Na,K Pump Stimulation by Intracellular Na in Isolated, Intact Sheep Cardiac Purkinje Fibers

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ABSTRACT Regulation of the Na,K pump in intact cells is strongly associated with the level of intracellular Na^+. Experiments were carried out on intact, isolated sheep Purkinje strands at 37°C. Membrane potential (V_m) was measured by an open-tipped glass electrode and intracellular Na^+ activity (a^i_{Na}) was calculated from the voltage difference between an Na^+-selective microelectrode (ETH 227) and V_m. In some experiments, intracellular potassium (a^i_K) or chloride (a^i_{Cl}) was measured by a third separate microelectrode. Strands were loaded by Na,K pump inhibition produced by K^+ removal and by increasing Na^+ leak by removing Mg^{++} and lowering free Ca^{++} to 10^{-8} M. Equilibrium with outside levels of Na^+ was reached within 30–60 min. During sequential addition of 6 mM Mg^{++} and reduction of Na^+ to 2.4 mM, the cells maintained a stable a^i_{Na} ranging between 25 and 90 mM and V_m was -30.8 ± 2.2 mV. The Na,K pump was reactivated with 30 mM Rb^+ or K^+. V_m increased over 50–60 s to -77.4 ± 5.9 mV with Rb^+ activation and to -66.0 ± 7.7 mV with K^+ activation. a^i_{Na} decreased in both cases to 0.5 ± 0.2 mM in 5–15 min. The maximum rate of a^i_{Na} decline (maximum $\Delta a^i_{Na}/\Delta t$) was the same with K^+ and Rb^+ at concentrations >20 mM. The response was abolished by 10^{-5} M acetylstrophantidin. Maximum $\Delta a^i_{Na}/\Delta t$ was independent of outside Na^+, while a^i_K was negatively correlated with a^i_{Na} ($a^i_K = 88.4 - 0.86 \cdot a^i_{Na}$). a^i_{Cl} decreased by at most 3 mM during reactivation, which indicates that volume changes did not seriously affect a^i_{Na}. This model provided a functional isolation of the Na,K pump, so that the relation between the pump rate ($\Delta a^i_{Na}/\Delta t$) and a^i_{Na} could be examined. A Hill plot allowed calculation of V_{max} ranging from 5.5 to 27 mM/min, which on average is equal to 25 $pmol \cdot cm^{-2} \cdot s^{-1}$. $K_{0.5}$ was 10.5 ± 0.6 mM (the a^i_{Na} that gives $\Delta a^i_{Na}/\Delta t = V_{max}/2$) and n equaled 1.94 ± 0.13 (the Hill coefficient). These values were not different with K^+ or Rb^+ as an external activator. The number of ouabain-binding sites equaled 400 $pmol \cdot g^{-1}$, giving a maximum Na^+ turnover of 300 s^{-1}. The Na,K pump in intact Purkinje strands exhibited typical sig-

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J. GEN. PHYSIOL. © The Rockefeller University Press · 0022-1295/88/03/0445/21 $2.00 445
Volume 91 March 1988 445–466

moidal saturation kinetics with regard to a^i_{Na} as described by the equation $v/V_{max} = a^{i(1.94)}_{Na}/(95.2 + a^{i(1.94)}_{Na})$. The maximum sensitivity of the Na,K pump to a^i_{Na} occurred at ~6 mM.

INTRODUCTION

Maintenance of low intracellular Na^+ activity (a^i_{Na}) is a basic feature of most living cells. The Na gradient across the cell membrane provides an energy source for a variety of transmembrane processes, including the action potential and several transport mechanisms. The low a^i_{Na} is a consequence of outward pumping of Na^+ by the Na,K pump. Precise maintenance of a low and stable a^i_{Na} requires a pump capacity in excess of the maximum Na influx and requires a high sensitivity of the Na,K pump to a^i_{Na}. The present experiments were carried out to quantify the pump capacity and to describe the relationship between the pump rate and a^i_{Na} in intact cells.

Since Skou (1957) described the requirements for Na,K and Mg by an ATPase from crab nerves, it has been clear that this enzyme, identical to the Na,K pump, has an intracellular site with high specificity for Na^+. This property has been extensively investigated on isolated membrane fractions and purified enzymes (for review, see Robinson and Flashner, 1979; Jørgensen, 1980; Schuurmans Stekhoven and Bonting, 1981). For several reasons, these data might not be directly applicable to the intact cell. First, in the cell, the pump carries out a translocation between two separate compartments. Second, the extracellular and intracellular sites are exposed to solutions of widely different ionic compositions. Third, cellular processes might regulate the pump.

Most studies of the pump rate have been carried out on red blood cells, which have very low pump activity. In other tissues, measurements of pump rates have been based on ^{22}Na or ^{24}Na fluxes or a decline of a^i_{Na} upon reactivation of the Na,K pump or pump current. Whereas there is evidence for saturation of the pump by internal Na^+ in some studies (Mullins and Frumento, 1963; Nelson et al., 1980; Gadsby and Nakao, 1986), other investigators have not been able to detect saturation even at very high intracellular Na^+ concentrations (Brinley and Mullins, 1974; Brink, 1983), and in many cases an estimate of maximum pump rates could not be derived from the data (Deitmer and Ellis, 1978; Gadsby and Cranefield, 1979; Glitsch et al., 1981; Eisner et al., 1981*a*). One barrier to obtaining this information in cardiac tissue has been the difficulty of raising a^i_{Na} above 20–30 mM, even by complete inhibition of the pump by digitalis (Eisner et al., 1981*a*, *b*). Hence, pump rates after reactivation have in many cases been examined within too narrow a range of a^i_{Na} to give maximum pump rates.

Since estimating $K_{0.5}$ (the a^i_{Na} at which the pump rate is half-maximal) requires knowledge of the maximum pump rate, such data on intact cells are scarce. Furthermore, the extent of sigmoidicity at low a^i_{Na} has been poorly described, which means that it is not known at which a^i_{Na} the pump is most sensitive to changes in a^i_{Na}.

The present experiments were therefore designed to isolate the Na,K pump functionally in intact sheep cardiac Purkinje strands. The pump rate was estimated from the change in a^i_{Na} measured by an ion-selective microelectrode. High

intracellular a^i_{Na} was obtained by omitting K^+ and Mg^{++} from the superfusate and lowering Ca^{++} to 10^{-8} M as described by Chapman et al. (1986). The pump was subsequently reactivated by K^+ or Rb^+. The data indicate saturation and a typical sigmoidal dependence of the Na,K pump rate on a^i_{Na}.

