Effect on Membrane Potential and Electrical Activity of Adding Sodium to Sodium-Depleted Cardiac Purkinje Fibers

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ABSTRACT Canine cardiac Purkinje fibers exposed to Na-free solutions containing 128 mM TEA and 16 mM Ca show resting potentials in the range −50 to −90 mV; if the concentration of Na in the perfusate is raised from 0 to 4 to 24 mM, hyperpolarization follows. If the initial resting potential is low, the hyperpolarization tends to be greater; the average increase in the presence of 8 mM Na is 14 mV. Such hyperpolarization is not induced by adding Na to K-free solutions, is not seen in cooled fibers, or in fibers exposed to 10^{-3} M ouabain, nor is it induced by adding Li and thus may result from electrogenic sodium extrusion. Fibers exposed to Na-free solutions are often spontaneously active; if they are quiescent they often show repetitive activity during depolarizing pulses. Such spontaneous or repetitive activity is suppressed by the addition of Na. This suppression may or may not be related to the hyperpolarization.

Propagated action potentials dependent on Ca as a carrier of inward current can occur in canine cardiac Purkinje fibers exposed to sodium-free solutions (Aronson and Cranefield, 1973). Such action potentials presumably result from the flow of inward current through the so-called "slow channel." Voltage-clamp studies suggest that the slow channel is permeable both to Ca and to Na (Rougier et al., 1969; Vitek and Trautwein, 1971; for review see Reuter, 1973, and Trautwein, 1973). Since a very small amount of Na may remain in bundles of Purkinje fibers exposed to Na-free solutions (Aronson and Cranefield, 1973; Reuter, 1973), the action potentials seen in fibers exposed to Na-free solutions might depend at least in part on Na as a carrier of inward current. To examine this possibility we added small amounts of NaCl to initially Na-free perfusates. Such an addition of Na may transiently increase the overshoot of the action potential but the almost invariable result is the abolition of spontaneous activity followed by a marked increase in resting potential. The hyperpolarization suggests that the addition of Na to the previously Na-free perfusate induces electrogenic extrusion of sodium. We report below results consistent with this hypothesis, supporting the belief that electrogenic

sodium extrusion does occur in cardiac tissues. The effect of increasing Na_o on spontaneous activity and on excitability is less easily explained.

METHODS

Mongrel dogs of either sex weighing at least 26 kg were anesthetized with an intravenous injection of pentobarbital sodium (30 mg/kg). The heart was rapidly excised and immersed in a balanced salt solution (see below). Bundles of Purkinje fibers (false tendons) were removed from the right and left ventricles and incubated in the same solution at 36°. Bundles to be studied were placed in a tissue bath and perfused with solution maintained at 35–36°.

Electrical recording and current injection were achieved by means of glass microelectrodes filled with 3 M KCl (tip resistance 8–20 MΩ). Current was passed through intracellular electrodes by means of a high-voltage field-effect transistor circuit. External stimuli were delivered through bipolar, Teflon-coated silver wires (0.005 inches in diameter), insulated except at their tips. The timing of the pulses was achieved by means of a digital parallel timing system (Silverman and Eisenberg, 1971). All stimuli were isolated from ground. Some records were obtained by photographing an oscilloscope, others were recorded on a rectilinear polygraph (model ICT-5H; Gilson Medical Electronics, Inc., Middleton, Wis.). Many records were recorded on a Honeywell 5600 tape recorder (Honeywell, Inc., Test Instruments Div., Denver, Colo.) and were played back either on an oscilloscope (RM565 or R5103N, Tektronix, Inc., Beaverton, Ore.) or on the polygraph. The frequency response of the tape recorder at the speeds used was 5 kHz; that of the polygraph is 75 Hz. These frequency responses are adequate for recording diastolic depolarization, slow upstrokes seen in Na-free solutions, and slow changes in resting potential.

The Tyrode's solution contained (in mM): NaCl, 137; $NaHCO_3$, 12; NaH_2PO_4, 1.8; $CaCl_2$, 2.7; KCl, 4; $MgCl_2$, 0.5; dextrose, 5.5. The most frequently used Na-free solution contained 128 mM tetraethylammonium chloride (TEA), 16.2 mM $CaCl_2$, 2.7 mM KCl, 0.5 mM $MgCl_2$, 5.5 mM dextrose, and 5.0 mM tromethamine (Tris) adjusted to pH 7.2 to 7.4 with HCl. In Na-free solutions in which Ca was less than 16 mM the concentration of TEA was raised to preserve osmolarity. Addition of NaCl or reduction of KCl in such solutions was made without compensation for osmolarity. All chemicals were reagent grade except TEA (Eastman Organic Chemicals Div., Eastman Kodak Co., Rochester, N.Y.). TEA solutions were filtered before use. Glass condensed redistilled water was used. The Tyrode's solution was bubbled with 95% O_2 and 5% CO_2. The solutions that were buffered with Tris were bubbled with 100% O_2. Because of the temperature dependence of Tris buffer (Sigma Technical Bulletin № 106B), experiments in which temperature was changed required one solution buffered to pH 7.2–7.4 at 36° and another buffered to the same pH at 10°. Those solutions were heated or cooled to the required temperature before they reached the bath, and a change in temperature was accomplished fairly abruptly by switching from one solution to the other. Ouabain was used as the octahydrate (Sigma); fresh solutions were made shortly before they were used. Tetrodotoxin (Sigma) was dissolved in 2 ml of distilled water; the resulting solution was immediately added to the perfusate.

Several bundles were assayed for total tissue Na and K. The fiber was blotted

gently on ashless filter paper and a wet weight was obtained. The fiber was then dried for several hours under a heat lamp, after which a dry weight was obtained. The dried fiber was extracted with 3 ml of 0.1 N HNO_3 for 72 h. Na and K content was then determined by flame spectrophotometry.

In the text that follows, concentrations are designated simply by the chemical symbol and a subscript to indicate intracellular or extracellular concentration. For example, Ca_o is used instead of $[Ca^{++}]_o$.

RESULTS

Some cardiac Purkinje fibers exposed to Na-free solutions that contain Ca are spontaneously active (Aronson and Cranefield, 1973), others show sustained rhythmic activity only after having been "triggered" into such activity by a driven impulse (Cranefield and Aronson, 1974), and still others are quiescent but show repetitive activity during a depolarizing pulse (Aronson and Cranefield, 1974). Each type of sustained rhythmic activity depends, at least in part, on the fact that every action potential is followed by an oscillatory after-potential made up of an initial after-hyperpolarization and a delayed after-depolarization. The after-depolarization carries the membrane potential to the threshold potential and initiates the next action potential.

