Changes in External Na Induce a Membrane Current Related to the Na-Ca Exchange in Cesium-loaded Frog Heart Cells

DANIEL MENTRARD, GUY VASSORT, and RODOLPHE FISCHMEISTER

From the Laboratoire de Physiologie Cellulaire Cardiaque, INSERM U-241, Université Paris-Sud, F-91405 Orsay, France

ABSTRACT The effects of transient alterations in Na_o were investigated under voltage clamp conditions in frog heart cells previously loaded with Cs. Tetrodotoxin and Cs were used to inhibit Na and K currents. On applying a Na-poor solution (39.2 mM), an outward current was generated during both depolarizations and hyperpolarizations. The current amplitude described a U-shaped function of the membrane potential. On reapplying the standard solution after 15 min equilibration, an inward current was then induced that exhibited a bell-shaped function of the membrane potential. Current amplitude was sensitive to the external Ca concentration. Increasing pH_i by 10 mM NH_4Cl enhanced this current, while the internal acidification that occurred on switching back to the control solution greatly reduced it. Variations in the amplitude of this current during repetitive stimulations or long pauses are best explained by subsequent alterations in Na_i and pH_i; no evidence for a time dependence was found. This current was inhibited by La^{3+}, Co^{2+}, and D600, and was sensitive to adriamycin, quinidine, and disopyramide; lidocaine, another local anesthetic, and nifedipine had no effect. These observations extend previous work on intact heart cells and sarcolemmal vesicles. They suggest that the Na-Ca exchange may generate a current that is outward when Ca ions are moving into the cell.

INTRODUCTION

Calcium is an important regulator of cardiac muscle contraction. In addition to the inward movement of Ca during the initial phase of each action potential, the asymmetry of Ca concentrations on both sides of the membrane results in a leakage influx of Ca. To prevent Ca overloading, two mechanisms have been described in the cardiac cell membrane: a Na-Ca exchange (Reuter and Seitz,

Address reprint requests to Dr. Guy Vassort, Laboratoire de Physiologie Cellulaire Cardiaque, INSERM U-241, Université Paris-Sud, F-91405 Orsay, France.

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1968; Glitsch et al., 1970) and a Ca-ATPase (Caroni and Carafoli, 1980; Lamers and Stinis, 1981). In the frog heart, there is evidence favoring the hypothesis that during each cardiac cycle the Na-Ca exchange participates in the development of tonic tension as well as in the control of relaxation (Benninger et al., 1976; Horackova and Vassort, 1979; Roulet et al., 1979; see Chapman, 1979; Mullins, 1981).

The first quantitative expression of the opposing inotropic effects of Na and Ca ions on cardiac contractility was made by Wilbrandt and Köller (1948). Their model was developed by Lüttgau and Niedergerke (1958), who suggested that a carrier existed in the cell membrane that could convey one Ca or two Na and that the two ions competed. These authors also suggested that the carrier in its loaded form might still be negatively charged so that a reduction in membrane potential would increase Ca entry. The transformation of this scheme for Ca entry into Na-Ca countertransport was derived from flux experiments (see Reuter, 1974). These, as well as other investigations (Benninger et al., 1976), were thought to reinforce a 2:1 stoichiometry. However, besides the fact that such a 2:1 stoichiometry makes it difficult to account for the low myoplasmic Ca concentration, several observations cannot be easily explained. Alterations of the Ca/Na ratio were not predictive of the tension response (Tillisch and Langer, 1974; Miller and Moisescu, 1976). The voltage dependence of Ca influx, which was initially suggested in squid axons (Mullins and Brinley, 1975), was extended in heart muscle, where it was shown that a sustained component of tension continues to increase with extreme depolarizations at which Ca current declines (see Fozzard, 1979). Based on experimental observations in squid axon, Mullins (1977) proposed that four Na might be exchanged for each Ca. This was indirectly reinforced by analyzing the initial effect of Na_o alterations on tension development at high membrane polarizations (Horackova and Vassort, 1979). Reinterpreting the strength of Na withdrawal contractures, Chapman and Tunstall (1980) also found a dependence of the exchange on the membrane potential and suggested a 3:1 stoichiometry. If more than two Na are exchanged for one Ca and if no other ion is bound to the carrier, one would expect a current associated with the movement of these ions. Some evidence of such a current has been obtained by analyzing the hyperpolarization (or the outward current) induced by removal of external Na in frog and in mammalian heart (Horackova and Vassort, 1979; Coraboeuf et al., 1981). Recent findings on cardiac sarcolemmal vesicles have also suggested that three or more Na ions exchange for each Ca ion and that the Na-Ca countertransport generates a current (Pitts, 1979; Reeves and Sutko, 1980; Caroni et al., 1980; Lamers and Stinis, 1981; Ledvora and Hegyvary, 1983).

The aim of the present work is to confirm the electrogenic nature of the exchange on intact cardiac preparations and to determine some of its characteristics. Previous proposals of an actual exchange current, I_{ex}, outward during depolarization and inward during hyperpolarization, were questioned mainly because of the hypothetical presence, in frog heart as in other cardiac tissues, of a Ca-dependent K current (Meech, 1972) or of the I_f current (Di Francesco, 1981). Cs loading of frog heart cells makes it possible to get rid of these currents

(Mentrard et al., 1984), and the voltage dependence of I_{ex} has been investigated under these conditions. The alterations of I_{ex} brought about by intracellular pH changes are reported. A few drugs—quinidine, adriamycin, and amiloride—have been suggested to inhibit Na-dependent Ca movements in some preparations; their effects on I_{ex} were also investigated in frog heart fibers. A preliminary report of these results has appeared (Mentrard and Vassort, 1983).