METHODS

Experimental Set-Up

Sheep hearts were obtained from a slaughterhouse and transported to the laboratory in cold Tyrode's solution. Free-running Purkinje strands were removed and mounted as

TABLE I

Composition of Superfusing Solutions

	Na^+	K^+/Rb^+	$Choline^+$	TMA^+	Mg^{++}	Ca^{++}	Cl^-	$CH_3SO_3^-$	EGTA	Sucrose
	mM	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*	*mM*
Tyrode's solution	161	5.4	—	—	1	1.8	146	—	—	—
Loading solutions	161	—	—	—	—	0.16	137	—	5	—
	161	—	—	—	—	0.16	—	137	5	—
High-Mg solutions	163	—	—	—	6	—	121	—	—	—
	2.2	—	20	—	6	—	12	—	—	216
	2.4	—	22	139	6	—	151	—	—	—
	2.4	—	22	139	6	—	—	151	—	—
Reactivating solutions	133	30	—	—	6	—	151	—	—	—
	2.2	27	20	—	6	—	39	—	—	171
	2.4	30	22	109	6	—	151	—	—	—
	2.4	30	22	109	6	—	—	151	—	—
	2.2	27	20	—	6	—	—	39	—	171

All solutions contained in addition 22 mM HCO_3, 5.5 mM glucose, and 2.4 mM phosphate and were bubbled with a gas mixture of 5% CO_2 and 95% O_2, providing a pH of 7.4 at 37°C. Some experiments were carried out with 22 mM Na replacing choline.

described previously (Wasserstrom et al., 1982). Strands were pinned at both ends to the bottom of a beeswax-paraffin chamber and superfused at 37°C with Tyrode's solution bubbled with 95% O_2, 5% CO_2 (Table I). The flow rate was ~10 ml/min. The Purkinje strands were impaled by conventional voltage-recording glass electrodes (pulled from thick-walled borosilicate tubing; WPI-1B 150 F4, World Precision Instruments, Inc., New Haven, CT) filled with 3 M KCl and having tip resistances of 5–15 MΩ. An Na-sensitive ion-selective microelectrode (Na-ISE) was then inserted, in some experiments together with a second ISE. The two electrodes were connected to electrometers (750 and FD 223, World Precision Instruments, Inc.) and the signals were displayed on both an oscilloscope and a stripchart recorder. After 20–30 min, the strand was stimulated for 2–5 min at 1 Hz through silver wires insulated except at the tips. The pulse duration was 1

ms and the voltage was adjusted to about twice threshold. Purkinje strands were discarded if the resting membrane potential was less than −75 mV, a^i_{Na} was higher than 13 mM, or the action potentials were longer than 500 ms.

Data Acquisition

The voltage signals from the electrometers were led into an Apple II computer equipped with a 12-bit AI 13 A/D interface providing a resolution of 0.05 mV (Interactive Structures, Inc., Bala Cynwyd, PA), and an Apple clock (Mountain Computer, Inc., Santa Cruz, CA). By means of an AMPRIS program (Interactive Structures, Inc.), 5,000 samples were collected from two or three channels at 10 kHz at given intervals and the mean values were stored on a diskette. The sampling interval was 10 s during periods of rapid changes in membrane potential or a^i_{Na}.

Construction and Calibration of ISEs

Micropipettes were pulled from thin-walled borosilicate tubing (WPI-TW 150 F4, World Precision Instruments, Inc.) using a vertical pipette puller (700B, David Kopf Instruments, Inc., Tujunga, CA) dried, silanized, filled with neutral ion exchanger, and backfilled with an electrolyte solution as described previously for Na-ISEs (Sheu and Fozzard, 1982; Wasserstrom et al., 1982), K-ISEs (Baumgarten et al., 1981), and Cl-ISEs (Baumgarten and Fozzard, 1981). Briefly, the glass micropipettes were dried at 150°C for ~30 min and thereafter exposed to dichlorodimethylsilane vapor for 30 min at the same temperature. The tips of the Na-ISEs were filled with neutral Na-ion exchanger (ETH 227; Steiner et al., 1979) by suction and backfilled with a 300 mM NaCl solution. The tips of the K-ISEs and Cl-ISEs were filled with ion-exchange resins 477315 and 477913 (Corning Glass Works, Medfield, MA), respectively, by dipping the tip into the solution and then placing a small drop as far down the shank of the electrode as possible. They were both backfilled with 300 mM KCl. Air bubbles were gently removed using a microforge after introducing a glass fiber into the electrode. All ISEs were left overnight in a dry environment before use. Tip resistances varied from 1 to 50 GΩ.

Calibration of the Na-ISEs and K-ISEs was carried out by the "unorthodox" method of using a series of solutions where concentrations of NaCl and KCl totaling 150 mM were varied reciprocally. Experiments were discarded if the before and after calibrations differed by more than 2 mV at 10 mM of the ion species. The Nicolsky equation describing the electrode response can be written as

$$E = E_0 + s \cdot \ln[a_x + k_{xy}\,(a_y)^{z_x/x_y}],$$

where E_0 is the standard potential, s equals RT/zF for a perfect electrode, a_x is the principal x ion activity, a_y is the interfering y ion activity, k_{xy} is the selectivity coefficient, and z is the valence. A best fit to a straight line utilizing this equation was obtained by an iterative procedure varying k_{xy} in a stepwise fashion. The correlation coefficient always exceeded 0.99. Activity coefficients of 0.76 and 0.73 were used for Na^+ and K^+, respectively (Pitzer and Mayorga, 1973).

In some experiments, Rb^+ replaced K^+ in the superfusing solution. Hence, calibration of the Na-ISEs was also carried out in the presence of Rb^+. Cl-ISEs were calibrated in pure KCl solutions and, assuming Nernstian behavior, a linear regression was calculated by taking into account the fact that the activity coefficient for Cl^- is approximately linearly related to the logarithm of the ionic strength within the range of interest (Pitzer and Mayorga, 1973).

Electrodes were discarded if the experimentally derived slope (s), which theoretically has a value of 61.5 mV at 37°C, was <53 mV for the K-ISEs and Na-ISEs and −48 mV

for the Cl-ISEs. Characteristics of the electrodes are presented in Table II. The selectivity of the Na-ISEs relative to K^+ equaled, on average, 44:1 (calculated from k_{xy}), with a range from 21:1 to 93:1. For the K-ISEs, the selectivity was higher vs. Na^+, averaging 61:1. The electrical response times of electrodes were reduced to <1 s by capacitance compensation with a DC amplifier (FC-23B, World Precision Instruments, Inc.).

Experimental Protocol

After an initial equilibration period, the Tyrode's solution was replaced by a solution containing 0 K^+, 0 Mg^{++}, and 10^{-8} M free Ca^{++} (loading solution; see Table I). As described by Chapman et al. (1986), this procedure caused an abrupt depolarization and an increase in a^i_{Na} to high levels within 30–60 min. When the desired level for a^i_{Na} had been reached, 6 mM Mg^{++} was added to prevent a^i_{Na} from rising further (high-Mg solutions). After 5–10 min, the NaCl in the superfusate was in some experiments replaced by either equiosmolal concentrations of sucrose or tetramethylammonium-Cl (TMA-Cl) (low-Na_o solution). Finally, after 5–10 min to ensure stable conditions, K^+ or Rb^+ was added (reactivating solutions). The detailed compositions of all solutions are given in Table I.

TABLE II

Characteristics of Ion-selective Microelectrodes

	Slope (s)	E_0	Selectivity coefficient (k_{xy})
	mV	*mV*	
Na-ISE (n = 28)	58.2±2.7	−87.2±9.5	0.023±0.007
K-ISE (n = 11)	55.5±1.9	−84.0±4.2	0.016±0.004
Cl-ISE (n = 6)	−51.7±1.1	167.1±8.8	—

Values are means ± SD derived from the "unorthodox" calibration procedure described in the Methods for Na-ISEs and K-ISEs. The Cl-ISE was calibrated in pure KCl solutions.

The following series of experiments was carried out. Recovery of a^i_{Na} was followed at normal and low extracellular Na using 30 mM K^+ or Rb^+ as activators of the Na,K pump. In some experiments with K^+, both a^i_{Na} and a^i_K were measured. The same experiments were carried out replacing Cl^- with methanesulfonate ($CH_3SO_3^-$). In some of these experiments, a^i_{Na} and a^i_{Cl} were monitored simultaneously.