Fig. 1 A shows action potentials recorded from a fiber that was spontaneously active when exposed to Na-free solution containing 16 mM Ca. Elevation of Na_o from 0 to 8 mM (arrow) is followed by an increase in maximal diastolic potential and by a slight decrease in rate; in some fibers, an

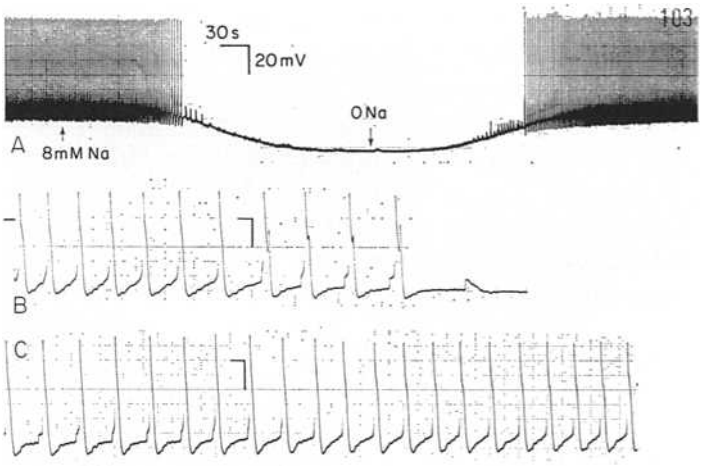

FIGURE 1. Panel A shows the effect of readmission of Na to a spontaneously active Na-depleted Purkinje fiber exposed to 16 mM Ca. Na was raised to 8 mM for the period indicated. In B and C the cessation and resumption of spontaneous activity are shown at a higher sweep speed to show the behavior of the phase of spontaneous depolarization. Calibrations in B and C: horizontal, 1 s; vertical, 10 mV. The horizontal line of each calibration grid marks the level of "zero" membrane potential.

increase in overshoot is seen. Less than 1 min after the addition of Na, spontaneous activity ceases at a level of membrane potential that is positive to the maximum diastolic potential. Continuing hyperpolarization carries the membrane potential to a level well negative to the maximum diastolic potential seen when the perfusate was Na free. In general fibers thus exposed to Na become quiescent at a level of membrane potential less negative than the maximum diastolic potential, as might be expected from the fact that the maximum diastolic potential is determined by an after-hyperpolarization. Values for hyperpolarization given in the present article are referred to the membrane potential seen at the moment the fiber becomes quiescent rather than to the preceding maximum diastolic potential. The maximum hyperpolarization attained in Fig. 1 A is 23 mV. Soon after that maximum was attained, Na_o was lowered to zero (arrow) whereupon the membrane potential declined and spontaneous activity reappeared.

In Fig. 1 B, the last 11 action potentials seen before spontaneous activity stopped after the addition of Na are shown at a faster recording speed to show the behavior of the diastolic depolarization. Between the first and second action potentials shown in Fig. 1 B there is a smooth continuous depolarization that begins at the level of the maximum diastolic potential and eventually merges with the upstroke. After Na is added two changes are seen: (*a*) there is a beat-to-beat increase in the maximum diastolic potential and (*b*) there is a gradual disappearance of the delayed after-depolarization. As the after-depolarization vanishes the decay of the after-hyperpolarization is followed by a period of constant transmembrane potential. After this flattening has occurred the upstroke appears as an abrupt deflection of the sort expected when activation at the recording site results from propagated activity. These records indicate that the pacemaker has shifted from the recording site to another site. Some of the upstrokes also show a small step or notch. In Fig. 1 A a few low amplitude deflections are seen after the last full upstroke; the first of these is shown in Fig. 1 B where it can be seen to correspond to that part of the action potential that precedes the notch. Such notches are an inconstant finding; they suggest the presence of impaired conduction in the vicinity of the recording site. The sequence of events just described is reversed during the return of spontaneous activity after Na_o is reduced to zero (Fig. 1 C). The earliest responses show a brief after-hyperpolarization declining to a constant level of membrane potential; that constant level is interrupted by a propagated upstroke. Later responses show diastolic depolarization merging smoothly into the upstroke.

Frank hyperpolarization does not always precede the loss of spontaneous activity. In some fibers the onset of quiescence is preceded by a gradual decline in the slope of the diastolic depolarization without an increase in the maximum diastolic potential. In such fibers spontaneous activity ceases at

the level of the maximum diastolic potential and hyperpolarization follows. The decline in the slope of diastolic depolarization before block obtains is, of course, equivalent to a gradual increase in average membrane potential during diastole.

Hyperpolarization induced by addition of Na to the perfusate is to some extent proportional to the extent to which Na_o is increased. Fig. 2 A shows the effect of increasing Na_o from 0 to 8 mM. After the hyperpolarization reached its maximum the bundle was again exposed to Na-free perfusate; the hyperpolarization vanished and spontaneous activity reappeared. After the fiber had been reexposed to Na-free solution for about 20 min Na_o was raised to 24 mM (Fig. 2 B). The maximum hyperpolarization was 40 mV during exposure to 24 mM Na_o as against 20 mV during exposure to 8 mM Na_o and the hyperpolarization developed far more rapidly.

The hyperpolarization induced by adding Na does not persist at its peak level indefinitely even if the fiber remains exposed to Na. Fig. 3 A shows a

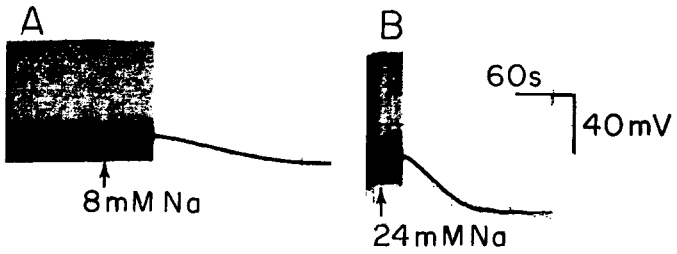

FIGURE 2. In A and B the initial portion of the records shows spontaneous activity in a fiber exposed to a solution containing 0 Na and 16 mM Ca. The cessation of activity and hyperpolarization shown in A resulted from an increase in Na_o to 8 mM; after a period of exposure to Na-free solution the same fiber was exposed to 24 mM Na. The level of the horizontal line represents zero membrane potential.