METHODS

Materials and Methods

The materials and methods are essentially the same as previously described in Mentrard et al. (1984). Briefly, the experiments were performed under voltage clamp conditions by the double sucrose gap method in frog atrial trabeculae previously loaded with Cs by soaking the whole atria 2–3 h in 40 Cs, 0 K Ringer. Under these conditions, the intracellular K content is decreased to ~25%. The standard solution, derived from the Ringer, contained (mM): 110.5 NaCl, 2.4 $NaHCO_3$, 1.8 $MgCl_2$, 1.8 $CaCl_2$, and 20 CsCl, instead of 2.5 KCl (osmolarity was not compensated). Tetrodotoxin (TTX; 5×10^{-6} g/liter) and 4-aminopyridine (2 mM) were added. In such a solution and after Cs loading, it was necessary to hyperpolarize the preparations by ~15 mV to bring them to or close to their original resting potential (E_R was estimated to be −80 mV). Na-poor (39.2 mM) solution was made by substituting LiCl, choline chloride in the presence of atropine, or D-glucosamine hydrochloride for ⅔ NaCl. High Ca (5.4 mM) and low Ca solutions (0.6 mM) were obtained by adding $CaCl_2$ to, or removing it from, the standard solution. The internal pH was varied as follows: alkalinization was induced by adding NH_4Cl (10 mM) to the standard solution while internal acidification was expected on return to the control solution (Boron and De Weer, 1976). Quinidine (Searle, Montrouge, France), disopyramide (Roussel-UCLAF, Romainville, France), and lidocaine (Roger Bellon, Neuilly, France) were used at 4×10^{-4} M, amiloride (Merck, Sharp and Dohme-Chibret, Paris, France) at 1×10^{-4} M, and D600 (Knoll Pharmaceutical, Inc., Whippany, NJ) at 2.2×10^{-6} M by adding given amounts from stock solutions. Nifedipine (The Bayer Co., New York) and adriamycin (Sigma Chemical Co., St. Louis, MO) were freshly prepared, protected from light, and used at 7×10^{-7} and 4×10^{-5} M, respectively. In all solutions, the pH was adjusted to 7.4 and the temperature was 18 ± 2°C.

Current traces were generally digitized at 1 kHz (unless otherwise stated) and stored on a 10-M octet disk for subsequent analysis on a minicomputer (Plurimat S; Intertechnique, Plaisir, France). Variations in current amplitude were estimated at 100 and 480 ms after altering the external Na content on averaging 10 ms of tracing to increase precision.

Definition of Experimental Conditions

To demonstrate the existence and define some characteristics of the exchange current, I_{ex}, we focused first on a reduction of the other currents and then on a way to induce a specific change in I_{ex}. The latter task is, unfortunately, difficult, since any alteration in I_{ex} will change Ca_i, which, in one or several ways, controls other membrane conductances. The effectiveness of cellular Cs loading in reducing K currents has been detailed in a previous paper (Mentrard et al., 1984). The linearity of the *I-V* relationship, the low K content, and the very slight effect of Co suggested that any current carried by K ions in frog cells would be markedly reduced after Cs loading. In the control solution, the maximal Ca inward current was about four times the leakage current. Only experiments

where the preparations exhibited such large inward currents for at least 45 min were considered.

Considering that the Na-Ca exchange mechanism is sensitive to the membrane potential and to Na ions on both sides of the membrane, several possibilities are offered for its study. However, a specific approach is difficult to undertake by varying E_m, since this can induce changes in several ionic conductances. Varying the external Ca concentration is also not a useful procedure because it changes the Ca current, membrane surface charges, and resting membrane resistance. A change in the external Na concentration from 112.9 to 39.2 mM was chosen. Such a reduction in Na significantly alters the reversal potential of the exchange mechanism, but should not significantly alter its binding on the carrier sites, because the reported values of the apparent affinity constant are of the order of 25 mM with a high Hill coefficient (Wakabayashi and Goshima, 1981; Mullins et al., 1983). Furthermore, Na_o can be varied for a very short period to induce a minor change in Na_i. A complete replacement of the solution in the test compartment was achieved in <1 s, and the washout of extracellular space occurred with a half-time ($t_{1/2}$) of ~6 s, as indicated by the reduction of I_{Na} to a steady state value upon low Na Ringer application or after TTX addition (Horackova and Vassort, 1979). The low Na solution was generally applied for 25 s, allowing the current trace elicited during the third pulse (stimulation frequency 0.125 s^{-1}) to be compared with the one just before switching to low Na_o. Such a protocol was necessary because of the relatively low variations in current amplitude on altering external Na. Also, this differential method eliminates a possible distortion induced by the voltage-dependent and use-dependent block of both TTX and Cs, since at each membrane potential the effects of Na_o were checked on applying the same voltage step at a constant frequency. Then the preparation was switched back to the standard solution for 5 min before a new transient application of the low Na solution was made during repeated application of another membrane polarization. This was attempted in order to avoid much change in Na_i, which was shown to be highly sensitive to the external Na (Ellis and Deitmer, 1978; Chapman et al., 1984). Since the low Na solution was applied for short periods, the change in Na_i should have been limited; moreover, since the durations of these Na_o alterations were all identical, they were expected to induce similar changes in Na_i, and thus they allowed for comparison in the successive medium alterations.

RESULTS

Variations in current amplitude were analyzed during 500-ms pulses at different membrane potentials soon after (25 s) changing the external Na solutions. Current traces elicited in normal solution and in the low Na solution are compared in Fig. 1 for step pulses at $E_m = E_R - 60$ mV, $E_R + 40$ mV, and $E_R + 80$ mV. The variations in current always had a low amplitude, at most one-tenth of the maximal slow calcium current. Independently of the clamp potential, the differences (upper traces, amplified fivefold) were always positive over the range of voltage tested. Fig. 1 also shows, on a faster time scale and digitized at 10 kHz, that there was an initial fast transient lasting for only 3 ms before the nearly steady value was reached during hyperpolarization. Decreasing Na_o might also reduce the current carried by the slow inward channel, which might not be highly selective to Ca ions (Rougier et al., 1969; Reuter and Scholz, 1977). This could explain why the largest variation was obtained after ~100 ms in the depolarizating range. In the following experiments, variations in current amplitude were thus estimated at 480 ms to avoid much of the interference with the

slow inward current. More complete effects on the amplitude and the time course of the current were examined on applying Na-poor solutions at different membrane potentials and for different durations.

