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Direct measurement of changes in sodium pump current in canine cardiac Purkinje fibers

(electrogenic sodium extrusion/voltage clamp/acetylstrophanthidin)

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ABSTRACT **Purkinje fibers from dog hearts may have either a "high" resting potential of about −90 mV or a "low" resting potential of about −40 mV when immersed in low-Cl^- solution containing 4 mM K^+. Brief exposure of Purkinje fibers at the low level of resting potential to K^+-free fluid causes further depolarization, and return to K^+-containing solution elicits a transient hyperpolarization which reaches a peak within a few seconds and then declines within a few minutes. Repeating these changes in K^+ concentration after clamping the membrane potential at its steady resting level in K^+-containing fluid allows the changes in net membrane current presumably underlying the depolarization and transient hyperpolarization to be measured. Net inward current is recorded when the fiber is exposed to K^+-free solution, and a transient net outward current arises when it is returned to K^+-containing solution. The transient net outward current reflects a temporary increase in the rate of electrogenic Na^+ extrusion caused by the rise in intracellular Na^+ concentration that occurs while the sodium pump is slowed in K^+-free fluid. Sodium extrusion remains enhanced, presumably until the internal Na^+ concentration has been brought back to its resting level. The transient outward current is completely abolished by the cardiac steroid acetylstrophanthidin, and its amplitude is increased as the prior exposure to K^+-free fluid is prolonged. The decay of the transient outward current and the decline in intracellular Na^+ concentration both appear to follow first-order kinetics.**

Although it is widely believed that activity of an electrogenic sodium/potassium pump contributes directly to the membrane potential in a large number of cell types (1), in only a few of these have direct measurements of sodium pump current been made. Changes in sodium pump current have been measured in voltage-clamped *Helix* and *Aplysia* neurons after intracellular injection of Na^+ to stimulate pump activity (2, 3). In voltage-clamped Purkinje fibers from sheep hearts a net inward current, attributed to a reduction in outward sodium pump current, was seen on applying a cardiac steroid or lowering the temperature, both of which inhibit pump activity (4, 5).

We report here a technique for directly measuring the changes in sodium pump current which can be readily and reproducibly elicited by briefly exposing voltage-clamped Purkinje fibers from dog hearts to K^+-free fluid and then returning them to K^+-containing solution. Pump activity is slowed in the absence of extracellular K^+ so that the intracellular Na^+ concentration ($[Na]_i$) rises (6, 7), and, because the pump is stimulated by raised $[Na]_i$, the rate of sodium extrusion is temporarily enhanced on returning to K^+-containing fluid. This temporary increase in the rate of electrogenic Na^+ extrusion produces a transient net outward current in Purkinje fibers when those changes in K^+ concentration are repeated after clamping the membrane potential at its steady resting level in K^+-containing fluid. In the presence of acetylstrophanthidin, a specific inhibitor of the sodium pump, that transient outward current is completely abolished.

This method and some of the results were presented to The Biophysical Society at its 1978 Washington meeting (8).

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MATERIALS AND METHODS

Mongrel dogs weighing 5–15 kg were anesthetized by intravenous injection of sodium pentobarbital (30 mg/kg). The hearts were removed rapidly through a thoracotomy. Small Purkinje fiber bundles, not longer than 2 mm and not wider than 200 μm in overall diameter, dissected from the right ventricles of those hearts, were suspended between two fine pins in the narrow channel of a modified Hodgkin–Horowicz (9) fast perfusion system. Conventional glass microelectrodes filled with 3 M KCl were used both for potential recording and current injection; the current and voltage electrodes were inserted near the midpoint of the fiber and about one third of the distance towards the fiber end, respectively. A simple operational amplifier circuit was used to clamp the membrane potential at its initial resting level and another operational amplifier, in ammeter configuration, was used to monitor the applied current and to hold the bath at virtual ground. Currents were filtered (time constant 10 ms or 100 ms) before being displayed on oscilloscopes and pen recorder.

The low-Cl^- solution used throughout these experiments contained (in mM): Na isethionate, 146; K methylsulfate, 4; Hepes (pH 7.3), 5; $MgCl_2$, 0.5; Ca methanesulfonate, 2.7; dextrose, 5.5. K^+-free, low-Cl^- solution was made by replacing the 4 mM K methylsulfate with 4 mM Na isethionate. These solutions were prewarmed and equilibrated with pure oxygen. When required, the cardiac steroid 3-acetylstrophanthidin (kindly provided by Eli Lilly) was added from a refrigerated stock solution of 5 mM acetylstrophanthidin in ethanol; control experiments showed that 0.1% (vol/vol) ethanol had no effect on the resting membrane potential of Purkinje cells over the range of K^+ concentrations used in these experiments. A detailed description of the perfusion and recording systems has been published (10).