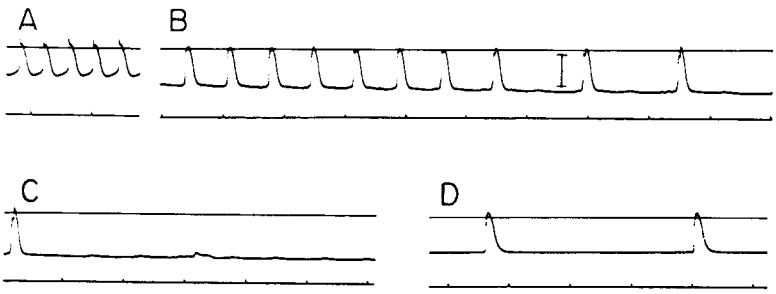

FIGURE 3. In A spontaneous activity is seen in a fiber exposed to Na-free solution; in B-D the perfusate contains 8 mM Na. Progressive hyperpolarization (B) leads to quiescence (C). Panel C is continuous with B. After 27 min the resting potential diminishes in the presence of Na and activity reappears (D). The upper line in all records represents zero membrane potential; the vertical calibration (in B) represents 60 mV. The time marks in the lower trace occur every 5 s.

fiber exposed to a Na-free solution containing 16 mM Ca; the maximum diastolic potential is −56 mV. The level of Na in the perfusate was increased from 0 to 8 mM and a gradual hyperpolarization ensued, initially causing flattening of diastolic depolarization and reduction of rate (Fig. 3 B) and then quiescence at a membrane potential of −83 mV (Fig. 3 C). The maximum hyperpolarization was attained 3 min after raising Na_o; thereafter the membrane potential slowly declined to less negative levels. Nearly 30 min after the moment of maximum hyperpolarization the membrane potential was −72 mV and slow spontaneous activity was seen (Fig. 3 D). A similar decline is seen in Figs. 7 and 8 below. The decline in resting potential has been followed for as long as 60 min in some fibers. In all fibers studied, resting potential always remained higher in the presence of Na than during perfusion with Na-free medium either before or after exposure to Na. Similarly, spontaneous activity, if present, was always slower than the control rate.

Purkinje fibers that are spontaneously active when exposed to Na-free, Ca-containing solutions usually show a maximum diastolic potential of about −60 mV. Fibers that show significantly higher resting potentials are usually quiescent but often show repetitive activity during the flow of a pulse of depolarizing current (Aronson and Cranefield, 1974). The tendency of a fiber to show repetitive activity during a pulse of depolarizing current is reduced or abolished by adding Na to the perfusate. Fig. 4 A shows a quiescent Purkinje fiber exposed to a Na-free solution containing 16 mM Ca. The fiber becomes active during a 12-s constant current depolarizing pulse. The first and each subsequent action potential during the pulse is followed by an after-hyperpolarization, the decay of which leads into a delayed after-depolarization that merges with the next action potential (Aronson and Cranefield, 1974). After Fig. 4 A was recorded, the level of Na in the perfusate was raised from 0 to 4 mM; the fiber remained quiescent and gradual hyperpolarization was seen. After 10-min exposure to 4 mM Na, the same applied current elicited only a local response (Fig. 4 B). A stronger pulse (Fig. 4 C) evoked a single regenerative response that appeared to have a higher threshold than the control. That response was not followed by the oscillatory after-potential associated with repetitive activity. On removal of Na from the perfusate the membrane potential returned to control values and repetitive activity was again seen during depolarizing pulses (not shown). It is usually possible to evoke one regenerative response during exposure to Na but some fibers become completely inexcitable during such exposure.

We have seen an inhibitory effect of Na on repetitive activity with concentrations as low as 1 mM and routinely see hyperpolarization using concentrations of 4–8 mM. Hyperpolarization induced by raising Na_o to 8 mM in solutions containing 16 mM Ca varies from about 8 mV to about 20 mV. The degree of hyperpolarization varies with the concentration of Na_o and also

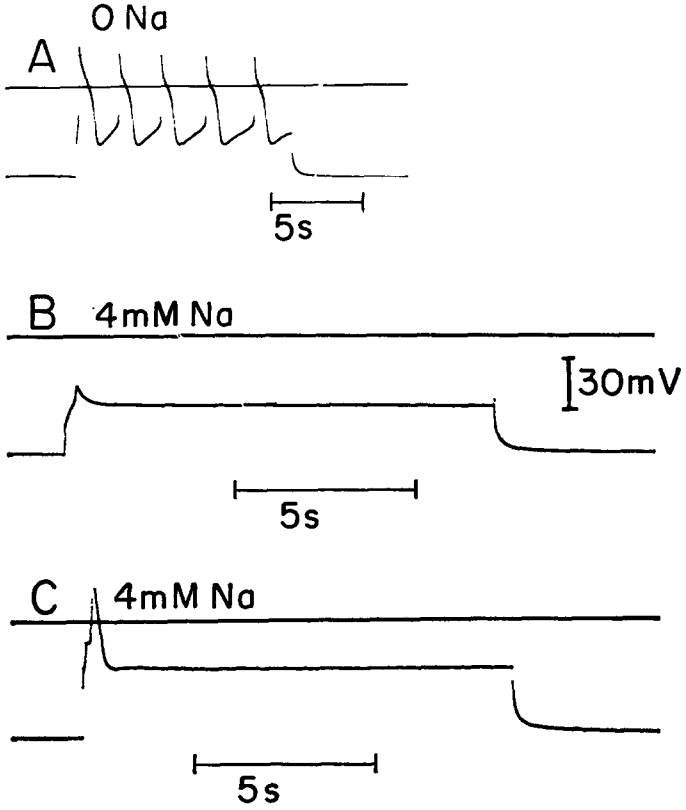

FIGURE 4. In A a fiber that was quiescent when exposed to a Na-free solution becomes active during a depolarizing pulse. Between A and B the level of Na in the perfusate was raised to 4 mM and hyperpolarization occurred after which a depolarizing pulse of the same strength evoked no activity (B). A much stronger depolarizing pulse (C) produced a single action potential. The upper line in all records represents zero membrane potential. The recording speed in B and C is twice that in A.

varies with the initial level of membrane potential. Greater hyperpolarizations are seen in fibers with a low initial resting potential (Fig. 5). In 3 fibers out of some 90 that were studied, elevation of Na produced little or no slowing and no hyperpolarization. By chance, none of those fibers was exposed to 8 mM Na, one having been exposed to 4 mM Na_o and two to 12 mM Na_o. It is perhaps significant that in those same fibers hyperpolarization induced by applied current produced relatively little slowing. One fiber exposed to 12 mM Na_o showed no hyperpolarization, but repetitive activity was abolished.