Effect of Altering Na_o at Different Membrane Potentials

Fig. 2 summarizes the results obtained in 11 fibers for short applications of a low Na solution during 500-ms membrane polarizations varying from E_R −80

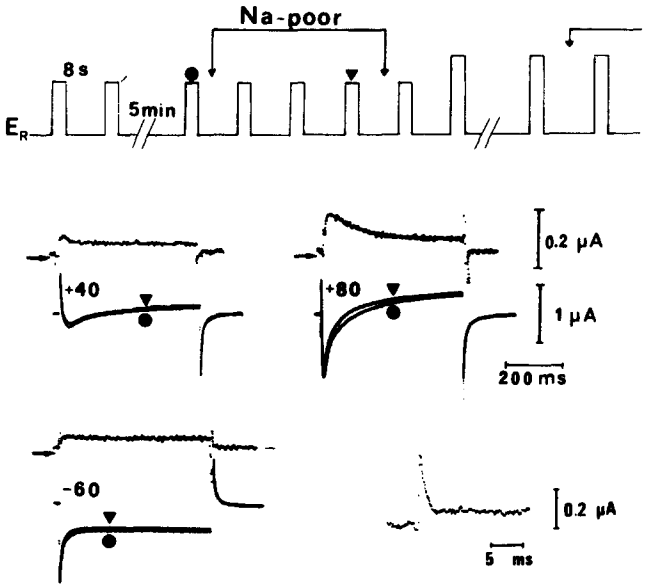

FIGURE 1. Effects of a reduction from 112.9 to 39.2 mM in the external Na (Li substituted) on the currents elicited by 500-ms steps of +40 and +80 mV in the depolarizing range and −60 mV in the hyperpolarizing range. In each set, the upper trace shows the difference amplified fivefold in the currents elicited in the normal solution (●) and during the third pulse in the low Na solution (▼). The difference at E_R −60 mV is observed after digitization at 10 kHz on a faster time scale (lower right). The experimental protocol is illustrated in the upper part. Stimulation frequency was 0.125 s^{-1}. Between two Na alterations, fibers were re-equilibrated during 5 min in standard solution to recover their initial Na gradient. In each amplified trace, the arrow on the left indicates the zero current level.

mV to E_R +80 mV in steps of 20 mV. The variation in current amplitude was a U-shaped function of the membrane potential. The average mid-value was −83.1 ± 2.4 mV, at which the minimal current difference elicited was 0.14×10^{-7} A. The maximal variation in current amplitude on changing the external Na was of the order of $1-1.5 \times 10^{-7}$ A during pulses of E_R ±80 mV. Notice that saturation generally occurred at E_R +80 mV. Smaller variations in current were observed at higher potentials, whereas this was not the case for negative potentials; however, a detailed study was not undertaken since the background *I-V* curve was no longer linear at these large potentials (Mentrard et al., 1984). Lower

alterations in Na_o (65.2 and 85.7 mM) were also checked; they induced much smaller changes in current amplitude—about one-third and one-fifth at ±60 mV, which made the analysis very imprecise at other potentials, but they had the advantage of inducing smaller variations in Na_i.

In most experiments, Na was replaced by Li. However, it has been shown that Li is a partially effective substitute for Na (Mullins, 1976; Ledvora and Hegyvary, 1983). Thus, in some experiments, Na was replaced by choline chloride ($n = 5$) or by D-glucosamine ($n = 3$). Similar U-shaped relations were observed, but the minimal currents were larger (see Fig. 7). Furthermore, to exclude the possibility that an electrogenic Na-K pump played a dominant role in the development of these current variations, similar Na alterations were elicited in trabeculae that had previously been bathed for 30 min in ouabain (10^{-7} M). This treatment did not significantly affect the U-shaped relations.

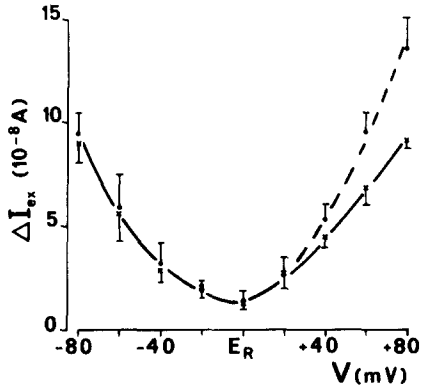

FIGURE 2. Variations in current amplitude following short applications of the low Na solution (as in Fig. 1) at different membrane potentials and estimated at 100 (●) and 480 ms (×) by averaging during 10 ms. Mean (and SEM) of 11 experiments. For each membrane potential, the fiber was re-equilibrated for ~5 min before applying the low Na solution over ~25 s.

Reserved Effects of Altering Na_o

Fig. 3 compares the variations in current amplitude obtained either on decreasing Na_o as previously or on increasing Na_o to the normal level after the preparation was equilibrated for 15 min in a low Na solution. In the latter case, variations in current amplitude were always observed in the inward direction during depolarizations or hyperpolarizations and now exhibited a bell-shaped function of E_m (Fig. 3*B*). In six fibers, the average mid-value was −75.0 ± 3.6 mV; the variations in current were of similar amplitude to those observed on switching from normal to low Na solution.

Effect of Time

In the following experiments, the effect of time on the variation in current amplitude was followed during repetitive applications of 500-ms step potentials for several minutes after switching to low Na or after equilibration in the low

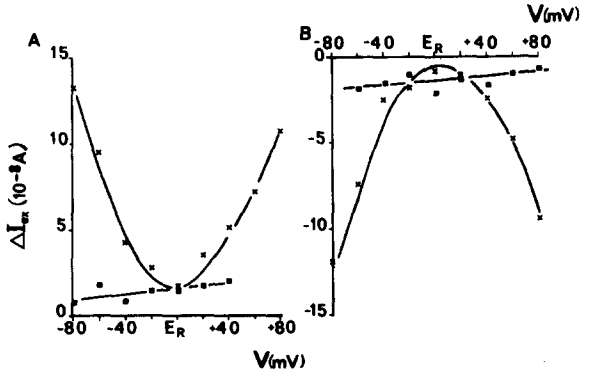

FIGURE 3. Variations in current amplitude at different membrane potentials obtained by briefly altering the external Na solutions either (*A*) by decreasing Na from 112.9 to 39.2 mM (replaced by Li) as in Fig. 2, or (*B*) on a different fiber after increasing Na back to control after the preparation has been soaked for 15 min in the low Na solution. The variations in current amplitude during each step were estimated at 480 ms. In both cases the application of 2 mM La^{3+} (■) suppressed the effects of the change in Na_o.