Calculations

Determination of a^i_x requires subtraction of the membrane potential (V_m) from the voltage signal of the ISE (V_{ISE}). However, the high resistance of the ISE makes accurate monitoring of the membrane potential component of V_{ISE} difficult (Cohen et al., 1982; Désilet and Baumgarten, 1986). Because V_m changed suddenly during exposure to the loading and reactivating solutions, changing the voltage without changing the ion activities, the data offered an opportunity to compare the voltage response of the two electrodes. From the digitized records, plots of V_m vs. V_{ISE} were made for each load-recovery cycle. During the sudden voltage changes, the relation between V_m and V_{ISE} was linear, with slopes varying between 0.9 and 1. In calculation of $V_{diff} = V_{ISE} - V_m$, this slope was used to adjust for non-ideal behavior of V_{ISE} by reducing V_m by a factor equal to the

regression slope for that experiment. After this correction, E was set equal to V_{diff} and a_x^i was calculated by means of the equations obtained from the calibration curves. Many experiments required no corrections, and in no case did the correction change the nature of the response.

In the experiments carried out with 2.4 mM extracellular Na, a_{Na}^i was not influenced by passive Na^+ influx. During the reactivation phase, the change of a_{Na}^i between successive points was calculated from the digitized data and a two-point smoothing was carried out in order to estimate da_{Na}^i/dt. Analysis of the relationship between da_{Na}^i/dt and a_{Na}^i was made according to the simple model described by the Hill equation:

$$\frac{v}{V_{max}} = \frac{[S]^n}{K' + [S]^n}.$$

The analysis was similar to that used by Karlish and Stein (1985). The Hill equation can be transformed to the linear form:

$$\ln[v/(V_{max} - v)] = n \cdot \ln[S] - \ln K',$$

where v is approximated by $\Delta a_{Na}^i/\Delta t$, V_{max} is the maximum reaction velocity, $[S]$ is the substrate activity (a_{Na}^i), and K' is a constant comprising the intrinsic dissociation constant and interaction factors between substrate binding sites. The best mean least-squares fit to the Hill equation between 2 and 25 mM was calculated by an iterative procedure changing V_{max} stepwise to search for the maximal correlation coefficient (r).

Hill plots of $\ln[v/(V_{max} - v)]$ vs. $\ln[S]$ provides the Hill coefficient n. The intercept of the plot provides the estimate of K', which is equal to $K_{0.5}^n$. While a_{Na}^i values >25 mM could be used, the measurement of $\Delta a_{Na}^i/\Delta t$ is less accurate at higher concentrations. Consequently, these data were added only if they increased the value of r. The range of r values for the 12 experiments with 2.4 mM extracellular Na was 0.939–0.998, with an average of 0.972. It should be noted that there were three extracted variables: n, V_{max}, and K'. The quality of the fit can be seen in Fig. 6, where the mathematically derived curve for one experiment is compared with the data from which it was derived.

Measurements of Ouabain-binding Sites

Vanadate-facilitated ouabain binding was measured on separate fibers. The technique was modified from Nørgaard et al. (1984). The fibers were kept at −70°C after they had been dissected out. The frozen fibers were cut into pieces weighing 3.6–6.9 mg and preincubated in 250 mM sucrose, 30 mM histidine, 6 mM $MgCl_2$, 7.5 mM Tris-HCl, and 1 mM $NaVO_3$ (pH 7.4) at 0°C for 60 min. The fibers were then transferred to an incubation medium with the same composition plus 2×10^{-6} M [^{3}H]ouabain (specific activity, 1.1 Ci/mmol) at 37°C for 240 min. Finally, unspecifically bound ouabain was washed out at 0°C in 30 mM histidine and 150 mM NaCl (pH 7.4) for 150 min. Specifically bound ouabain was released by transferring the cut fibers to 10% trichloroacetic acid (TCA) for 18 h at room temperature. The ^{3}H activity in the TCA extract was counted in a scintillation counter. In separate experiments, the optimal preincubation time and the incubation time producing maximum binding were ascertained. The washing procedure lowered the unspecific binding measured in the presence of 10^{-3} M unlabeled ouabain to 1–2% of total binding. The specific binding fell by 21% during washing. Based on measurement of the dissociation constant from Scatchard plots, a standard concentration of 2×10^{-6} mM ouabain was used, resulting in 90% saturation. In the calculation of the number of ouabain-binding sites, the latter two factors have been corrected for. There is ample evidence that ouabain binding to intact tissue provides an accurate estimate of the number of functioning pumps (Clausen, 1986).

Statistics

Sample means were compared using Student's t test. The null hypothesis was rejected when $p < 0.05$.

RESULTS

Na^+ *Loading and Pump Reactivation*

In the quiescent state after equilibration in normal Tyrode's solution, V_m was -77.7 ± 0.6 mV ($n = 21$), a^i_{Na} was 8.2 ± 0.4 mM ($n = 21$), a^i_K was 99.9 ± 5.1 mM ($n = 9$), and a^i_{Cl} was 14.7 ± 0.6 mM ($n = 5$).

When fibers were exposed to loading solution containing no K^+ or Mg^{++} and 10^{-8} M Ca^{++}, the fibers rapidly depolarized to -15 to 0 mV (Fig. 1). In some

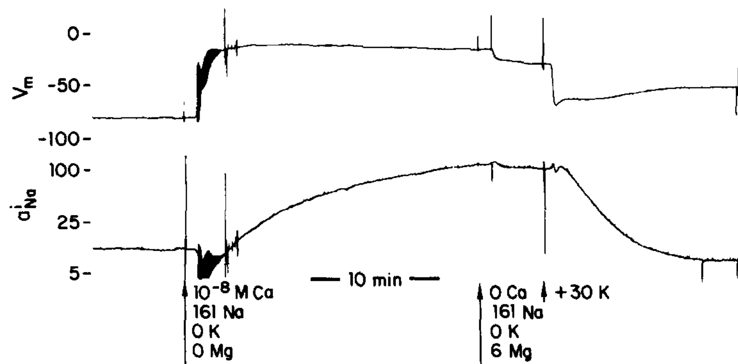

FIGURE 1. General outline of the experimental protocol. The top trace is the membrane potential, V_m, and the bottom trace is V_{diff}, which equals the voltage difference between the Na-ISE and V_m. By means of the calibration curve for the Na-ISE, the scale has been transformed to a^i_{Na}. Note that it is approximately logarithmic. Sheep Purkinje fibers were exposed to a loading solution until a^i_{Na} had reached a desired level. After addition of Mg^{++}, the cell maintained the high a^i_{Na}. Reactivation of the Na,K pump was subsequently accomplished by adding 30 mM K^+. Concentrations are given in millimolar except for Ca^{++} (molar).

fibers, V_m switched in the course of a few seconds, whereas in other fibers a train of spontaneous action potentials preceded the depolarized state. As a result of pump blockade by zero K^+ and leak of Na^+ as a consequence of low divalent cation concentrations, a^i_{Na} started to rise and reached levels close to the concentration in the loading solution over 30–60 min. After successively adding 6 mM $MgCl_2$ to block the Na^+ leak and lowering Na^+ to 2.4 mM, V_m was -30.8 ± 2.2 mV and a^i_{Na} ranged between 25 and 90 mM ($n = 12$). Illustrations of these changes and the experimental protocol are shown in Figs. 1 and 2.