RESULTS

We applied step changes in the extracellular K^+ concentration ($[K]_o$) to modify sodium pump activity in small Purkinje fibers, alternately inhibiting the pump in K^+-free fluid and restarting it by switching back to 4 mM $[K]_o$. Fig. 1*a* shows typical changes in membrane potential resulting from a 1-min exposure to K^+-free fluid, recorded in a fiber whose steady resting potential was initially −33 mV at a $[K]_o$ of 4 mM. In low-Cl^- solutions containing about 4 mM K^+, Purkinje fibers may have either a normal ("high") resting potential, near −90 mV, or a "low"

Abbreviations: $[K]_o$, extracellular concentration of K^+; $[Na]_i$, intracellular concentration of Na^+.

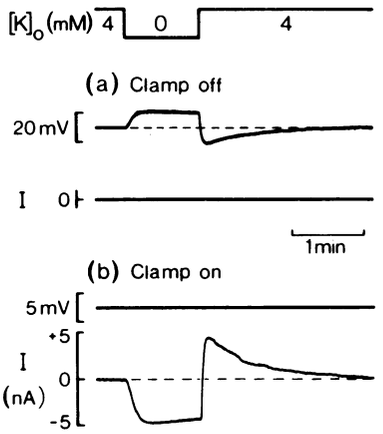

FIG. 1. Changes in membrane potential and in net membrane current (I) recorded in a Purkinje fiber in response to a 1-min exposure to K^+-free fluid. The changes in K^+ concentration ($[K]_o$) from 4 mM to zero and back are indicated by the top line. (*a*) Membrane potential changes (upper trace) in the absence of applied current (lower trace, labeled I). The broken line indicates the resting potential in 4 mM $[K]_o$, which was at the "lower level" of −33 mV (see text). (*b*) Corresponding changes in applied current (lower trace, labeled I) seen when potential (upper trace) is held constant at −33 mV. The broken line indicates zero net current. Current flowing outward across the cell membrane is defined as positive. Note different voltage calibrations in *a* and *b*.

resting potential, usually between −30 and −40 mV; reducing $[K]_o$ at that low level of resting potential causes membrane depolarization, not hyperpolarization (10). Thus, as seen in the upper trace in Fig. 1*a*, the switch to zero $[K]_o$ (indicated by the top line) resulted in a maintained depolarization of some 10 mV. On switching back to 4 mM $[K]_o$, a transient hyperpolarization was recorded which reached a peak within a few seconds and then more slowly declined, the membrane potential returning to its initial resting level after 2–3 min. These potential changes were recorded after the fiber had been impaled with a second, current-injecting, microelectrode, but, as the lower trace (labeled I) in Fig. 1*a* shows, they were recorded in the absence of any applied current.

To obtain the net changes in membrane current underlying these changes in potential, we applied the step changes in $[K]_o$ to the same fiber after switching on the voltage-clamp amplifier to hold the membrane potential at the steady resting level—i.e., zero net current level—in 4 mM $[K]_o$. The upper trace in Fig. 1*b* shows that the membrane potential remained constant during the changes in $[K]_o$; as seen in the lower record, net inward current corresponding to the depolarization in Fig. 1*a* was recorded on switching to zero $[K]_o$, and, on returning to 4 mM $[K]_o$, a transient net outward current corresponding to the transient hyperpolarization in Fig. 1*a* was recorded. The time courses of the changes in net current (Fig. 1*b*) corresponded closely to those of the changes in membrane potential (Fig. 1*a*). The transient net outward current is readily explained if activity of the sodium pump directly generates outward current across the membrane of Purkinje cells—i.e., if the pump is electrogenic. Thus, inhibition of the pump during exposure to zero $[K]_o$ causes $[Na]_i$ to rise, leading to pump stimulation on returning to 4 mM $[K]_o$, so that electrogenic sodium extrusion is enhanced until $[Na]_i$ has been brought back to its initial, steady-state level. The time taken for the transient outward current to decay presumably reflects the time required for the pump to extrude the excess $[Na]_i$. (Similar current changes, attributable to changes in the rate of electrogenic Na^+ extrusion, may be recorded in fibers clamped at the high resting potential, but these are partially obscured by changes in other currents, presumably K^+ currents.)