Na solution on returning to the standard solution. Fig. 4 shows the result of such an experiment when 60-mV depolarizing pulses were applied. On switching to low Na solution, there was a fast transient increase to a peak value that was reached in 45 s. Then the variation in current was smaller and decreased to one-third of its peak value in ~3 min. Similar time courses were obtained in several fibers whenever they were submitted to depolarizing or hyperpolarizing pulses of different amplitudes. After the same fiber was left 17 min more in the low Na solution, the effects of switching back to the normal Na solution were checked

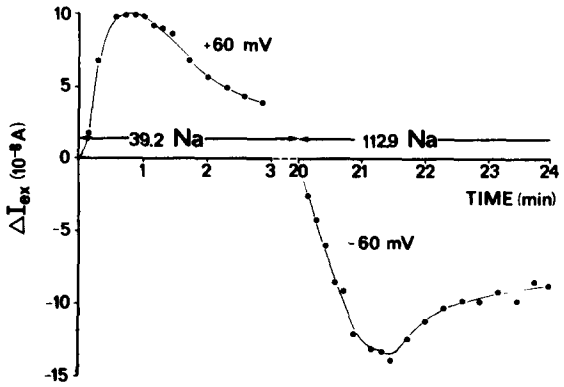

FIGURE 4. Time course of the variations in current amplitude. The fiber was first submitted to a series of 500-ms, +60-mV depolarizing pulses on reducing Na_o; then, after being equilibrated for 20 min in this low Na solution, the same fiber was submitted to a series of 500-ms, −60-mV hyperpolarizing pulses during which the normal Na solution was reapplied. Current variations were estimated at 480 ms.

during 60-mV hyperpolarizations. The variation in current amplitude also showed a peak value (inward in this condition) followed by a slow decrease. The time to peak was generally about two times longer in increased Na_o experiments than in reduced Na_o.

Fig. 5 reports a typical result of comparing current traces (digitized at 100 Hz) during 60-mV depolarizing pulses of 20 s duration. Two identical depolarizations were applied, one in normal solution and the other 0.5 s after switching to the low Na solution. The comparison between the two current traces shows that the difference (outward current) reached a peak value after 3–5 s and then declined toward zero. Notice that the time to peak is close to the time required for the extracellular diffusion of Na (Horackova and Vassort, 1979). Very similar time courses were seen on other fibers on applying depolarizations or hyperpolarizations, except that the difference between the two currents after the peak sometimes decreases to negative values, particularly when a large initial peak was induced by 80-mV depolarizations.

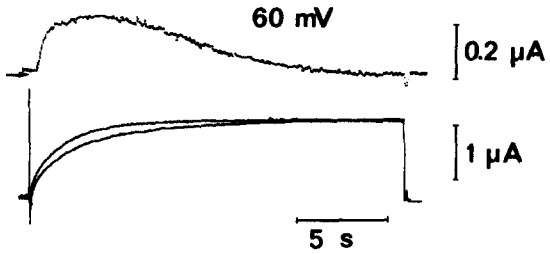

FIGURE 5. Digitized current traces (at 100 Hz) elicited by a 60-mV depolarizing pulse of long duration (20 s) applied before and just after switching from normal to low Na solution. The upper trace is the difference in the two traces on an expanded scale.

Effect of Altering Na_o at Different Ca_o

Step reductions in the external Na concentration have been repeated at two other levels of the external Ca concentration (0.6 and 5.4 mM $CaCl_2$). The fiber was initially equilibrated in the low (or high) Ca solution for 10 min before the same Ca-containing solution deprived of Na was applied. Fig. 6 reports plots of the current variations following Na_o alterations at different membrane potentials for these two Ca concentrations. The values are compared with those previously obtained in the normal Ca solution (1.8 mM). A U-shaped function of the current-voltage relationship induced by Na alteration is still observed. However, exposure to low Ca_o caused a decrease in the amplitude of the current variations at all membrane potentials. Exposure to high Ca_o induced a slight increase over the whole depolarizing range and at the minimum around E_R but induced, if anything, a decrease in the hyperpolarizing range. The potential at which the minimal currents occurred was not significantly different in the three Ca solutions.

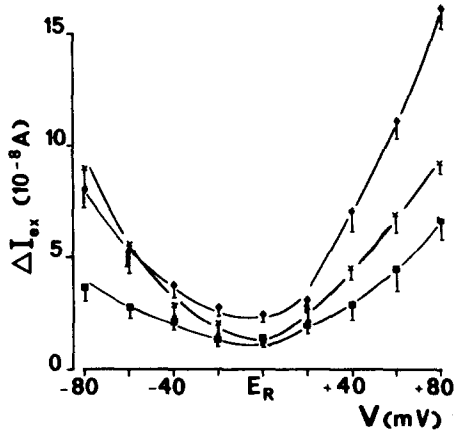

FIGURE 6. The variations in current amplitude (estimated at 480 ms) following short applications of the low Na solution (replaced by Li) at different membrane potentials are compared for three different Ca concentrations: ×, 1.8 mM (Fig. 2); ♦, 5.4 mM (n = 3); ■, 0.6 mM (n = 3). SEM, $P < 0.01$ at +40, +60, and +80 mV in Ca 5.4 mM and at +60 and −80 mV in Ca 0.6 mM; $P < 0.05$ at +40, +80, and −60 mV in Ca 0.6 mM when compared with values in Ca 1.8 mM.