Pump reactivation by 30 mM K^+ or 30 mM Rb^+ caused abrupt hyperpolarization before a^i_{Na} decreased rapidly (Figs. 1 and 2 *A*). In the experiment shown

in Fig. 1, extracellular Na^+ was kept at 161 mM throughout the load-recovery cycle. Therefore, when a^i_{Na} fell after pump reactivation, backleak of Na^+ increased until it equaled the pump rate, which occurred at a normal a^i_{Na} of ~10 mM in this particular fiber. In contrast, the top trace in Fig. 2 *A* shows that when extracellular Na^+ had been lowered to 2.4 mM, subsequent addition of 30 mM Rb^+ caused a rapid decline of a^i_{Na} to <1 mM over ~10 min. The average V_m reached -66 ± 8 mV using 30 mM K^+ as the activator ($n = 6$) and -77 ± 6 mV using 30 mM Rb^+ as the activator ($n = 6$) over a period of 50–60 s. The final a^i_{Na} was in both cases 0.5 ± 0.2 mM after 5–15 min. Although there is evidence that the Na,K pump is voltage sensitive (Gadsby et al., 1985),

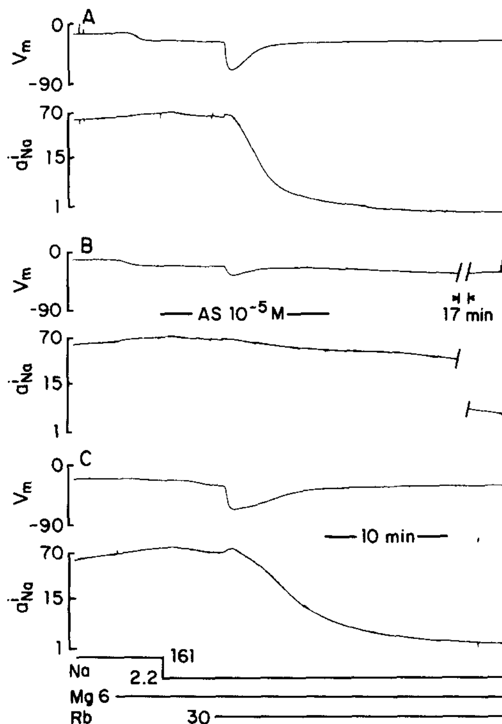

FIGURE 2. Effect of AS on the reactivation of the Na,K pump by 30 mM Rb^+. V_m and a^i_{Na} are shown for load-recovery cycles repeated three times in succession in the same fiber. Note that after addition of Mg^{++}, extracellular Na was lowered to 2.2 mM and the fibers maintained the high a^i_{Na} in the face of an outward concentration gradient. Only the parts of the strip-chart recordings starting with the addition of 6 mM Mg^{++} are shown. Panels *A* and *C* depict reactivation before and after the load-recovery cycle in panel *B*, where AS was added 5 min before 30 mM Rb^+.

no relationship could be found in these experiments between V_m and the rate of decline of a^i_{Na}.

Effect of Acetylstrophanthidin

The decline in a^i_{Na} was tentatively attributed to the activity of the Na,K pump. If this were true, then the response should be blocked by acetylstrophanthidin (AS). Fig. 2 shows the reactivation of Na,K pump activity by 30 mM Rb^+ three times in succession in the same fiber. Addition of Rb^+ was preceded by loading phases that increased a^i_{Na} to nearly 70 mM. The two traces in *A* show the normal response to pump reactivation.

After a^i_{Na} was again elevated to 70 mM, AS (10^{-5} M) was added to the superfusate simultaneously with the lowering of extracellular Na^+ to 2.4 mM. 3 min later, 30 mM Rb^+ was added again and caused a slight transient hyperpolarization, probably because inhibition of Na,K pump activity was not complete after only a few minutes of exposure to AS (*B*). This voltage response was abolished in other experiments with longer exposure. Addition of Rb^+ will not cause displacement of AS since it does not compete at the same binding site (Hansen, 1984). However, during the 1 min over which a^i_{Na} fell by 20 mM in the preceding recovery period, a^i_{Na} only declined by 3 mM in the presence of AS. When AS was removed, a^i_{Na} slowly fell during a 20-min period before the loading solution was added again. The two traces in *C* show that after 40 min of washout, the response to 30 mM Rb^+ was partially restored. The response to 30 mM K^+ or Rb^+ was similarly abolished by AS in three other experiments.

Extracellular Activator

As stated by Eisner and Lederer (1980), Rb^+ is preferable to K^+ as an activator since changes in K^+ will alter the shape of the current-voltage relationship. In

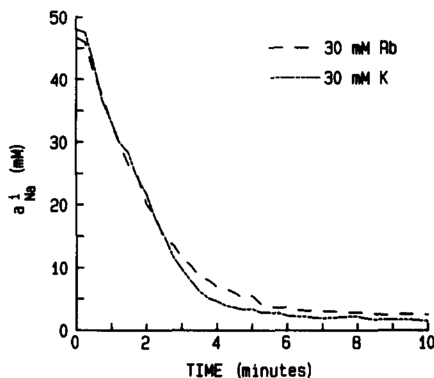

FIGURE 3. Comparison of recovery of a^i_{Na} during activation of the Na,K pump by Rb^+ and K^+ during two load-recovery cycles in the same Purkinje strand.

accordance with studies on the isolated enzyme and Purkinje fibers, we found that the pumping rate is the same whether Rb^+ or K^+ was used as the activator (Fig. 3) (Eisner and Lederer, 1980).

In contrast to experiments carried out on isolated cells or enzymes, concentrations much higher than 4–5 mM were required in the superfusing solution to obtain maximum stimulation by the extracellular activator. Fig. 4 shows that maximum rates of a^i_{Na} decline after addition of Rb^+ to the superfusate were reached only at Rb^+ concentrations exceeding 20 mM. Therefore, 30 mM of either K^+ or Rb^+ was used as the standard activator concentration.

The apparent concentration of extracellular K^+ or Rb^+ required to give 50% stimulation of the enzyme was ~9 mM. This high value is presumably due to a diffusion limitation between the surface of the preparation and the extracellular K^+ sites of the enzymes.

Analysis of Recovery of a^i_{Na} *in Terms of the Hill Equation*

Since the rapid decrease of a^i_{Na} was dependent on extracellular Rb^+ or K^+ and could be inhibited by AS, it was taken to reflect the activity of the Na,K pump.

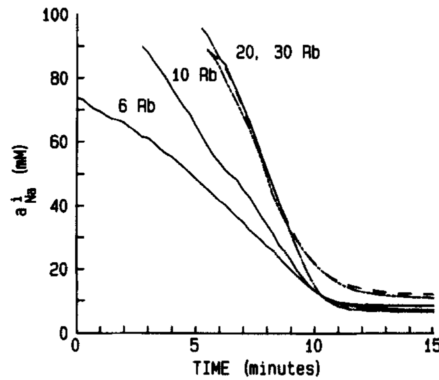

FIGURE 4. Recovery of a^i_{Na} during activation of the Na,K pump by different Rb^+ concentrations. Data from two Purkinje strands are shown. In one experiment, reactivation was performed three times in succession with 10, 6, and 20 mM Rb^+. In the second experiment, 20 and 30 mM Rb^+ were used.