An obvious explanation of the hyperpolarization described above is that the addition of Na to the perfusate leads to an increase in Na_i and thereby to the activation of an electrogenic Na-K pump. To test this interpretation we have examined the effect of adding Li instead of Na and we have examined

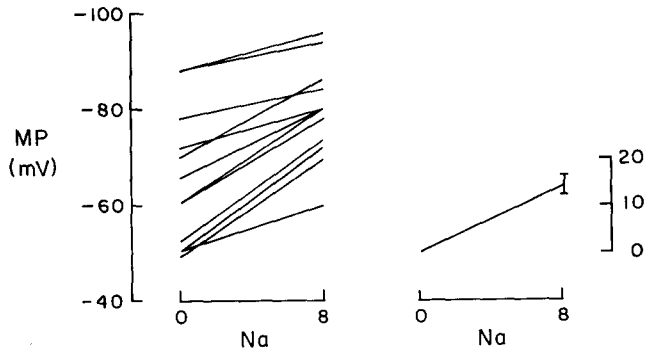

FIGURE 5. In the left-hand graph, each line connects the resting or maximum diastolic potential seen in a fiber exposed to Na-free solution and the resting potential seen at the level of maximal hyperpolarization during exposure to 8 mM Na. Each line represents a different fiber; all fibers were exposed to 16 mM Ca and 2 7 mM K throughout the course of the experiment. The right-hand graph shows the average increase (± SEM) in membrane potential for these 12 fibers.

the effects of adding Na to K-free perfusates, at low temperatures and in the presence of ouabain.

Effect of Li

Although Li can replace Na as a carrier of inward current it is not actively extruded from cardiac Purkinje fibers (Carmeliet, 1964). The addition of Li to a Na-free Ca-containing perfusate therefore should not induce electrogenic extrusion. Fig. 6 shows that a spontaneously active fiber (Fig. 6 A) is not affected by raising Li_o to 8 mM (Fig. 6 B), but that the subsequent increase of Na_o to 8 mM rapidly leads both to a loss of activity and to hyperpolarization (Fig. 6 C). In D the fiber remained exposed to 8 mM Na but Li_o was reduced to 0. Spontaneous activity and membrane potential rapidly return towards control levels after the fiber is exposed to a Na-free solution containing 8 mM Li (Fig. 6 E) and reach control levels after exposure to a Na-free, Li-free solution (Fig. 6 F).

Effects of K_o

In the most commonly postulated form of electrogenic extrusion of Na, potassium ions obligatorily enter the fiber. Such extrusion is inhibited by exposing the fiber to a K-free solution since no K is available to enter the fiber other than the quite small amounts that are present as the result of the leakage of K from the myoplasm. At the beginning of Fig. 7 A spontaneous activity is seen in a fiber exposed to a Na-free, K-free medium containing 16 mM Ca. The elevation of Na_o from 0 to 8 mM produced a flattening of spontaneous depolarization, and a cessation of spontaneous activity. The membrane

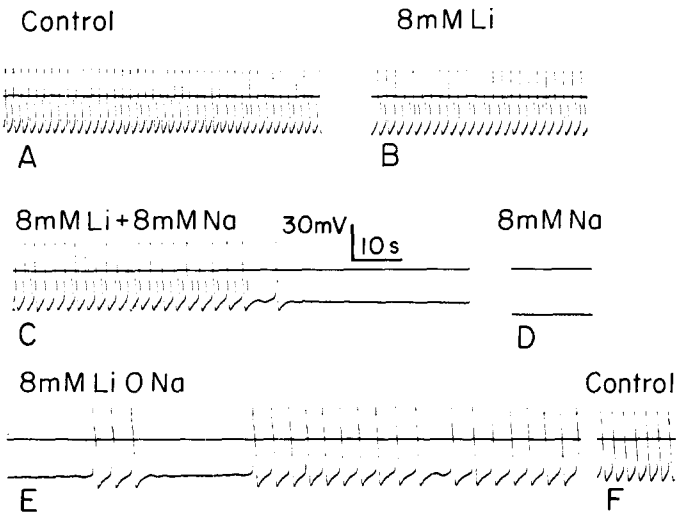

FIGURE 6. In A, spontaneous activity is seen in a fiber exposed to a solution containing 0 Na and 4 mM Ca. In B the perfusate contained 8 mM Li; electrical activity is unchanged after 10 min. In C, the perfusate contained 8 mM Li and 8 mM Na; the rate began to slow within 1 min after the addition of Na and hyperpolarization led to quiescence within 2 min. In D, the fiber has been exposed to Li-free solution containing 8 mM Na for 10 min; the hyperpolarization has attained a maximum. At this point, 8 mM Li was readmitted and Na was removed from the perfusate; spontaneous activity reappeared within 3 min (E). In F, the fiber is again exposed to a perfusate containing neither Na nor Li. The continuous line in each record represents zero membrane potential.

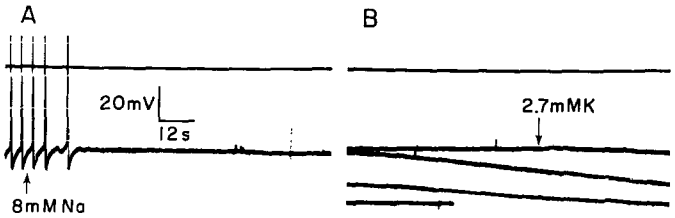

FIGURE 7. In A, the fiber is exposed to 16 mM Ca in a K-free, Na-free perfusate to which 8 mM Na is added after the second action potential. A slight degree of hyperpolarization accompanies a loss of spontaneous activity. Five minutes elapsed between the records shown in A and in B. Near the end of the first sweep in B, K_o was increased to 2.7 mM, leading to a progressive hyperpolarization seen during the next three sweeps. The uppermost line in both panels represents zero membrane potential.

potential becomes about 4 mV negative to the level at which quiescence appeared. This suggests that some active extrusion of Na occurred even when the perfusate was K free; as pointed out above, the fact that the perfusate was K free does not mean that there is no K whatever at the outer surface of the cell. Fig. 7 B shows the effect of increasing the level of K in the perfusate

from 0 to 2.7 mM; this was done near the end of the first sweep (arrow). The second and third sweeps and part of a fourth sweep show a hyperpolarization of over 32 mV to a level of −83 mV.