Effect of Altering Na_o in the Presence of Ca Antagonists

Nifedipine was used at 7×10^{-7} M, a concentration which in our experimental conditions reduced the slow inward current to about one-third of its initial amplitude (Fig. 7, inset). Variations in current amplitude on altering Na_o (re-

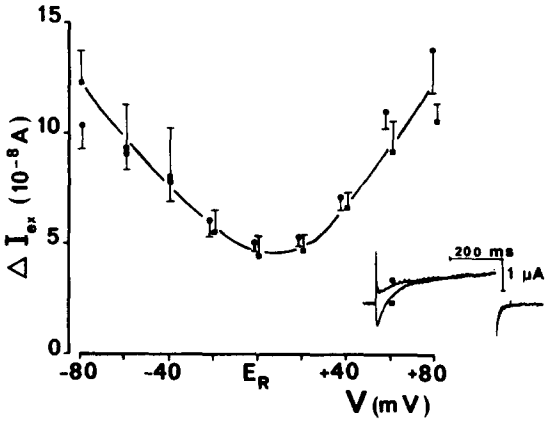

FIGURE 7. The variations in current amplitude induced by short applications of the low Na solution (Na replaced by choline chloride, ■) at different membrane potentials are not influenced by the addition of nifedipine (●; 7×10^{-7} M), although this drug has significantly reduced the slow inward current (inset) (n = 3 for each series, SEM).

placed by choline chloride) at hyper- or depolarized membrane potentials were not significantly affected by the presence of nifedipine in three experiments (Fig. 7). On regular stimulation at 0.125 s^{-1}, when D600 was added at 2.2×10^{-6} M it also reduced the slow inward current to about one-third. The variations in current induced by the low Na solution were reduced to about one-third after 5 min exposure to D600 and were negligible after 15 min exposure to the drug ($n = 3$).

La ions have been shown to inhibit the Ca-dependent Na efflux in squid axons (Baker, 1972). When our preparation was bathed in La^{3+} (2 mM), only a residual current appeared on switching to the low Na solution, which was increased by nearly a constant amount at each potential from E_R −80 to E_R +40 mV (see Fig. 3). Co ions added at 3 mM also markedly reduced the variations in current induced in the same conditions. Maximal effects of these ions were observed after ~1 min exposure.

Changes in the Effects of Altering Na_o Induced by Internal pH

NH_4 is known to cause multiphasic changes of pH_i; the rapid rise of pH_i caused by NH_3 entry into the cells is followed by a slower fall caused by NH_4^+ entry; on removal of external NH_4Cl, pH_i decreases markedly before recovery (Boron and De Weer, 1976; Deitmer and Ellis, 1980). In the following experiments, 10 mM NH_4Cl was added to both normal and low Na solutions and the variations in current amplitude were compared in the standard NH_4-containing solution and after NH_4 removal (i.e., supposedly at normal, alkaline, and acidic internal pH) for 500-ms, 60-mV depolarizing pulses applied over 100 s in the low Na solution. According to Boron and De Weer (1976), we estimate to ~0.4 pH unit the change in pH immediately following the application and removal of 10 mM NH_4Cl. In Fig. 8 are shown original current traces recorded just before and after the fifth pulse after applying the low Na solution in the three different conditions. It shows that the alkaline pH_i markedly increases (and acidic pH_i markedly decreases) the amplitude of the slow inward Ca current. It also shows that the variations in current amplitude induced by low Na are noticeably dependent upon pH_i. Whole series of estimations at 100 and 480 ms during 60-mV depolarizations are plotted in Fig. 8 for the fiber submitted over 100 s to low Na solution in the three conditions. Alkalinization increased and acidification decreased the electrogenic effects of altering Na_o. Similar results were obtained on three fibers during 60-mV depolarizations and on two fibers during 60-mV hyperpolarizations: in the latter case, the results could not be the result of alterations in the Ca current.

Changes in the Effects of Altering Na_o Induced by Quinidine and Other Local Anesthetics

After verifying that the low Na solution induced variations in current amplitude at two membrane potentials, a fiber was held for 15 min in the standard solution with 4×10^{-4} M quinidine added; then the low Na solution (plus quinidine) was applied for short periods at different polarizations. Notice that because of the duration of the experiments, pulses were successively positive and negative and

of increasing amplitude (+20, −20, +40, . . ., −80 mV) so as to compare the effects of the drug in the depolarizing and hyperpolarizing range. Fig. 9*A* shows that on three fibers at each membrane potential, quinidine sharply reduced the variations in current amplitude induced by applying the low Na solution. This effect was more marked in the hyperpolarizing range. It required 10–15 min to develop, but thereafter did not significantly increase with time. In reverse experiments, when the standard solution was applied for short periods after the fiber had been equilibrated in the low Na solution, less variation in (inward) current was observed in the presence of quinidine, particularly in the hyperpolarizing range. In both types of experiments, quinidine decreased by <20% the peak slow inward current (Fig. 9*A*, inset).

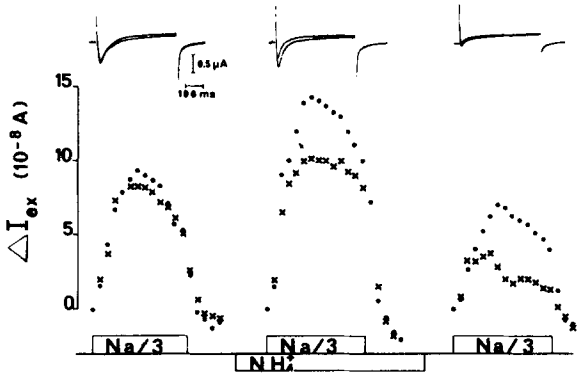

FIGURE 8. Variations in the current amplitude obtained during repetitive steps at E_R +60 mV applied every 8 s during Na_o alterations lasting 100 s before or after changing the internal pH by NH_4Cl (10 mM). In the presence of NH_4^+, the internal pH is increased, whereas on return to the standard solution an acidification is expected. Current variations were measured at 100 (●) and 480 ms (×). The upper traces show the currents recorded in the three conditions before and on the fifth pulse applied in low Na solution (stimulation frequency 0.125 s^{-1}).