Part of the net inward current seen on switching to K^+-free solution could thus be accounted for by a reduction in a steady-state component of outward pump current. The existence of a steady-state component of pump current in 4 mM K^+ solution is readily demonstrated by applying a maximal concentration of acetylstrophanthidin, a fast-acting cardiac steroid that specifically inhibits the sodium pump. As shown in Fig. 2*a*, the application of 5 μM acetylstrophanthidin to a Purkinje fiber clamped at its lower level of resting potential in 4 mM $[K]_o$ caused a net inward membrane current, which developed with a half time of about 30 s. When the current was approximately steady, after 2 min of exposure to acetylstrophanthidin, the drug was washed off and the net membrane current slowly returned to zero. In most other experiments the net current temporarily became outward during recovery from a brief exposure to acetylstrophanthidin (e.g., Fig. 2*b*, middle trace), presumably reflecting a transient increase in pump current secondary to the rise in $[Na]_i$ that is expected to occur while the pump is inhibited.

Complete inhibition of the pump by 2 μM acetylstrophanthidin is demonstrated in Fig. 2*b*. In this figure, the lower three traces show changes in net current recorded in the same fiber during consecutive 2-min exposures to K^+-free fluid, indicated by the upper line. Throughout the experiment the fiber was held at its steady low level of resting potential in 4 mM $[K]_o$, −40 mV. During the first run the usual net inward current was seen in zero $[K]_o$, and a transient net outward current appeared when the fiber was returned to 4 mM $[K]_o$. Just before the second run, acetylstrophanthidin was applied and a net inward current slowly developed. Four minutes after adding the drug, when the current had become steady, the fiber was again exposed to K^+-free fluid for 2 min (second trace). An increase in net inward current occurred, but only to about the same level previously obtained in zero $[K]_o$. When the $[K]_o$ was returned to 4 mM in the presence of acetylstrophanthidin, the net current returned monotonically to the level seen just before exposure to zero $[K]_o$; the absence of any transient overshoot of that level strongly suggests that electrogenic pump activity had been completely

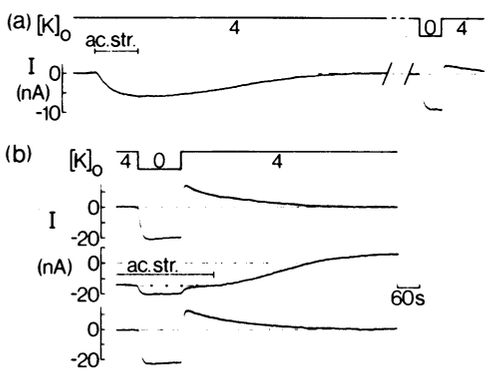

FIG. 2. Effects of acetylstrophanthidin on the net membrane current (I) in voltage-clamped Purkinje fibers. (*a*) Acetylstrophanthidin (5 μM) was applied for a 2-min period (indicated by the bar labeled ac. str.) to a fiber held at its steady resting potential in 4 mM $[K]_o$, −32 mV. After the net current had returned to zero (the break in the current trace indicates omission of a 5-min section of record), the fiber was exposed to zero $[K]_o$ for 1 min, as indicated by the top line. (*b*) Net current changes recorded in a fiber held at −40 mV during three consecutive 2-min exposures to K^+-free fluid. The changes in $[K]_o$ (in mM) are indicated by the upper line. Acetylstrophanthidin (2 μM) was added 3 min before the start of the second run and was washed out at the end of the bar (labeled ac. str.). The 60-s time calibration applies to both *a* and *b*. The broken lines in *a* and *b* indicate the levels of zero current, and the dot-dash line in *b* indicates the steady current level in 4 mM $[K]_o$ in the presence of acetylstrophanthidin.

abolished. When the acetylstrophanthidin was washed out, the net current slowly changed from inward to outward, as discussed above. The net current remained outward for a long time, returning to zero some 25 min after the drug was removed. The third record was obtained a few minutes later; once again, a transient net outward current appeared when the fiber was returned to 4 mM $[K]_o$ after a 2-min exposure to K^+-free fluid.