Since lowering of K_o to zero reduces P_K and thus causes depolarization in cardiac Purkinje fibers (Weidmann, 1956; Coraboeuf, 1960; Hoffman and Cranefield, 1960) the hyperpolarization shown in Fig. 7 may be caused in part by an increase in P_K induced by the increase in K_o from 0 to 2.7 mM. That the effect is not solely the result of the relationship between K_o, P_K, and resting potential is seen in Fig. 8. A fiber that was quiescent at a resting

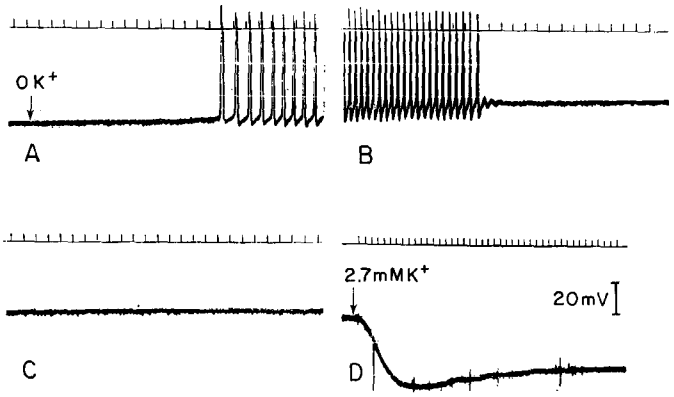

FIGURE 8. In A, a quiescent fiber exposed to 16 mM Ca, 2.7 mM K in a Na-free perfusate is suddenly exposed to a K-free medium. As K_o declines, the fiber depolarizes and spontaneous activity appears. Panel B shows electrical activity after 6 min of exposure to K-free medium; panel C is a continuation of B. Between the end of the record shown in C and the beginning of the record shown in D, Na_o was raised to 8 mM; at the beginning of panel D, the fiber had been exposed to K-free medium containing 8 mM Na for 10 min. Note the slight hyperpolarization. At the arrow in D, the level of K in the perfusate was increased from 0 to 2.7 mM. Note that the prompt increase in resting potential is not maintained but declines slowly in time. The uppermost line in each panel represents zero membrane potential; time marks on this line are 5 s apart. Sweep speed in D is slower than in A-C.

potential of −60 mV when exposed to a Na-free solution containing 16 mM Ca depolarized and became spontaneously active when K_o was reduced from 2.7 mM to 0 (Fig. 8 A). Further loss of resting potential to about −40 mV was accompanied by loss of spontaneous activity (Fig. 8 B, C). Between Fig. 8 C and D the level of Na in the perfusate was raised from 0 to 8 mM; at the beginning of Fig. 8 D the fiber had been exposed to 8 mM Na for 10 min and had shown a very small increase in resting potential. Raising the level of K in the perfusate from 0 to 2.7 mM produced a marked and fairly rapid hyperpolarization to a membrane potential of about −85 mV (Fig. 8 D). At the peak of the hyperpolarization the membrane potential is nearly 50 mV more

negative than it was in Fig. 8 C when the fiber was exposed to Na-free, K-free solution. More importantly, it is almost 25 mV more negative than it was in Fig. 8 A when the fiber was exposed to Na-free solution containing 2.7 mM K.

Fibers exposed to Na-free, K-free solutions are sometimes spontaneously active. Elevation of Na from 0 to 8 mM causes only a small increase in resting potential but may make the fiber wholly inexcitable so that not even a single initial action potential is seen during passage of a strong depolarizing pulse. This finding is not consistent with attributing the Na-induced inhibition of activity solely to electrogenic extrusion of Na because it implies inhibition of excitability without a marked increase in the membrane potential.

Low Temperature

Cooling fibers exposed to Na-free, Ca-containing solutions causes a remarkable increase in the overshoot and the duration of the action potential; the resting potential often increases but in some fibers it decreases. The rate slows at low temperatures and spontaneous activity may become intermittent or may cease. The addition of Na at low temperature has little effect on rate or on membrane potential.

The effect of a sudden change in temperature on a spontaneously active fiber exposed to a Na-free solution containing 16 mM Ca is shown in Fig. 9 A. As the temperature is lowered from 36 to 16° the rate slows, the overshoot and duration of the action potential increase markedly, and there is an increase in maximum diastolic potential; these effects are rapidly reversed when the temperature is increased to 35°C. If 8 mM Na is present, however, the effect of cooling is entirely different. In Fig. 9 B the same fiber shows quiescence at 36° when exposed to 8 mM Na; as the fiber is cooled to 13° a transient increase in resting potential is followed by a 10-mV decrease and by the appearance of spontaneous activity. The fall in resting potential and the change from quiescence to spontaneous activity suggest that cooling has inhibited electrogenic sodium extrusion.

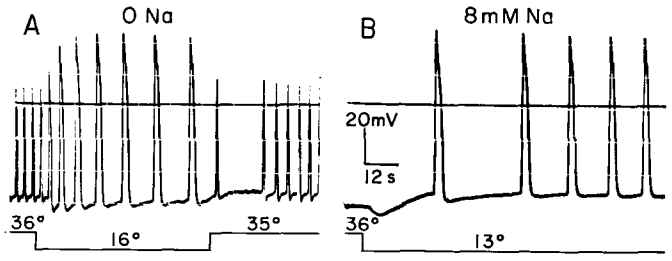

FIGURE 9. The effect of cooling on a spontaneously active fiber exposed to 16 mM Ca in Na-free (A) and Na-poor (B) medium is shown. In A, the fiber is cooled to 16°. In B, during exposure to 8 mM Na, it is cooled to 13°. The upper line in both records represents zero membrane potential.

The tracing shown in Fig. 10 was obtained from a fiber that showed sustained rhythmic activity at 36° when exposed to a Na-free solution containing 16 mM Ca. The recording speed is too slow to separate the action potentials that are seen at the beginning and end of the record. When the temperature was lowered to 9°C, a moderate hyperpolarization accompanied the disappearance of spontaneous activity. After cooling the level of Na_o was raised from 0 to 8 mM, a change that had no effect on the resting potential for 4 min. Perfusion with a warm (36°) solution containing 8 mM Na led to hyperpolarization within 30 s; the hyperpolarization attained a maximum of 14 mV, carrying the resting potential to −82 mV. Finally, perfusion with a warm (36°), Na-free solution caused the resting potential to fall slowly until spontaneous activity resumed. A further gradual loss of membrane potential was

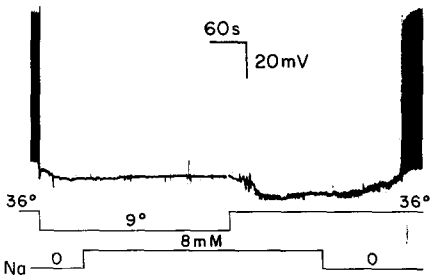

FIGURE 10. A fiber that is spontaneously active at 36° in Na-free solution containing 16 mM Ca is suddenly cooled to 9°. While cooled 8 mM Na is introduced. In the continued presence of Na, the fiber is warmed to 36°, and finally, Na-free perfusate of 36° is introduced. The horizontal calibration marks the level of zero membrane potential

manifested by a shift of the maximum diastolic potential to less negative levels during the first 60 s of spontaneous activity seen at the end of the record.