During reactivation, extracellular Na^+ was kept low to avoid significant backleak into the cell at low a^i_{Na}. Furthermore, at high a^i_{Na}, Na leaked out of the cells very slowly, despite the favorable gradient (Fig. 2). Na^+/Ca^{++} exchange could not contribute to Na^+ efflux, since the reactivation solution contained no Ca^{++} for

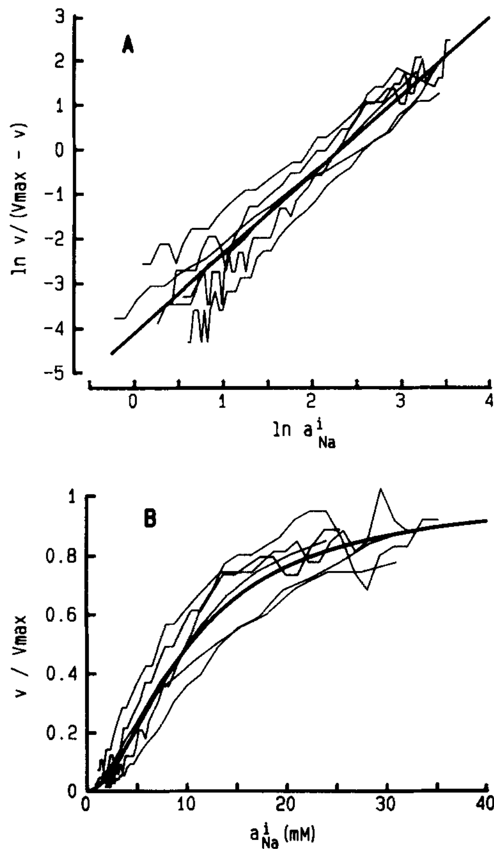

FIGURE 5. Panel *A* shows Hill plots constructed as described in the Methods for six individual experiments where reactivation was carried out with 30 mM Rb^+. The straight line represents the mean slope (the Hill coefficient, *n*) and intercept ($\ln K'$). In panel *B*, original data showing the rate of a^i_{Na} decline during recovery, normalized for V_{max} (v/V_{max}), are shown as a function of a^i_{Na}. The smooth full drawn line represents the Hill equation with the mean constants obtained from *A*.

exchange. Hence, during activation, the fall in a^i_{Na} seems to be almost entirely due to Na^+ transport by the Na,K pump.

The rate of decline of a^i_{Na} was calculated for each 10-s interval providing v. By an iterative procedure varying V_{max} stepwise, a best fit to the linear version of the Hill equation could be obtained as described in the Methods. Fig. 5 *A* shows individual best fits for six experiments where Rb^+ was used as the external activator. Table III gives the means of derived data for six experiments using Rb^+ and six experiments using K^+. In all experiments, good linear fits were obtained, with r values exceeding 0.94. Hence, in these intact cells, the relationship between a^i_{Na} and its rate of decline is well described by the Hill equation. The Hill coefficient for all 12 preparations was 1.94 ± 0.13 and the $K_{0.5}$ (the a^i_{Na} at which the rate of a^i_{Na} decline was half-maximal) was 10.5 ± 0.6 mM. The asymptotic value V_{max} (describing the maximum rate of a^i_{Na} decline) varied between 5.5 and 27.0 mM·min^{-1}, with an average of 13.5 ± 2.3 mM·min^{-1}.

TABLE III

Values Derived from Fitting the Hill Equation in Linear Form to the Derivative of the a^i_{Na} *Data*

Activator	Hill coefficient	$K_{0.5}$	V_{max}	Correlation coefficient
		mM	*mM/min*	
K^+	2.12±0.21	10.6±0.7	9.7±2.2	0.958±0.008
Rb^+	1.77±0.12	10.3±0.9	17.3±3.6	0.986±0.005
p	NS	NS	0.002	NS

The Hill equation is $v/V_{max} = (a^i_{Na})^n/[K' + (a^i_{Na})^n]$ where $v = da^i_{Na}/dt$, here approximated by the change in a^i_{Na} over 10-s intervals. $K_{0.5}$ is the a^i_{Na} value where $v = V_{max}/2$. Values were obtained during reactivation of the Na,K pump by 30 mM K^+ or Rb^+ in the medium containing 2.4 mM Na^+. Values are means ± SE.

In Fig. 5 *B*, the individual V_{max} values obtained from the linear fitting procedure have been used to calculate the relative pumping rates so that ordinary substrate activation curves could be plotted for the six Rb^+ experiments. The thick line represents the Hill equation with a Hill coefficient of 1.77, and is thus a mean curve for the six experiments. The inflection point of the curve, the point where v is most sensitive to a^i_{Na}, can be calculated by means of the constant K' obtained from the linear regression analysis (Segel, 1975). On average, for all 12 experiments, the inflection point was 6 mM.

To illustrate the fit of the derived Hill curves to the original data, the Hill equation was integrated. Fig. 6 shows original data points obtained at 10-s intervals from one experiment. The continuous line is the integrated Hill equation with inserted constants obtained for this particular experiment by means of the Hill plot. In addition, integration requires another constant that had to be determined separately (the mean distance of the curve from the data points when this constant was set to zero). It is evident that the fit is good, well beyond the concentration range for a^i_{Na} of 2–25 mM chosen for the calculations. The

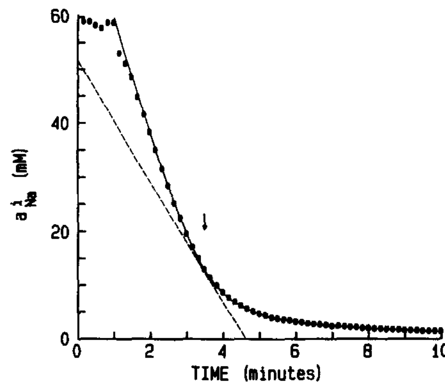

FIGURE 6. a^i_{Na} measurements from one experiment shown as data points (open circles) obtained at 10-s intervals during reactivation of the Na,K pump by 30 mM Rb^+. The solid curve was obtained by inserting the Hill coefficient, V_{max}, and K' obtained from the Hill plot of this experiment into the integrated form of the Hill equation. The derived curve therefore represents the best fit to the original data. The dashed line has a slope equal to $V_{max}/2$ and is a tangent to the curve at $K_{0.5}$ = 13.8 mM.

dashed line indicates $V_{max}/2$ and is the tangent to the original curve where a^i_{Na} = $K_{0.5}$, 13.4 mM in this experiment (indicated by an arrow).

Effect of Varying Extracellular Na^+

Since the experiments allowing construction of Hill plots were carried out in 2.4 mM extracellular Na^+, it was important to ascertain that Na,K pump activity was unaffected by extracellular Na^+. However, comparison of the rates of a^i_{Na} decline at various extracellular Na^+ concentrations can only be carried out if backleak of Na^+ is of little importance. Only experiments where backleak can be disregarded can be fitted to the Hill equation in a meaningful way and will result in linear relationships in double-reciprocal plots as exemplified in Fig. 7. At physiological concentrations of extracellular Na^+, backleak will increase gradually and reach its maximum when a^i_{Na} stabilizes at the normal level (Fig. 1). Comparison of pumping rates at high and low extracellular Na^+ can only be done when a^i_{Na} is so high that the pump rates are close to maximum. Therefore,

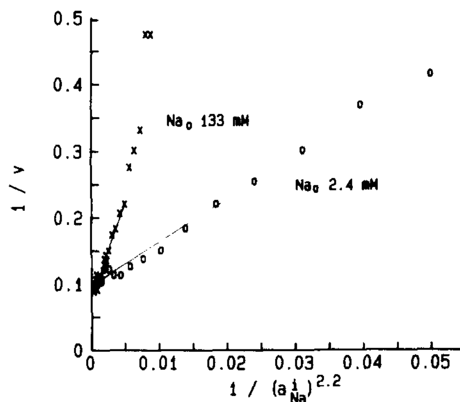

FIGURE 7. Data from one experiment exemplifying how V_{max} was compared in recoveries carried out with 30 mM Rb^+, but different extracellular Na^+ concentrations in the superfusing solution. After raising a^i_{Na} to the power of the Hill coefficient obtained from the Hill plot of the experiment carried out in low extracellular Na^+, linear regression analysis was carried out on the double-reciprocal data from the two conditions over the same range of v. V_{max} was obtained from the intercepts with the y axis.