Fig. 2*a* shows that, after the acetylstrophanthidin had been washed out for some 15 min, exposure to zero $[K]_o$ caused the appearance of a net inward current about 50% larger than that caused by the addition of acetylstrophanthidin. If the pump was completely inhibited by the concentration of drug used here, this result suggests that, in this fiber, about two-thirds of the change in net current seen on switching to zero $[K]_o$ is due to reduction of steady-state pump current and the remaining one-third is due to reduction of some other component of outward current, most probably K^+ current (see *Discussion*). The latter explanation presumably also applies to the further increase in net inward current obtained on reducing $[K]_o$ in the presence of acetylstrophanthidin (Fig. 2*b*, middle trace).

If the transient net outward current reflects enhanced electrogenic Na^+ extrusion caused by raised $[Na]_i$, then prolonging the exposure to zero $[K]_o$ should result in a greater increment in $[Na]_i$ and hence, on returning to 4 mM $[K]_o$, a greater increment in pump rate and, therefore, a larger outward current. In Fig. 3, current changes recorded in the same fiber, in response to six exposures of different durations to K^+-free fluid, have been photographically superimposed. The upper line indicates the timing of the exposures to zero $[K]_o$ which lasted 15, 30, 60, 90, 120, and 180 s, respectively. Although the inward current seen on switching to zero $[K]_o$ was the same in each case, the peak amplitude of the outward current transient on returning to 4 mM $[K]_o$ increased progressively as the prior exposure to K^+-free fluid was prolonged. The net outward currents decayed from their peaks with exponential time courses. The declining phases of the outward current transients in Fig. 3 are shown in the semilogarithmic plots of Fig. 4; each plot is reasonably well approximated by a single exponential. The straight lines were fitted to the experimental points by eye. The time constant of exponential current decay is seen to change very little as the exposure to K^+-free fluid is prolonged, in spite of a large increase in the peak amplitude of outward current. The half time of the decline of outward current in 4 mM $[K]_o$ was determined from semilogarithmic plots like those in Fig. 4 in experiments on 16 preparations in which an average of more than three measurements per fiber were made; the mean half time was 52 s (SD, ±13 s) corresponding to an average time constant of 75 s.

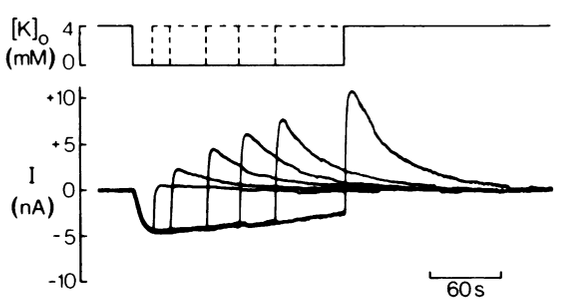

FIG. 3. Photographically superimposed current traces (I) showing the increase in amplitude of the transients of net outward current, recorded in 4 mM $[K]_o$, obtained by prolonging the prior exposure to K^+-free solution. The six different exposures to zero $[K]_o$ are indicated by the upper lines. The membrane potential was clamped at its lower resting level in 4 mM $[K]_o$, −33 mV.

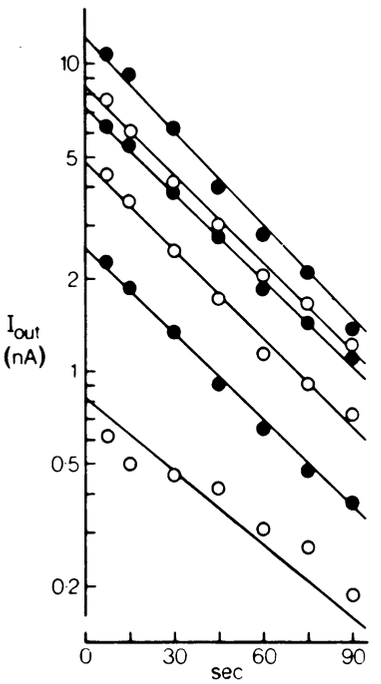

FIG. 4. Semilogarithmic plots of the decay of the transients of net outward current (I_{out}) illustrated in Fig. 3. The points were measured from enlarged records of the current changes. The straight lines were fitted to the points by eye. The abscissa indicates the time from the step-change in $[K]_o$ from zero to 4 mM at the end of the exposures to K^+-free fluid, which lasted 15, 30, 60, 90, 120, and 180 s, respectively. Empty and filled circles serve merely to distinguish adjacent plots.