Ouabain

It ought to be possible to inhibit electrogenic sodium extrusion by ouabain and it is well known that rather small concentrations of ouabain cause a loss of resting potential in Purkinje fibers exposed to normal Tyrode's solution. We have not been able to suppress Na-induced hyperpolarization in fibers showing Ca-dependent action potentials except by use of 1 mM ouabain. Fig. 11 A shows the effect on spontaneous activity and membrane potential of increasing Na_o from 0 to 8 mM in a solution containing 16 mM Ca. The expected effect is seen, the maximum hyperpolarization being 27 mV. After return of Na_o to zero the fiber was exposed to 1 mM ouabain for 20 min and Na_o was again increased to 8 mM. During the interval shown in Fig. 12 B there was a decline in overshoot and in maximum diastolic potential; the electrode was dislodged just after the end of the record but impalements made

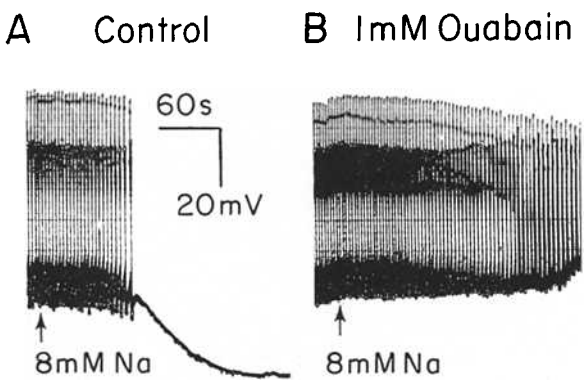

FIGURE 11. In A, a spontaneously active fiber exposed to 16 mM Ca rapidly becomes quiescent and hyperpolarizes when Na_o is increased to 8 mM In B, this fiber has been exposed to 10^{-3} M ouabain for 20 min before elevation of Na_o to 8 mM The horizontal line marks the level of zero membrane potential.

as much as 20 min after Na_o had been raised to 8 mM showed that although the action potential had suffered a fairly marked loss in amplitude and duration spontaneous activity persisted and there was no hyperpolarization. When 0.1 mM ouabain was used, partial inhibition of the effect of increasing Na_o was obtained; spontaneous activity ceased but the maximum hyperpolarization was less than half of that seen under control conditions.

Effect of the Length of Exposure to Na-Free Solutions

If a bundle of Purkinje fibers is initially exposed to Tyrode's solution and then to Na-free solution, typical Ca-dependent action potentials can be obtained within 15 min after the beginning of perfusion with Na-free, Ca-containing solutions. The Na concentration of the entire bundle falls to less than 3 mM by the end of 15-min exposure to Na-free solutions, i.e. to the same level found after 2-h exposure to Na-free solution. Nevertheless the elevation of Na_o from 0 to 8 mM 15 min after the bundle has been exposed to a Na-free perfusate may provoke little or no reduction in rate and induce little or no hyperpolarization. Fig. 12 A shows such an elevation of Na_o from 0 to 8 mM 18 min after the bundle had been exposed to a Na-free solution. Almost no change is seen for 5 min whereupon a comparatively small hyperpolarization is seen. After the same bundle is again exposed to a sodium-free perfusate for about 1 h an increase in Na_o readily evokes hyperpolarization, as shown in Fig. 12 B in which the onset of marked hyperpolarization occurs almost immediately. This full effect can be obtained reproducibly only after the bundle has been exposed to Na-free solution for 90 min. The control rate is far lower in Fig. 12 B than in Fig. 12 A; such slowing of rate is usually seen during the first hour of exposure to Na-free solutions but the rate is usually stable for

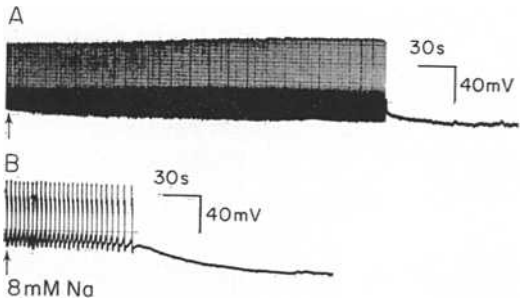

FIGURE 12. A spontaneously active fiber was exposed to 16 mM Ca TEA Tyrode's solution for 18 min before the beginning of the record shown in A. Na_o is increased from 0 to 8 mM at the beginning of this record. In B, the same fiber has been exposed to Na free solution for a total of 75 min. Na_o is again increased from 0 to 8 mM at the beginning of the record. The horizontal bar marks the level of zero membrane potential.

prolonged periods thereafter. There is, throughout the course of Fig. 12 A, a gradual increase in the amplitude of the action potential; this increase in amplitude results from an increase in both the reversal and in the maximum diastolic potential. The increase in maximum diastolic potential seen in Fig. 12 A may reflect the presence of electrogenic extrusion.

Two Levels of Resting Potential

Anodal hyperpolarization sometimes results in a permanent shift of resting potential in fibers exposed to Na-free, Ca-containing solutions; this effect can be obtained with some regularity in fibers that have been exposed to Na-free solutions for only 15 min and only rarely in fibers that have been exposed to Na-free solutions for 60 min or longer. The transition, which often carries the membrane potential from about −60 mV to about −80 mV, occurs far more rapidly than the hyperpolarization induced by the addition of Na. The rapidity of the change and the fact that it can be evoked only at a time when the Na-induced hyperpolarization cannot be obtained indicate that it is a phenomenon distinct from Na-induced hyperpolarization. Its existence nevertheless shows that one must use caution in attributing Na-induced hyperpolarization solely to electrogenic Na extrusion.

DISCUSSION

An obvious conclusion to be drawn from the results described above is that the action potentials seen in Na-free, Ca-containing solutions are indeed Ca spikes. Were they action potentials caused by minute amounts of Na one would not expect the addition of very small amounts of Na to the perfusate to inhibit them. Apart from that, the results described above raise four questions: (*a*) Is the hyperpolarization caused by electrogenic sodium extrusion? (*b*) If so, how does Na enter the fiber sufficiently rapidly and in sufficient quantites

to evoke that extrusion? (*c*) Is the inhibition of spontaneous and induced activity caused by the hyperpolarization or by some quite different mechanism? (*d*) And what is the effect of Na on the presumably Ca-dependent action potentials that can be induced in the presence of low concentrations of Na?