The effects of disopyramide were very similar to those of quinidine at the same concentration, although slightly less pronounced. A more marked inhibition was also observed in the hyperpolarizing range than in the depolarizing range (50–70% and 20–30% inhibition, respectively).

The effects of lidocaine (4×10^{-4} M) were also checked on three fibers. At each membrane potential, the low Na solution induced similar variations in current amplitude before and after ≤20 min of lidocaine application.

Changes in the Effects of Altering Na_o Induced by Adriamycin

The experimental protocol was the same as with quinidine. Fig. 9*B* shows the effects of 4×10^{-5} M adriamycin on two fibers: ~50% inhibition was observed in the hyperpolarizing range with less effect in the depolarizing range, even after more than 30 min in the presence of the drug. Incomplete series, confirming the inhibition in the hyperpolarizing and weaker effect in the depolarizing range,

have been obtained on three other fibers. No significant variation in the Ca current has been observed even after 30 min of application of the drug.

Changes in the Effects of Altering Na_o Induced by Amiloride

After ≥10 min of application of 10^{-4} M amiloride, the variations in current amplitude, induced by applying the low Na solution for short periods of time at different membrane potentials (ranging from E_R −80 to E_R +80 mV), were not significantly changed (three fibers).

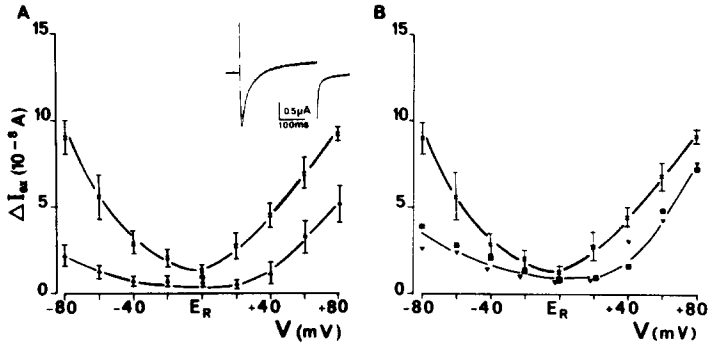

FIGURE 9. (*A*) Effects of quinidine (4×10^{-4} M) on the variations in current amplitude obtained at different membrane potentials. After 15 min of quinidine application, the low Na solution was applied briefly during positive or negative polarizing pulses of increasing absolute amplitude (+20, −20, +40, . . ., −80 mV) to compare the effects in the depolarizing and hyperpolarizing range at about the same time. I_{ex} was measured at 480 ms. For comparison, the variations in current amplitude observed in the absence of drugs are reproduced (see Fig. 2). Mean ± SEM, $n = 3$; $P < 0.01$ at all potentials but E_R −20, E_R, and E_R +20 mV. The effects of quinidine on the slow inward current are also shown (inset). (*B*) Effects of adriamycin (4×10^{-5} M) on the variations in current amplitude obtained at different membrane potentials when altering Na_o. Same protocol as in *A*. The values obtained at 480 ms on two fibers are compared with the relation in the absence of drugs (see Fig. 2).

DISCUSSION

The main observation reported in this paper is that a decrease in the external Na always induced an outward current whose amplitude increased with hyper- and depolarizations applied to the membrane. Thus, current amplitude was a U-shaped function of the membrane potential; it was also sensitive to Ca_o and could be reversed on increasing Na_o.

The above experiments were performed in the presence of TTX to abolish the fast Na current and in the presence of Cs to tentatively inhibit the K currents. In these conditions and in the presence of Co, which is known to block the slow inward current, the membrane conductance appeared independent of voltage and time; the current was reduced mostly to nonspecific leakage (Mentrard et al., 1984).

Although the variations in current were small compared with the nonspecific leakage current, they seem to be attributable to the Na_o alterations since they were independent of the Na substitutes (Li, choline, or D-glucosamine); moreover, they were observed with very similar amplitudes on every preparation that develops a large slow inward current. Substantial support for the existence of a genuine current related to Na_o alterations is also provided by the effects of some drugs. Nifedipine, at a concentration that strongly depressed I_{Ca}, did not affect this current, whereas adriamycin or quinidine and disopyramide, which hardly reduced I_{Ca}, significantly antagonized the effects of Na alterations. Ouabain did not alter this current, as was expected from a preparation with a partially inhibited Na-K ATPase caused by the replacement of K by Cs. The question of a secondary conductance activation by increased Ca_i might arise. A U-shaped *I-V* relationship is rather difficult to attribute to a single conductance change, even if complex rectifications are involved. In particular, the recently described Ca-dependent channel, which discriminates poorly between Na and K ions (Kass et al., 1978; Colquhoun et al., 1981), could not be a candidate because inward or less outward currents would be expected on hyperpolarizations. Rather than involving simultaneous and opposite changes of two or more conductances, the above observations are more likely to be related to an electrogenic Na-Ca exchange. Indeed, lowering Na_o facilitates coupled Na efflux/Ca influx during depolarizations to potentials larger than the reversal potential of the exchange and reduces Na influx/Ca efflux with lower potentials; both alterations would give rise to an outward current if the exchange mechanism carried more than two Na for one Ca ion.

Following Mullins' (1976, 1977) model, the current I_{ex} generated by the Na-Ca exchange at steady state can be described by

$$I_{ex} = k \sin h[(E_m - E_{ex})\, F/RT], \tag{1}$$

where k is a scaling factor proportional to the number of functionable carriers. The zero current potential, E_{ex}, is a function of the carrier coupling ratio, n, and of the Nernst potentials for Na and Ca ions such that:

$$E_{ex} = (nE_{Na} - 2E_{Ca})/(n - 2). \tag{2}$$

A mathematical simulation of I_{ex} and its variations, ΔI_{ex}, upon modifications of Na_o was attempted assuming that k and n are not sensitive to the external Na concentration in the range investigated; a sudden decrease (or increase) in Na_o will initially induce a shift of E_{Na} and result in a decrease (or increase) in E_{ex}: this will lead to a shift of I_{ex} on the voltage axis toward negative (or positive) potentials so that ΔI_{ex} is a U-shaped (or bell-shaped) function of membrane potential. This was observed experimentally in the present study (Figs. 2 and 3). Under these conditions, the U-shaped and bell-shaped curves present extrema at a membrane potential, E_{mid}, equal to the mid-value between E_{ex} and its new value obtained immediately after the variation of Na_o. This allows us to calculate n:

$$n = 4\ (E_{Ca} - E_{mid})/(2E_{Na} + \Delta E_{Na} - 2E_{mid}), \tag{3}$$

if the intracellular Na and Ca concentrations are known. Finally, the scaling factor k is estimated so that, at −160 mV, ΔI_{ex} = +0.1 μA (or −0.1 μA) for the U-shaped (or bell-shaped) curve, a value close to the average value obtained experimentally at that membrane potential (e.g., Fig. 2). On switching from normal (112.9 mM) to low Na (39.2 mM) in 11 fibers, E_{mid} was −83.1 ± 2.4 mV; assuming Na_i = 19.8 mM (calculated from Chapman et al., 1984) and Ca_i = 260 nM (Marban et al., 1980; Dahl and Isenberg, 1980), Eq. 3 gives n = 3.43 and k = 2.87 × 10^{-3} μA. The calculated ΔI_{ex} in Fig. 10*A* is very similar to the

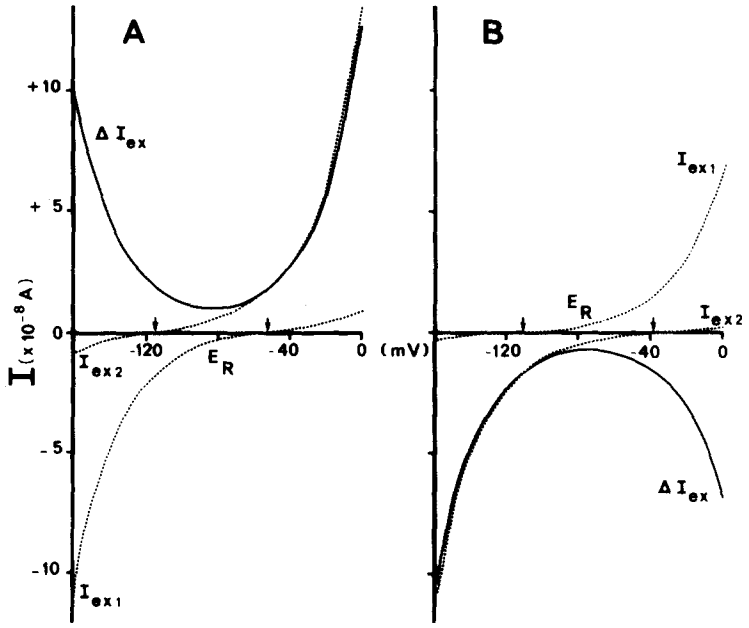

FIGURE 10. Mathematical simulation of the current-voltage (*I-V*) relationships for the Na-Ca exchange current, I_{ex}, and its modification induced by a change in the external Na concentration. (*A*) I_{ex1} is the current in normal conditions, I_{ex2} is the current immediately after reducing $[Na]_o$ from 112.9 to 39.2 mM, and $\Delta I_{ex} = I_{ex2} - I_{ex1}$. The zero current potentials are −50.95 and −115.05, respectively. E_R is the resting potential (−80 mV). (*B*) I_{ex1} is the current after equilibration in the low Na solution (39.2 mM), I_{ex2} is the current immediately after restoring the normal Na concentration (112.9 mM), and $\Delta I_{ex} = I_{ex2} - I_{ex1}$. The zero current potentials are −111.09 and −38.91 mV, respectively (for further details, see text).

experimental one in Fig. 2. Fig. 10*A* also shows the computed exchange current I_{ex} in normal conditions and immediately after reducing Na_o: I_{ex} reverses at −50.9 and −115.1 mV, respectively. In normal conditions, the exchange current would be 4 × 10^{-8} A/cm^2 at rest (−80 mV) and 10^{-6} A/cm^2 at 0 mV (since the area of the surface membrane in the test gap was estimated from the capacitive current to be 0.1 cm^2), as compared with a maximal Ca current of 5–10 × 10^{-6} A/cm^2. It should be noted that if, inversely, we had fixed n at 2.5, 3, or 4, together with the above values of ionic concentrations, I_{ex} would reverse at,

respectively, −226.9, −91.5, and −23.8 mV; for these values, E_{mid} would be, respectively, −293.7, −131.6, and −50.5 mV.

After equilibration in the low Na solution (39.2 mM), Na_i decreased to ~12.3 mM (Chapman et al., 1984). On switching back to normal Na in six fibers, E_{mid} was −75.0 ± 3.6 mV. With Ca_i unchanged, this now gives $n = 3.17$ and $k = 1.74 \times 10^{-3}$ μA, i.e., a slight reduction in the carrier coupling ratio and a large decrease (40%) in the number of functionable carriers induced by a ~40% reduction in Na_i. The decrease in k is in line with the decrease in the mobile form of the carrier when Na was decreased (see Fig. 2 in Mullins, 1977). ΔI_{ex} computed with these values (Fig. 10*B*) is similar to the experimental curve (Fig. 3*B*). However, an increase in Ca_i after equilibration in low Na_o is not excluded. For that reason, we have also considered a two- and fivefold increase in Ca_i. This leads to $n = 3.03$ and $k = 1.51 \times 10^{-3}$ μA and $n = 2.83$ and $k = 1.16 \times 10^{-3}$ μA, respectively. Nevertheless, the calculated ΔI_{ex} remains superimposable on the curve drawn in Fig. 10*B*. For the three increasing intracellular Ca concentrations, the calculated E_{ex} values are −111.1, −114.1, and −120.6 mV in the low Na_o and become −38.9, −35.6, and −29.4 mV immediately after restoring the normal Na solution. Such negative E_{ex} values in the low Na solution would imply that the Na-Ca exchange contributed to an influx of Ca ions. Then, and *a fortiori* in a Na-free solution, the intracellular Ca concentration should be maintained by one or several other mechanisms (Busselen and Van Kerkhove, 1978).