Since Na^+ influx into voltage-clamped fibers exposed to K^+-free fluid may be expected to remain approximately constant, at least during the first few minutes, the increment in $[Na]_i$ should be proportional to the duration of the exposure to zero $[K]_o$. Fig. 5 shows that the "maximum" amplitude of the transient outward current [obtained by extrapolating the ex-

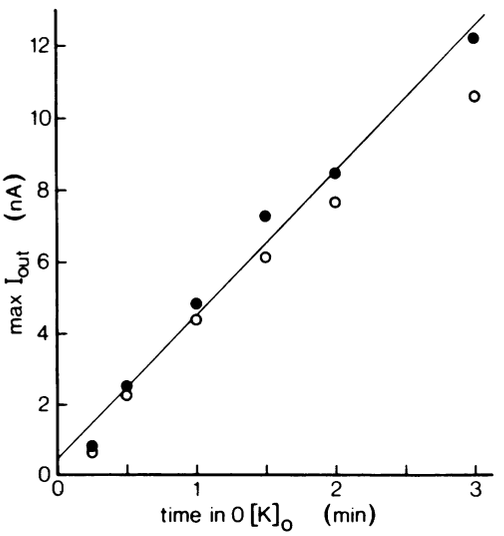

FIG. 5. Plot of maximum net outward current (max I_{out}) from the experiment of Figs. 3 and 4 versus the duration of the preceding period of exposure to zero $[K]_o$, indicated on the abscissa. ○, Peak amplitudes of the outward current transients shown in Fig. 3; ●, values obtained by extrapolation of the semilogarithmic plots of those transients in Fig. 4 to zero time—i.e., to the end of the exposure to zero $[K]_o$. The straight line is a least-squares fit to the latter values.

ponential current decline back to "zero time"—i.e., to the end of the exposure to K^+-free fluid—(see Fig. 4)] increases approximately linearly with the duration of the prior exposure to zero $[K]_o$, suggesting that the increment in pump current is proportional to the increment in $[Na]_i$. If that is so, then the exponential decay of outward current (Figs. 3 and 4) must reflect an exponential decline in $[Na]_i$, which means that the rate of recovery of $[Na]_i$ is proportional to the level of $[Na]_i$ above its resting concentration. Furthermore, since the rate of decline of $[Na]_i$ is proportional to the increase in the rate of Na^+ extrusion above the resting rate, it may be concluded that the increment in outward pump current is linearly related to the increment in rate of Na^+ extrusion. This indicates that a constant fraction of the Na^+ moved by the pump is extruded as net charge; in other words, the Na^+ to K^+ coupling ratio of the pump remains constant under these conditions.

DISCUSSION

The present results indicate that canine cardiac Purkinje cells are rapidly "sodium-loaded" during brief exposures to K^+-free fluid, causing enhancement of electrogenic Na^+ extrusion after the cells are returned to K^+-containing solution. Noma and Irisawa (11, 12) similarly used brief exposures to K^+-free fluid to cause stimulation of the sodium pump in small preparations of cells from the rabbit sino-atrial node. By using Na^+-sensitive microelectrodes, Ellis (7) directly measured the rise in intracellular Na^+ activity occurring in sheep Purkinje fibers during a 10-min exposure to K^+-free fluid and the subsequent fall in Na^+ activity on returning the cells to K^+-containing fluid. That fall in Na^+ activity was accompanied by a transient hyperpolarization similar to, but slower than, that shown in Fig. 1*a*. In voltage-clamped fibers, the transient net outward current underlying that hyperpolarization can be monitored directly (Fig. 1*b*). That no transient overshoot of the steady level of net current is seen after brief periods of exposure to K^+-free fluid in the presence of acetylstrophanthidin, which specifically inhibits the sodium pump (Fig. 2*b*), is strong evidence that the transient net outward current in Fig. 1*b* results from temporary pump stimulation and not, for example, from a temporary change in membrane conductance, or from a reduction in Na^+ influx secondary to the decrease in Na^+ gradient across the cell membrane caused by the rise in $[Na]_i$. Furthermore, neither the hyperpolarization nor the net outward current can be attributed to K^+ depletion just outside the cell membrane due to rapid, pumped K^+ movements, because Fig. 1 shows that lowering $[K]_o$ causes monotonic depolarization or the appearance of a net inward current, respectively.