Electrogenic Sodium Extrusion

Previous reports of electrogenic sodium extrusion in cardiac cells (see Thomas, 1972) include those of Page and Storm (1965) who found that cat papillary muscle exposed to cold Ringer's solution for 1–2 h regained resting potential to levels apparently exceeding E_K when warmed; the increase in resting potential was ouabain sensitive. Hyperpolarization to extraordinarily high resting potentials was found by Tamai and Kagiyama (1968) on warming cat ventricular muscle that had been loaded with Na by prolonged incubation at low temperatures; those hyperpolarizations were also ouabain sensitive. Hiraoka and Hecht (1971) reported similar findings in cardiac Purkinje fibers. Other findings that point to electrogenic sodium extrusion in cardiac cells are those of Glitsch (1969, 1972) and Haas (see Haas, 1972). Vassalle has argued persuasively that overdrive suppression and certain of the effects of epinephrine on overdrive suppression can best be explained by assuming that electrogenic sodium extrusion exists in cardiac Purkinje fibers and is enhanced by epinephrine (Vassalle, 1970; Vassalle and Carpentier, 1971; Carpentier and Vassalle, 1971). It should also be noted that by far the most plausible explanation of the loss of resting potential in ouabain-toxic cardiac tissues is partial inhibition of sodium extrusion.

Our finding of a sodium-induced hyperpolarization is most simply explained by assuming that the elevation of Na_o from 0 to 8 mM leads to an increase in Na_i and thus to electrogenic sodium extrusion. The Na-induced hyperpolarization is inhibited by cooling, by ouabain, and by zero K_o as would be expected of electrogenic sodium extrusion. Findings such as the marked increase in the rate and level of hyperpolarization seen when the fiber is exposed to 24 mM Na instead of 8 mM Na (Fig. 2) and the induction of hyperpolarization by elevation of K_o in fibers exposed to K-free, Na-containing solutions (Fig. 8) seem to us to argue rather strongly for electrogenic sodium extrusion as the primary cause of the Na-induced hyperpolarization.

The highest resting potentials that we have observed in the course of Na-induced hyperpolarization are about −95 mV. We have not attempted to show that electrogenic sodium extrusion leads to resting potentials in excess of E_K because the ratio of extracellular space to intracellular space in bundles of Purkinje fibers is so large and so hard to determine with accuracy (Vick et al., 1970) that it is difficult to assign a meaningful value to K_i. The ability to carry the membrane potential to levels negative to E_K is not a necessary

property of electrogenic ion pumping. Hyperpolarization beyond E_K is taken as evidence of electrogenic extrusion because no plausible explanation in terms of changes in permeabilities can be adduced to explain such a degree of hyperpolarization. The essential characteristic of electrogenic ion pumping is the generation of a net transmembrane current; the phenomenon might better be called rheogenic, as suggested by Schwartz (1971) and Schultz (1973).

It is known that i_{K_2} is "inactivated" in Na-free solutions and our findings do not rule out a contribution to the hyperpolarization of a Na-dependent change in P_K of the sort that might result from a Na-induced activation of i_{K_2}. Neither do our results rule out the possibility that a hyperpolarization caused by electrogenic sodium extrusion is enhanced by a voltage-dependent increase in P_K. The fact that the hyperpolarization is blocked by 1 mM ouabain appears to argue against Na- or voltage-dependent changes as the primary cause of the hyperpolarization. The finding that the hyperpolarization declines with time also seems to rule out Na_o-dependent changes in P_K and voltage-dependent changes in P_K as the primary cause of the hyperpolarization since neither would be expected to decline with the mere passage of time in the face of an unaltered Na_o. The possibility that either might change with a time-dependent change in Na_i is considered below.

Finally, one must consider the possibility that the Na-induced hyperpolarization results from the activation of a Na-dependent electrogenic calcium extrusion. Such extrusion would, in terms of our findings, have to be inhibited at low temperatures, be inhibited by zero K_o, be inhibited by ouabain, and be initiated by Na but not by Li. There is no a priori reason why electrogenic Ca extrusion could not have those characteristics.

Entry of Na Into the Fiber

If electrogenic Na extrusion causes the marked hyperpolarization seen on adding Na, one must ask how Na can enter the fiber with sufficient speed and in sufficient quantities to stimulate that extrusion. It is important to note that the presence of action potentials is not needed to permit Na to enter the fiber since hyperpolarization is evoked by elevation of Na_o with equal ease in active and quiescent fibers. We have also found, in a single experiment, that exposure of a quiescent fiber to 10 mg/liter of tetrodotoxin does not prevent Na-induced hyperpolarization; this suggests that Na does not enter via a tetrodotoxin-sensitive channel. The hyperpolarization seen on readmission of Na occurs within 1 or 2 min of adding Na, suggesting that Na_i increases rapidly. There is initially a large electrochemical gradient favoring diffusion of Na into the cell. However, for the rapid influx of Na to be caused by diffusion alone, P_{Na} must be high. If P_{Na} is high, at the moment Na_o is increased and before electrogenic extrusion begins, a significant depolarization should

be seen. In fact, although occasional fibers did show a slight (2–4 mV) depolarization, in most fibers the resting or maximal diastolic potential remained unchanged until the Na-induced hyperpolarization began. The entry of Na into the cell is thus an electroneutral event.

An electroneutral calcium-sodium exchange has been identified in many tissues, including mammalian atrium (Reuter and Seitz, 1968) and frog atrium (Chapman, 1974) but has not been demonstrated in cardiac Purkinje fibers. Many of our observations can be explained or interpreted by assuming that the normal mechanism for extrusion of Ca from Purkinje fibers involves Ca-Na exchange. If this normal exchange mechanism is not available in Na-free solutions, Ca_i will rise. The readmission of Na would cause Na_o to exchange for Ca_i in a rapid manner and the resultant rise in Na_i would stimulate electrogenic Na extrusion. Since the fibers contract and relax in Na-free solution, ionized Ca_i must remain low which means that the postulated rise in Ca_i would have to be a rise in stored Ca_i. On the basis of this hypothesis the fact that readmission of Na provokes hyperpolarization only after prolonged exposure to Na-free solutions would be explained by assuming that time is required for Ca_i to rise to the level needed to permit a rapid and large Ca-Na exchange. The decline of the hyperpolarization with time would be explained by assuming that once the initial Ca-Na exchange occurred the influx of Na would be reduced. The lack of a response to the readmission of Na at low temperatures could result both from the fact that Ca-Na exchange is reduced by cooling (Reuter and Seitz, 1968) and by the fact that electrogenic Na extrusion is inhibited by cooling. If the hypothesis is correct, it implies, as noted by Reuter and Seitz (1968), that there is no similar exchange between Li and Ca. If there were such an exchange the readmission of Na to a fiber already exposed to Li would not result in entry of Na by rapid Ca-Na exchange.

Inhibition of Repetitive Activity

The readmission of Na causes spontaneous activity to slow and then stop; it also affects the response to a depolarizing pulse so that, in general, a depolarizing pulse will evoke a single response but not repetitive responses. These effects are associated with a reduction of the steepness of phase 4 depolarization; that depolarization is part of an oscillatory after-potential. There are a number of possible explanations for the inhibition of repetitive activity.