V_{max} was compared in four fibers in which recovery of a^i_{Na} occurred with both 2.4 and 133 mM extracellular Na^+ solutions. The contribution of backleak is evident from the much steeper curve and significant deviation from linearity when extracellular Na^+ was high. However, backleak should be small at high a^i_{Na} so that calculation of the intercept with the ordinate should provide estimates of V_{max} in both conditions (Karlish and Stein, 1985). These V_{max} values averaged 17.8 ± 2.6 and 19.1 ± 1.6 mM/min (n = 4) with high and low extracellular Na^+, respectively.

Cell Volume Changes during Recovery of a^i_{Na}

Even though the recovery of a^i_{Na} can be ascribed entirely to the Na,K pump, the fall of a^i_{Na} is not necessarily linearly related to the amount of Na carried by the pump. The calculated v values therefore directly reflect the pump rates only if cell volume remained unchanged.

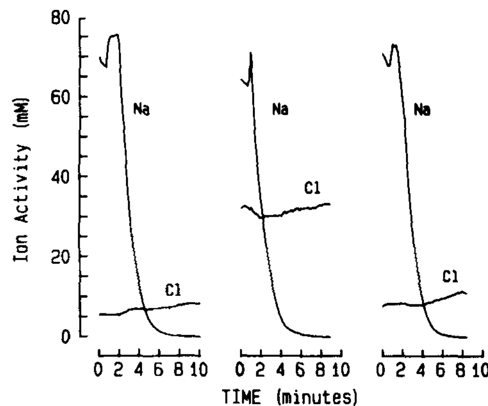

FIGURE 8. a^i_{Na} and a^i_{Cl} in one Purkinje strand during reactivation by 30 mM Rb^+ in three successive load-recovery cycles. The first and third cycles were carried out with no extracellular Cl^- in the superfusate, whereas extracellular Cl^- was 151 mM during the second cycle.

A change in cell volume is associated with altered a^i_{Cl} because of the large amount of fixed intracellular anions. Hence, two approaches were taken to identify a possible influence of volume changes on the rate of decline of a^i_{Na}. First, load-recovery cycles were carried out in two experiments with and without extracellular Cl^-, with methanesulfonate substituting for Cl. No differences in the recovery rate of a^i_{Na} could be observed (Fig. 8). During loading in the presence of extracellular Cl^-, a^i_{Cl} increased from 14.7 ± 0.6 to 29.1 ± 3.7 mM (n = 5), indicating a significant swelling. Provided the concentration of fixed intracellular anions is in the range of 100 mM (including proteinates, organic acids, and maybe some phosphate), a 1 mM increase in a^i_{Cl} will correspond to a 1% increase in cell volume. Hence, a^i_{Cl} is a sensitive indicator of cell volume, and in this case the cells swelled by ~15% during loading. In the absence of extracellular Cl^-, a^i_{Cl} declined. As pointed out by Baumgarten and Fozzard (1981), a^i_{Cl} measured by the Cl-ISE never reaches zero, probably because of interference from other anions at the electrode. Since methanesulfonate is probably restricted to the extracellular volume, the fall in a^i_{Cl} may be parallel to a cellular volume contrac-

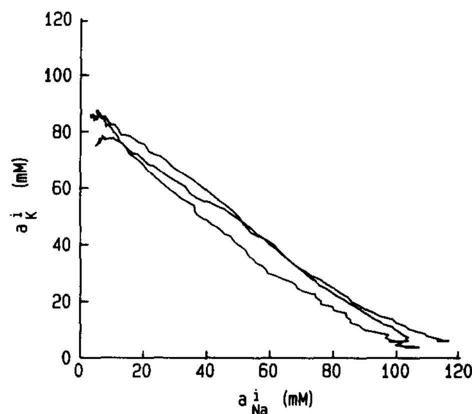

FIGURE 9. Comparison of a^i_K and a^i_{Na} from three Purkinje strands during reactivation by 30 mM K^+ in a superfusate containing 24 mM external Na^+.

tion of ~10% compared to the control volume. Even so, at these two different cellular volumes, the rate of a^i_{Na} decline after reactivation of the Na,K pump was almost the same (Fig. 8). For the same reason, coupled Na^+, Cl^-, and water movement as a significant efflux mechanism is ruled out by pump rates that were not affected by methanesulfonate substituting for Cl.

Second, during recovery in high extracellular Cl, a^i_{Cl} fell by ~3 mM, indicating a shrinkage of ~3%. However, this volume contraction occurred before a^i_{Na} had reached 25 mM and hence would not influence calculations of V_{max} and the Hill coefficient (Fig. 8). In two other experiments, no changes in a^i_{Cl} were detected during recovery.

It is therefore reasonable to conclude that the observed decline in a^i_{Na} is probably proportional to the amount of Na^+ transported by the Na,K pump and that cell volume changes are of little importance for interpretation of the data.

Relationship between a^i_{Na} and a^i_K

In three experiments, a^i_K fell from 99.2 ± 8.7 to 5.3 ± 0.8 mM during loading and a^i_{Na} rose from 7.4 ± 0.4 to 105.5 ± 4.2 mM. During recovery with 30 mM K^+ as the activator and an extracellular Na^+ concentration of 24 mM, a^i_K rose again to 82.5 ± 3.8 mM when a^i_{Na} fell to 4.2 ± 0.6 mM (Fig. 9). Hence, the sum $a^i_{Na} + a^i_K$ fell during reactivation by ~20 mM.

Over the range of a^i_{Na} values used for calculating the Hill equations, the relationship between a^i_{Na} and a^i_K was close to linear and can be expressed as $a^i_K = 88.4 - 0.86 \cdot a^i_{Na}$ ($r = 0.97$).

TABLE IV

Characteristics of Ouabain Binding to Intact Sheep Purkinje Fibers

K_{diss} (10^{-7} M)	Ouabain binding at 2×10^{-6} M	
	Range	Mean ± SE
	pmol·g⁻¹	*pmol·g⁻¹*
1.8	220–650	401±24

Binding was facilitated by 1 mM vanadate ($n = 33$).

Ouabain-binding Sites and Maximum Pump Rate

Binding of [^{3}H]ouabain at 2×10^{-6} M was measured on separate fibers in the presence of 1 mM vanadate. Table IV shows that binding varied by a factor close to 3, with a mean of 400 pmol·g^{-1}.

When ouabain binding is compared with V_{max} values, it can be seen that the range of V_{max} values is also quite large, with a mean of 13.5 mM·min^{-1} (Table III). Assuming an intracellular fluid volume-to-weight ratio of 0.5 as found in ventricular tissue (Page and Page, 1968), the rate of outward Na pumping will equal 6.8×10^{-6} mol·g^{-1}·min^{-1}. Hence, the maximum rate at which each enzyme can transport Na^+ is close to 300 s^{-1}. Provided the stoichiometry is 3 Na:1 ATP, the turnover rate per enzyme will average 100 s^{-1}.