The very slow transient of net outward current usually seen during recovery from exposure to acetylstrophanthidin (Fig. 2*b*) would be expected to result from the rise in $[Na]_i$ that occurs during pump inhibition (7), leading to enhanced Na extrusion as the drug unbinds from sodium pump sites. The apparent absence of net outward current after removal of acetylstrophanthidin in some experiments (Fig. 2*a*) might merely reflect the difficulty of accurately recording very small changes in current over a long period of time. Alternative possible explanations, including a temporary reduction in the Na^+ to K^+ coupling ratio of the pump in the presence of acetylstrophanthidin, must be tested by additional experiments.

Since pump activity is, apparently, completely abolished by 2 μM acetylstrophanthidin (Fig. 2*b*), it seems likely that the further increase in inward current seen on lowering $[K]_o$ in the presence of acetylstrophanthidin (Fig. 2*b*) results from reduction of a component of outward current other than that generated by the sodium pump. Fig. 2*a* shows that the net inward membrane current seen after switching from 4 mM $[K]_o$ to zero $[K]_o$ is greater than that seen after applying 5 μM acetylstrophanthidin in the presence of 4 mM $[K]_o$, again suggesting that removal of external K^+ not only abolishes pump current but also reduces another outward current, presumably K^+ current. A similar reduction in outward K^+ current in depolarized cells, when $[K]_o$ is lowered, has been well documented as a characteristic property of "inwardly rectifying" K^+ channels in, for example, skeletal muscle fibers (13, 14). Since the membrane of cardiac Purkinje cells also is believed to contain inwardly rectifying K^+ channels (10, 15), it would be reasonable to expect K^+ current to decline on lowering $[K]_o$ from 4 mM to zero at the low level of resting potential.

The current applied via the voltage clamp does not remain steady in K^+-free fluid but decreases slowly (Figs. 1–3). This slow change varies in steepness from preparation to preparation and persists, at least to some extent, in the presence of acetylstrophanthidin. It is not clear, at present, whether the change results from gradual reduction in inward Na^+ current as $[Na]_i$ rises or from an increase in outward current, possibly residual pump current.

The exponential decline of net outward current seen in Figs. 1–3 closely resembles the exponential decay of the current transient recorded by Thomas (2) in voltage-clamped snail neurons after rapid injection of Na^+. Just as Fig. 4 above shows that the time constant for decay of net outward current was approximately independent of the duration of the period of Na^+ loading in K^+-free fluid, Thomas found that the time constant for current decay in snail neurons was independent of the quantity of Na^+ injected. Direct measurements of intracellular Na^+ activity in those snail neurons showed that the decay of clamp current was accompanied by a parallel exponential decline of the increment in Na^+ activity (2), demonstrating that both the transient current and the rate of Na^+ extrusion are proportional to the increase of internal Na^+ activity above the resting level. Such a correlation implies that, under those conditions, a constant fraction of the extruded Na^+ appears as net charge. Our results (see Figs. 3–5) strongly suggest that in voltage-clamped canine cardiac Purkinje fibers, as in snail neurons, the increment in pump current is proportional to the increment in $[Na]_i$ and that both decline exponentially after switching back from K^+-free to K^+-containing fluid. This, in turn, suggests that the increment in rate of Na^+ extrusion is proportional to the increment in $[Na]_i$ and, taken together with the results of Fig. 4, suggests that, at constant $[K]_o$, the rate constant for Na^+ extrusion is independent of $[Na]_i$ over the range encountered in these experiments. Deitmer and Ellis (16) have recently reported that intracellular Na^+ activity in sheep Purkinje fibers declines exponentially after Na^+ loading in K^+-free fluid, although the membrane potential was not held constant. Thus the rate of Na^+ efflux has now been shown to decline exponentially in several preparations after varying degrees of Na^+ loading. However, the time constants for the decline vary considerably, being about 5 hr for squid axons (17), 1.5 hr for isolated fibers from frog skeletal muscle (18), 4.8 min for snail neurons (2), and 5.3 min for sheep Purkinje fibers (16). The present results suggest that the time constant for decline of the rate of Na^+ extrusion in canine Purkinje fibers, after modest Na^+ loading, has an average value of 1.3 min. The large variability of these values presumably reflects differences not only in the surface-to-volume ratio, but possibly also in the density of sodium pump sites in the surface membrane of those different types of cells.

The present techniques for rapidly altering activity of the sodium/potassium pump while monitoring the resulting changes in pump current under different experimental conditions should permit detailed characterization of the sodium

pump in heart cells and of its interaction with cardiac steroids.

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