(*a*) Hyperpolarization induced by electrogenic Na extrusion could suppress spontaneous activity by preventing the depolarizing phase of the after-potential from reaching threshold. That would be consistent with the fact that activity may return after prolonged exposure to Na, just as the hyperpolarization declines after such prolonged exposure. If suppression of repeti-

tive activity is caused only by the current generated by Na extrusion, it should be possible to relieve the inhibition by passing a depolarizing pulse. In most fibers, however, a 12-s pulse as strong as 5×10^{-6}A elicited only a single action potential after readmission of Na. Fibers exposed to K-free solutions sometimes become wholly inexcitable upon readmission of Na, although such loss of excitability may be related in part to the loss of resting potential evoked by exposure to K-free solutions. It seems unlikely that electrogenic extrusion is the sole cause of the depression of repetitive activity although it may contribute to that depression.

(*b*) The depression of repetitive activity might be a direct effect of increasing Na_o, but an increase in Na_o does not depress activity in cooled fibers nor in fibers exposed to ouabain.

(*c*) The inhibition of repetitive activity might result from an increase in Na_i. If that is so the fact that readmission of Na before 90-min exposure to Na-free solutions leads neither to inhibition of activity nor to hyperpolarization would be explained on the grounds that time is required for Ca to rise to a level that will permit a rapid Ca-Na exchange. This interpretation would also imply that readmission of Na to a K-free solution inhibits excitability by causing a rise in Na_i, i.e. that the Ca-Na exchange occurs in K-free solutions. It would also, however, require that readmission of Na in cooled fibers or ouabain-poisoned fibers does not lead to a rise in Na_i, i.e. that the Ca-Na exchange does not occur under those conditions.

(*d*) The inhibition of repetitive activity might result from some other event related to the Ca-Na exchange. A fall in stored Ca_i does not seem to be a likely cause of an inhibition of activity. If the Ca-Na exchange requires that bound Ca_i become ionized before leaving the fiber the resulting rise in ionized Ca_i might reduce excitability by lowering the Ca_o/Ca_i gradient, or by a direct effect of Ca_i on membrane properties. An explanation based on some event related to the Ca-Na exchange would be subject to the same reservations as those given above in relation to a rise in Na_i as the cause of inhibition.

(*e*) There is a final possibility that cannot be ruled out. If one assumes that electrogenic sodium extrusion is the crucial event in the sodium-induced hyperpolarization it is nevertheless possible that a Na-induced increase in P_K and a voltage-dependent increase in P_K are also present. An increase in P_K could contribute to a reduction in the steepness of phase 4 depolarization. We have suggested that the decline in hyperpolarization with the passage of time may be caused by a gradual decline in the entry of Na and by a gradual fall in Na_i. The decline in the hyperpolarization might lead to a voltage-dependent fall in P_K; the decline in Na_i would partially reverse the Na-dependent increase in P_K. These decreases in P_K could contribute to the reappearance of spontaneous activity at a low rate. This explanation would assign a role to electrogenic extrusion, to Na-dependent changes in P_K, and to voltage-

dependent changes in P_K both with respect to the hyperpolarization and with respect to the inhibition of repetitive activity. This is a complex explanation but the phenomenon is a complex one and the explanation is not inherently impossible.

On the whole, we must regard as unknown the mechanism by which readmission of Na inhibits repetitive activity. This becomes particularly clear if one notes that none of the above explanations readily explains the fact that cooling to 13° causes activity to reappear in a fiber in which Ca-Na exchange, electrogenic extrusion, and inhibition of repetitive activity all must be assumed to be present at 36° (Fig. 10 B), although one could suppose that the inhibition of electrogenic extrusion caused by cooling was enough to permit spontaneous activity to reappear.

Effect of Na on Ca-Dependent Action Potentials

We sometimes note an increase in overshoot when Na or Li is added to the perfusate; this effect is seen before inhibition of activity or hyperpolarization appear. After inhibition of activity and hyperpolarization are established, the action potentials that can be evoked by a depolarizing pulse appear at a less negative threshold potential and may have a slower upstroke and a reduced amplitude (Fig. 4 C). Similar effects can be seen when fibers producing Ca spikes are exposed to Mn or to verapamil (Aronson and Cranefield, 1973; Cranefield et al., 1974). If sodium moves through the slow channel but does so less freely than Ca, the presence of small amounts of Na could reduce the inward current since Na would impede the flow of Ca without itself carrying a comparable current. Vitek and Trautwein (1971) found that the slow inward current in canine Purkinje fibers is carried by Na in Ca-free solutions and by Ca in Na-free solutions and that the level of Ca affects the permeability of the slow channel to Na. Our findings suggest that the level of Na may affect the permeability of the slow channel to Ca. If all of these interactions are in fact present it explains the great difficulty of analyzing the slow inward current by voltage-clamp techniques. The idea that Ca and Na may each impede the movement of the other through a current carrying channel has been suggested by Stanley and Reiter (1965) and by Matsubara and Matsuda (1969). An alternative explanation is that Ca must occupy a site on the membrane before it can move through the membrane and that Na competes with Ca for such sites. This sort of competition has been postulated by Vereecke and Carmeliet (1971) to explain the inhibition of Sr-dependent action potentials by Ca and by Hagiwara et al. (1974) to explain the interactions of various divalent ions that carry inward current during the action potential of barnacle muscle. It is interesting to note that virtually all of our observations concerning the effects of sodium on repetitive activity and on the action potential can

be explained by assuming that inhibition occurs only if Na is present and able to compete with Ca on both sides of the membrane.

Other Questions

We can offer no explanation for the marked increase in the amplitude of Ca-dependent action potentials at low temperatures other than to make the obvious suggestion that cooling changes the kinetics of the permeability change so that the "slow channels" remain permeable to Ca for a longer time and so that more of them are permeable to Ca at the same time. That phenomenon has, in any event, no apparent direct bearing on the subject of the present article.

Our results cannot be applied directly to the question of the role played by electrogenic sodium extrusion in fibers exposed to normal Tyrode's solution. It might, however, be noted that the interaction between Na_o and resting potential that we have said might result in part from a Na-induced increase in i_{K_2} could equally well be held to raise questions about whether certain properties of i_{K_2} that supposedly depend on Na_o are in fact Na-dependent properties of some other current or permeability.

We wish to thank Dr. C. M. Connelly, Dr. Frank Brink, and Dr. Roger Thomas for invaluable suggestions and criticism, Dr. Paul Hurlbut for his help in measurements of the sodium content of the fibers, and Mrs. Joan Leary for her excellent technical assistance.
Supported by a grant from The National Heart and Lung Institute (HL-14899).

Received for publication 21 March 1974.

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