DISCUSSION

Rate of Outward Na^+ *Pumping as a Function of* a^i_{Na}

The approach used in these experiments allowed an accurate description, in terms of the Hill equation, of the decline in a^i_{Na} from high levels to very low levels after reactivation of the Na,K pump in cardiac tissue by Rb^+ or K^+. The rate of a^i_{Na} decline is probably linearly related to the Na,K pump rate, first, because no fall in a^i_{Na}, or only a minor one, was observed after addition of Rb^+ or K^+ to fibers exposed to AS. Second, interference from backleak of Na into the cells was prevented by keeping extracellular Na^+ low. Reversal of the Na^+ gradient across the sarcolemma also caused Na^+ to diffuse out of the cell in parallel with the active transport. This was not a significant problem, because the passive efflux rate was too small to interfere significantly with our estimates of the pump rate (Fig. 2). In addition, when the pump was reactivated, a hyperpolarization occurred that opposed the passive Na^+ efflux. Third, although small volume changes seemed to occur, as judged from a^i_{Cl} measurements, the rates of a^i_{Na} decline were not significantly affected.

The constants derived from fitting the Hill equation to the data therefore probably closely reflect properties of the Na,K pump. The analysis indicated that the pump exhibited typical saturation kinetics and the relationship to a^i_{Na} was sigmoidal, with a Hill coefficient close to 2. The $K_{0.5}$ of 10.5 mM expressed as a^i_{Na} is equivalent to 14 mM in terms of concentration.

This description of pump activation by a^i_{Na} requires that other factors that might influence the pump rate be constant or vary little. In the present experiments, these factors include the concentration of the external activator, intracellular K^+, Rb^+, Mg^{++}, or pH, the supply of ATP, and membrane potential.

Estimates of the $K_{0.5}$ for the extracellular K^+ site are in the range of 0.5–1.8 mM (Schuurmans Stekhoven and Bonting, 1981). This is in close agreement with the observation that the half-maximum pump current was obtained in isolated voltage-clamped myocytes at 0.8 mM (Cohen et al., 1987). However, in multicellular prepartions such as cardiac muscle, quite variable responses to external K^+ have been reported. As pointed out by Cohen et al. (1984), part of this variability can probably be explained by the fact that these analyses did not take into account the fact that the internal Na^+ site might be saturable. The present

study was not designed to estimate the affinity for K^+ at the external site of the pump. Clearly, at the high pump rates presently observed, an extracellular depletion of activator may occur in the narrow extracellular clefts, as pointed out by Eisner and Lederer (1980). Thus, a considerable diffusion gradient probably exists between the surface of the fiber and the K^+ site. The diffusion rate is linearly related to the concentration gradient so that by elevating the K^+ concentration sufficiently, saturating concentrations should be achieved for all K^+ sites. The high K^+ concentration in the bath therefore probably ensured maximum K^+ activation of a majority of the pump sites.

Relationship between a^i_{Na} and a^i_K

The relationship between a^i_{Na} and a^i_K during reactivation was almost linear so that $\Delta a^i_{Na}/\Delta a^i_K$ was -1.2. The Na,K pump carries out an Na,K countertransport with a ratio of 1.5:1 (Glynn and Karlish, 1975). This means that in the present experiments, K^+ also entered the cell through a separate pathway, probably a K^+ channel. However, even though a^i_{Na} reached levels well below the control activity of 8.2 mM, a^i_K did not recover completely, but remained 15–20 mM below control. Because a^i_{Cl} and osmolality did not change significantly, the difference between a^i_K and a^i_{Na} that developed during reactivation must have been made up by another cation in order to preserve electroneutrality. A likely possibility is that this intracellular cation gap was filled by Mg^{++}, which was present in the superfusate at a concentration of 6 mM.

It is well established that the affinity for K^+ at the intracellular Na^+ site of the pump is high enough for K^+ to act as a competitive inhibitor at normal intracellular concentrations (Knight and Welt, 1974; Glynn and Karlish, 1975). Thus, it is clear that the quantitative description of Na^+ activation of the Na,K pump provided here incorporates a variable inhibition by K^+ or Rb^+. The present use of the Hill equation, therefore, does not describe a simple single-substrate enzymatic reaction, but rather the composite effect of simultaneous changes of both a^i_{Na} and a^i_K that occur normally in a cell. The range of a^i_{Na} values used for the calculation was from 2 to 25 mM, or ~10-fold. At the same time, a^i_K varied between 65 and 90 mM, or 1.4-fold. The affinity constants for Na^+ and K^+ at the intracellular sites have been estimated as 0.19 and 9 mM, respectively, in red cell ghosts (Garay and Garrahan, 1973). However, the activation curves providing these constants are less sigmoidal and $K_{0.5}$ is 2.5 mM lower than we observed in sheep Purkinje fibers. Hence, the relative inhibition caused by the presence of K^+ cannot be estimated on this basis. On the other hand, studies on the Na,K pump in inside-out cardiac vesicles (Phillipson and Nishimoto, 1983) or vesicles reconstituted with pig kidney Na,K-ATPase (Karlish and Stein, 1985) have provided activation curves obtained with only Na^+ present at the intracellular cytoplasmic sites. These results indicate Hill coefficients of 2.8 and 1.9 and $K_{0.5}$ values of 9 and 7.1. Compared with the activation curves obtained without cytoplasmic K^+, the pump rates we observed were lower by ~15% at an a^i_{Na} of 20 mM and 40–55% at 5 mM. Thus, an a^i_K of 80 mM will cause ~50% inhibition of the pump rate at an a^i_{Na} of 5 mM. The inhibition of Rb^+ would be of the same magnitude.

Possible Influence of Intracellular Mg^{++}, ATP, and pH on the Pumping Rate

The Na,K pump has an absolute requirement for Mg^{++} on the intracellular side (Schuurmans Stekhoven and Bonting, 1981). The loading of the fibers with Na^+ took place in an Mg^{++}-free solution and intracellular stores might have become depleted. However, Mg^{++} was added to the superfusate at a concentration of 6 mM 10–15 min before reactivation by K^+ or Rb^+, and was kept at this concentration throughout the recovery period. There was surprisingly little splay on the a^i_{Na} curve after reactivation, which suggests that the pump rate was maximal almost from the moment the fall in a^i_{Na} became detectable. Thus, a variable effect of substrates or activators seems to be ruled out. Furthermore, intracellular Mg^{++} levels were probably high enough for full enzymatic activity, i.e., >3–4 mM and rising throughout recovery.

Intracellular pH might have fallen owing to the reversed Na^+ gradient across the sarcolemma or to lactate accumulation. Chapman and Suleman (1986) have recently reported minimal pH changes during similar loading in ferret papillary muscles. Intracellular acidosis only occurred upon readdition of Ca^{++} and the development of a contracture. In our experiments, all solutions were kept virtually Ca^{++}-free, and no tension developed. However, the cells could become loaded with protons when extracellular Na^+ was lowered. Since V_{max} was not different in high and low extracellular Na^+, it can be concluded that any intracellular acidosis caused by reversal of the Na^+/H^+ exchanger was not of sufficient magnitude to detectably inhibit the pump. Since the metabolic requirement of the Na,K pump is quite small (see below), lactate accumulation seems unlikely.

If the ATP supply became limited, not only would anaerobic glycolysis be stimulated, but the pump rate might be reduced owing to a lack of a high-energy substrate. Normally the intracellular ATP concentration is ~6 mM (Sellevold et al., 1986), which is several times higher than the highest apparent K_m of 0.48 mM for ATP at the intracellular site (Schuurmans Stekhoven and Bonting, 1981). A reduction in the availability of ATP is not reconciliable with repeated load-recovery cycles without any change in the pump rates. Finally, at V_{max}, the energy consumption of the pump would amount to a maximum of 10% of the energy consumption of the intact beating heart, which would strain the ATP-producing processes very little. Thus, the long periods of stable, maximum pump rates observed in several fibers are good evidence that the ATP supply was sufficient throughout repeated load-recovery cycles.

