. The variable response possibly arises from polarization effects at unstirred interfaces (Nakao & Gadsby, 1989).

The far more striking effect of reducing $[Na^+]_o$ was to lower the positive slope conductance over the range of negative membrane potentials. This datum is consistent with observations in cardiac myocytes (Gadsby & Nakao, 1987; Nakao & Gadsby, 1989), squid giant axon (Rakowski et al., 1989), and *Xenopus* oocytes (Béhé & Turin, 1984; Rakowski & Paxson, 1988) and contrasts strongly with the effects of changing external K^+ ($[K^+]_o$). Reducing $[K^+]_o$ over the range from 25 to 2.5 mM reduces I_{Str} at 0 mV by about a third without substantially altering the maximum slope conductance. The simplest and most direct interpretation is that Na^+ translocation is directly involved in the voltage-sensitive step, and K^+ translocation is not. This interpretation is consistent with measurements of current, ionic fluxes, and transient charge movements in other whole-cell and reconstituted-membrane preparations (Nakao & Gadsby, 1986; Borlinghaus, Apell & Läuger, 1987; Fendler, Grell & Bamberg, 1987; Goldshlegger et al., 1987; Bahinski, Nakao & Gadsby, 1988; De Weer et al., 1988).

In principle, the charge movement could reflect translocations of the Na^+-binding site, migration of Na^+ in access channels from the external medium

to the binding site within the membrane, and rotations of dipolar groups within the plasma membrane (Läuger & Apell, 1986). The first of these possibilities has long been considered of likely importance. Specifically, physiological outward charge movement could be associated with the conformational change of the $(Na_3)E_1$-P occluded form to the P-$E_2 \cdot Na_3$ deoccluded form of the enzyme (Glynn, 1984). Flux studies in vesicle suspensions and measurements of transient charge movements in cardiac myocytes and reconstituted membrane systems have strongly supported that concept (De Weer et al., 1988). The forward translocation of 3 Na^+ ions, together with two negative charges of the enzyme binding sites, are thought to produce outward movement of a single positive charge per cycle.

More recently, Läuger and his colleagues have pointed out that part of the transmembrane potential might be imposed between the external medium and the alkali-ion binding sites within the membrane and close to the cytoplasmic phase (Läuger & Apell, 1986; Läuger, 1990). Voltage drops across such deep access channels could provide the basis for the observed effects of $[Na^+]_o$ on the slope conductance of I_{Str} (Läuger & Apell, 1986; Nakao & Gadsby, 1989; Läuger, 1990). The higher external Na^+ concentrations would favor voltage-sensitive backflux through the access channels, whereas such a phenomenon would be lost in Na^+-free solutions.

Potassium exchanging between the external bath and the binding sites would also be subject to the voltage across the access channels. Nakao and Gadsby (1989) found little change in the voltage sensitivity of I_{Str} when $[K^+]_o$ was varied between 2.7 and 5.4 mM. They concluded that the effect of $[K^+]_o$ on I_{Str} over this concentration range must reflect cycle properties other than voltage-dependent K^+ binding. As documented by Fig. 11, a similar finding was observed with *Rana* oocytes bathed with a considerably wider range of external K^+ concentrations, from 2.5 to 25 mM. However, lowering $[K^+]_o$ further to 1.0 mM reduced the slope conductance at negative membrane potentials, as well as the magnitude of I_{Str} at $V_m = 0$ mV. A similar effect is apparent in the data of Nakao and Gadsby (their Fig. 9*B*, 1989). At this concentration, K^+ migration in the access channels may become rate limiting.

Significance of Voltage Sensitivity of Pump Current

The voltage sensitivity of the pump current is of computational and physiologic importance. Until recently, investigators have frequently modeled the transport characteristics of the pump as a constant current generator independent of membrane potential (e.g., Lew, Ferreira & Moura, 1979; Lindemann, 1979; Civan & Bookman, 1982). This computational approach is no longer appropriate. The monotonic increase in pump current with increasing depolarization has now been noted (for negative membrane potentials) in all preparations whose voltage dependence has been seriously studied over the past five years. Furthermore, the magnitude of the effect is not inconsequential, increasing by a little less than 1% mV^{-1} between -165 and -30 mV (*see* Fig. 8). One simple approach for simulating current through the pump (Civan et al., 1989) is to consider I_p as a product of three factors: the maximum current, a term dependent on the Na^+ and K^+ activities on the two surfaces of the membrane, and a voltage-sensitive term $[g(V_m)] \cdot g(V_m)$ can be most simply approximated as a linear term $[(165 + V_m)/165]$ over the range $-165 < V_m \leq 0$ mV and can be fixed at one [for $V_m > 0$ mV].

The physiologic implications of an electrogenic, rather than an electroneutral pump [such as that incorporated in the original model of Koefoed-Johnsen and Ussing (1958)], are less obvious. Depending on the parallel membrane conductance, increased turnover of the pump can certainly hyperpolarize some cells significantly (e.g., Nagel et al., 1980), thereby activating or inactivating parallel voltage-dependent processes. Perhaps more importantly, the voltage sensitivity may permit the pump to respond rapidly to changes in the external environment, which would otherwise be detected much more slowly and indirectly through changes in the intracellular activities of Na^+ and K^+. For example, rapid elevations in the extracellular $[K^+]$ have little direct effect on pump cycling because physiologic levels are several-fold times greater than the K_m (of $\simeq$1.7 mM) for the external activating site (*see* Fig. 12). On the other hand, depolarizations produced by sudden increases in external $[K^+]$ should immediately stimulate Na^+-K^+ exchange through the pump. This characteristic of the pump may permit buffering of the potentially lethal effects produced by rapid elevations in plasma $[K^+]$.

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