Symmetrical U-shaped *I-V* relationships were obtained upon decreasing or increasing Na_o in normal Ca solutions (Fig. 2); they suggest that the exchange mechanism encounters its peak activation energy just midway through the electrical field. This is in agreement with Mullins' (1977) first approach, whereas Baker and McNaughton (1976) have presented evidence that Ca-dependent Na efflux was much more voltage sensitive than Na-dependent Ca efflux. However, in our case at least, the symmetry might be fortuitous, not only because Na_i could not be constant on decreasing Na_o (although, because of the experimental protocol, we were expecting very similar alterations at the successively applied polarizations), but mostly because the genuine effect of the carrier leads to alterations in the ionic concentrations that counteract the carrier activity. These ionic alterations are opposite in the depolarizing and the hyperpolarizing ranges and thus should alter the symmetry. Besides, *I-V* relationships are not symmetrical in high Ca solutions (Fig. 6).

Another important feature reported above is the time course of the effects of applying the low Na solution. No clear evidence has been obtained for a direct time dependence of I_{ex}. The difference between current traces elicited in standard solution and by the third pulse in the low Na solution was generally constant during the 500-ms duration of the pulse. Two exceptions were (*a*) for the 60- and 80-mV depolarizations at which a Ca current was elicited and whose kinetics might be indirectly influenced by low Na (Mentrard et al., 1984) or by a slight alteration of Na_i, and (*b*) for the first 3 ms (Fig. 1), which could be accounted for by the difference in the time constants of loading the membrane capacitance as a result of a minor (<1%) change in the series resistance in the low Na solution. The complex time course of I_{ex} change observed during repetitive stimulations

(Fig. 4) may be due to a decrease in Na_i on prolonged application of low Na_o. A further effect is expected during the long pulse to account for the decrease or even inversion of I_{ex}: in addition to the decrease in Na_i, there should be an increase in Ca_i whenever Ca influx is increased or Ca efflux is decreased. Furthermore, such an increase in Ca_i will induce internal acidification (Meech and Thomas, 1977; Ahmed and Connor, 1980; Mullins et al., 1983; Vaughan-Jones et al., 1983), which is shown to inhibit the Na-Ca exchange mechanism (Wakabayashi and Goshima, 1981; Philipson et al., 1982; Mullins et al., 1983; this paper, Fig. 8). A reduction in Na_o might also induce internal acidification if a Na-H mechanism exists in frog heart cells as in other excitable cells; however, this mechanism could not be involved in the variation in current induced by altering Na_o since the Na-H exchange was expected to be electroneutral (Thomas, 1977) and since amiloride had no effect (these results).

A method often advanced to identify ionic currents is the use of inhibitors. Very few drugs have been reported to alter the Na-Ca exchange mechanism. Recently, Caroni et al. (1981) showed that the cardiotoxic antibiotic doxorubicin (adriamycin) inhibits the Na-Ca exchange of dog heart sarcolemmal vesicles. Our results on frog atrial cells (Fig. 9*B*) only partially confirm their observations. Some inhibition was obtained with this drug as well as with the local anesthetics in the hyperpolarizing range, i.e., there was a larger inhibition of the variation in inward current (related to Na-dependent Ca efflux) than of the outward current. One might thus consider that these drugs are acting asymmetrically, blocking preferentially on the external Na sites. This effect might not have been detected on sarcolemmal vesicles because of their nonuniform sideness. Amiloride, a drug initially shown to block Na movements related to Na^+-H^+ exchange (Aickin and Thomas, 1977), was reported to completely block the external Na-stimulated Ca efflux and external Na-inhibitable Ca influx during differentiation of murine erythroleukemia cells (Smith et al., 1982) and the Na-Ca exchange in brain vesicles (Schellenberg and Swanson, 1982). However, using amiloride to change the pH, Philipson et al. (1982) failed to observe a significant alteration of the Na-dependent Ca uptake on cardiac sarcolemmal vesicles. Under our experimental conditions, amiloride for short periods of application was ineffective on the variation in I_{ex} induced by lowering Na_o.

The first reported drug inhibition of Ca influx and Ca-activated Na efflux was in red blood cells by the local anesthetic quinidine (Parker, 1978). At the relatively high concentration used in this study, quinidine inhibits the exchange current induced by altering Na_o, particularly in the hyperpolarizing range. This result is in agreement with the recent observation of Ledvora and Hegyvary (1983) on dog sarcolemmal vesicles. Disopyramide, a synthetic agent that is considered a quinidine-like drug in the treatment of cardiac arrhythmias, also mimics quinidine in inhibiting I_{ex}. In recent studies on Purkinje fibers, lidocaine and quinidine were reported to have similar (Carmeliet and Saikawa, 1982) or different (Colatsky, 1982) sites of action for the control of action potential duration and automaticity. Furthermore, lidocaine was also reported to inhibit the transient inward current, TI (Eisner and Lederer, 1979), which was proposed in a mathematical model to be attributable to the Na-Ca exchange process

(DiFrancesco et al., 1982). On frog atrial cells, lidocaine, in contrast to quinidine at the same concentration, was ineffective in inhibiting the change in I_{ex} induced by Na_o alteration. The inhibitory effect of quinidine and disopyramide on the Na-Ca exchange allows us to interpret their higher potency, compared with lidocaine, in decreasing the strength of heart contractile force (Hauswirth and Singh, 1979), while these drugs only slightly reduced the Ca current and had a tendency to lengthen action potential.

In conclusion, a current is generated by altering Na_o, although the previously described Na-sensitive currents were inhibited; its amplitude exhibits a U- or bell-shaped function of membrane potential. This current has been tentatively related to Na-Ca exchange. Thus, besides playing an important role in regulating myocardial contractility, the Na-Ca exchange should also affect the electrical activity. From mathematical simulations (Fischmeister and Vassort, 1981), it was anticipated that increasing the exchange current would prolong and accelerate electrical activity and even induce re-excitation, although its instantaneous amplitude is weak compared with the passive currents.

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