Voltage Dependence of the Na,K Pump

Since the Na,K pump carries out a 3:2 translocation, one of the intermediate reaction steps must be sensitive to the membrane potential. Only recently has this voltage dependence been demonstrated in isolated heart cells, which indicates a reversal potential of −150 mV when the chemical Na^+ gradient amounted to −130 mV (Gadsby et al., 1985). At a voltage of −80 mV, those results would predict ~40% inhibition of pump activity. In our preparations, the membrane potential rapidly hyperpolarized during reactivation, but depolarized again much faster than the pump rate declined (see Figs. 1 and 2). Hence, we could not detect any voltage effect. An explanation for this is pro-

vided by the recent finding of Gadsby et al. (1987) that the pump becomes insensitive to membrane potential at low extracellular Na^+. We therefore conclude that under our experimental conditions, the pump rate was not significantly affected by the changing membrane potential.

Kinetics of the Na,K Pump in Intact Cells

A $K_{0.5}$ for the Na^+ site of 10.5 mM (14 mM intracellular Na^+) is close to estimates on the isolated enzymes (Schuurmans Stekhoven and Bonting, 1981). This is somewhat surprising in light of the high a^i_K in these experiments. Estimates of $K_{0.5}$ depend on the accuracy of the V_{max}, which was obtained by fitting data to the Hill equation.

In all experiments where a^i_{Na} was increased enough, saturation or v values close to V_{max} were obtained. This contrasts with several previous reports on skeletal muscle and cardiac tissue, where saturation was difficult to detect (Brinley and Mullins, 1974; Deitmer and Ellis, 1978; Gadsby and Cranefield, 1979; Glitsch et al., 1981; Eisner et al., 1981*a;* Brink, 1983). Several investigators have described the fall in a^i_{Na} in a Purkinje fiber preparation similar to ours as monoexponential (Deitmer and Ellis, 1978; Eisner and Lederer, 1981*a*; Glitsch et al., 1981). In those experiments, a^i_{Na} was elevated to 20–30 mM by removing external K^+ for a variable period before the Na,K pump was reactivated. External Na^+ was maintained at 137–145 mM. Those conditions were not conducive to the measurement of the maximal pump rate because of the lesser level of a^i_{Na} and the presence of significant backleak of Na^+.

In other experiments, the pump rate has been inferred from measurements of pump current (Gadsby and Cranefield, 1979; Eisner and Lederer, 1980). Only recently has the introduction of the internally perfused single-cell preparation allowed assessment of pump current over a wide range of a^i_{Na} values in guinea pig ventricle tissue (Gadsby and Nakao, 1986). In that preliminary report, saturation of pump current at high a^i_{Na} was observed. In epithelial tissues, saturation has been observed and $K_{0.5}$ values close to 14 mM have been reported (Lewis and Wills, 1983).

The observed V_{max} values showed large variability between fibers (Table III), as did the number of ouabain-binding sites. Since these measurements were not obtained in the same fibers, we cannot estimate the correlation. However, comparison of the mean values of pump rate and ouabain binding indicate a maximum ATP turnover of 100 s^{-1}. This value is close to the maximum pump rate calculated for renal tissue, but slightly lower than estimates on the isolated enzyme (Jørgensen, 1975; Sejersted et al., 1985*b*). A maximum pump rate of 6.8×10^{-6} $mol \cdot g^{-1} \cdot min^{-1}$ is equal to 25 $pmol \cdot cm^{-2} \cdot s^{-1}$ using the surface-to-volume ratio of 0.46 μm^{-1} provided by Mobley and Page (1972). This value fits reasonably well with a maximum pump rate of 30 $pmol \cdot cm^{-2} \cdot s^{-1}$ in guinea pig auricles (Glitsch et al., 1976), 17 $pmol \cdot cm^{-2} \cdot s^{-1}$ in frog sartorius muscle (Mullins and Frumento, 1963), and 33 $pmol \cdot cm^{-2} \cdot s^{-1}$ in the giant axon of the squid (De Weer et al., 1986). In terms of current, the pump rate will equal 5.2×10^{-6} mol $charges \cdot g^{-1} \cdot min^{-1}$ or 8.4 $mA \cdot g^{-1}$, equal to 1.8 $\mu A \cdot cm^{-2}$, which is close to the maximum pump current reported by Gadsby et al. (1985) in isolated

guinea pig heart cells. It seems reasonable to conclude that the observed V_{max} was close to a true maximum pumping rate for the Na,K pump in Purkinje fibers.

Few data are available on the amount of ouabain binding to cardiac tissue. The K_{diss} we found is close to that reported for ouabain binding to skeletal muscle from both rat and man (Erdmann et al., 1976; Nørgaard et al., 1984). The total ouabain-binding capacity has been reported to be 250 pmol/g wet wt in human heart (Erdmann and Brown, 1983) and 670 pmol/g wet wt in porcine ventricular tissue (Sejersted et al., 1985*a*). We are not aware of other measurements of binding in Purkinje fibers, but our results seem to agree reasonably well with the binding to ventricular tissue. The pump density will be in the range of 5×10^{10} pumps/cm^2.

Apart from studies on red cells (Garay and Garrahan, 1973), attempts at estimating the extent of sigmoidicity, for instance as the Hill coefficient, have been based on studies of the isolated enzyme (see, e.g., Lindenmayer et al., 1974) or Na,K pumping in vesicles (Philipson and Nishimoto, 1983; Karlish and Stein, 1985), where Na^+ and K^+ at the intracellular site could not be varied reciprocally. Karlish and Stein (1985) reported a transmembrane allosteric effect of extracellular Na^+. When extracellular Na^+ was elevated from 0 to 100 mM, the $K_{0.5}$ for intracellular Na^+ increased from 7.1 to 10.1 mM and the Hill coefficient fell from 1.9 to 1.3. In the present experiments, the measured $K_{0.5}$ might be on the low side and the Hill coefficient could be slightly overestimated, because of our use of 2.4 mM extracellular Na^+. The sigmoidal response that we observed has been difficult to detect in living cells and many investigators have assumed simple Michaelis-Menten kinetics or a linear relationship between the pump rate and a^i_{Na} in models of the pump, perhaps because of the limited range of a^i_{Na} they studied.

In conclusion, the Na,K pump rate in intact Purkinje fibers where a^i_{Na} and a^i_K vary reciprocally is a sigmoidal function of a^i_{Na}:

$$v/V_{max} = [a_{Na}^{i(1.94)}]/[95.2 + a_{Na}^{i(1.94)}],$$

where V_{max} (in terms of the Na pumping rate) equals 6.8 $\mu mol \cdot g^{-1} \cdot min^{-1}$ or 25 $pmol \cdot cm^{-2} \cdot s^{-1}$ and, in terms of current, 1.8 $\mu A \cdot cm^{-2}$. This suggests that at an a^i_{Na} of 8.2 mM, which is in agreement with previous estimates in resting Purkinje fibers (Lee, 1981), the pump will operate at 40% of its maximal Na-stimulated pumping rate. The resting pump rate will then be close to 10 $pmol \cdot cm^{-2} \cdot s^{-1}$.

We wish to thank Odd Vaage, Einer Jebens, and Per Kristian Lunde for expert advice and technical help with data analysis and ouabain-binding measurements.

This work was supported by the Norwegian Research Council for Science and the Humanities (OMS), by the National Institute of Occupational Health, Oslo, Norway (OMS), and by U. S. Public Health Service grants HL-20592 (H. A. Fozzard) and HL-30724 (J. A. Wasserstrom) and a grant-in-aid from the American Heart Association (J. A. Wasserstrom).

Original version received 23 January 1987 and accepted version received 1 October 1987.

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