Sodium-Calcium Exchange in Regulation of Cardiac Contractility

Evidence for an Electrogenic, Voltage-Dependent Mechanism

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ABSTRACT The origin and regulatory mechanisms of tonic tension (Ca current-independent component of contractility) were investigated in frog atrial muscle under voltage-clamp conditions. Tonic tension was elicited by depolarizing pulses of 160 mV (E_m = +90 mV, i.e., close to E_{Ca}) and 400–600 ms long. An application of Na-free (LiCl) or Ca-free Ringer's solutions resulted in a fast (<120 s), almost complete abolition of tonic tension. When $[Na]_o$ was reduced (with LiCl or sucrose as the substitutes), the peak tonic tension increased transiently and then decreased below the control level. The transient changes in tonic tension were prevented by using low-Na, low-Ca solutions where the ratios $[Ca]_o/[Na]_o^3$ to $[Ca]_o/[Na]_o^5$ were kept constant (1.1×10^{-8} mM^{-3} to 8.7×10^{-13} mM^{-5}). Na-free (LiCl) solution elicited contractures accompanied by a membrane hyperpolarization or by an outward current even when the Na-K pump was inhibited. 15 mM MnCl$_2$ (or 3 mM LaCl$_3$) inhibited the development of the Na-free contracture and the related part of hyperpolarization or the outward current. In conclusion, our results indicate that tonic tension is regulated by a Na-Ca exchange mechanism. Furthermore, they suggest that this exchange could be electrogenic (exchanging three or more Na ions for one Ca ion) and thus voltage dependent. The possible contribution of an electrogenic Na-Ca exchange in the maintenance of cardiac membrane potential is discussed.

INTRODUCTION

Since the voltage-clamp technique with simultaneous measurement of tension was introduced in several laboratories (Beeler and Reuter, 1970; Goto et al., 1971; Morad and Orkand, 1971; Ochi and Trautwein, 1971; Einwächter et al., 1972; Vassort and Rougier, 1972), the process of excitation-contraction coupling in cardiac muscle has been studied intensively (see review by Fozzard, 1977). Although the picture varies according to the species used, it is generally believed that the cardiac mechanical activity elicited by depolarization consists of two components: (a) phasic tension related to the Ca inward current, and (b) tonic

tension, which increases with the amplitude of depolarization and relaxes upon repolarization, i.e., independently of calcium inward current (for more detail, see Coraboeuf, 1974). The second component is well developed in frog myocardium (Goto et al., 1971; Vassort and Rougier, 1972; Vassort, 1973 a; Benninger et al., 1976) but is also present in mammalian cardiac muscle (Morad and Trautwein, 1968; Ochi and Trautwein, 1971; Gibbons and Fozzard, 1975; Trautwein et al., 1975). Tonic tension has been studied less than phasic tension, and the origin and regulative mechanisms involved in its development are not yet understood.

It has been shown that the tonic tension is suppressed in Na-free solutions (Vassort, 1973 a; Benninger et al., 1976); furthermore, this tension can also be modified by variation in $[Na]_i$, i.e., it could be increased by the elevated $[Na]_i$ (Horackova and Vassort, 1974). These findings suggest some interrelation between Na and Ca ions in activating this component of contraction. There is increasing evidence that the transport of Ca in various tissues involves a carrier-mediated transmembrane exchange of Na ions for Ca ions (reviewed by Blaustein, 1974). The existence of such a Na-Ca exchange mechanism in cardiac muscle was first suggested by Reuter and Seitz (1968) and Glitsch et al. (1970). Their studies suggested that such a transport mechanism could be important in determining the intracellular concentration of free calcium, with the Ca transport at rest being directed predominantly outward, i.e., Ca efflux linked to Na influx. A countertransport in the opposite direction, i.e., Ca influx linked to Na efflux, was also demonstrated although it was relatively small at resting potential (Glitsch et al., 1970). However, this Ca entry combined with a decreased Na-dependent Ca efflux could become an important source of Ca ions for activating tonic tension during depolarization if the Na-Ca transport mechanism is voltage dependent. This possibility was also considered in a recent study of Benninger et al. (1976). Most of the proposed models of this transport system (see reviews by Blaustein, 1974, and Reuter, 1974; see also Blaustein, 1977) assume a possibility of the competition between Na and Ca ions for a carrier at both sides of the membrane whereby the resulting flux depends on the respective concentrations of both ions at either side of the membrane. This assumption is also used in the present study; however, a possibility of two kinds of specific binding sites for Na and Ca ions as suggested by Mullins (1977) may exist.

Thus, the present voltage-clamp study on frog atrial muscle was undertaken to investigate whether the activation of tonic tension is affected by the various changes in extra- and intracellular concentrations of Na and Ca ions as would be expected if the Na-Ca exchange were responsible for the origin and regulation of this tension. In contrast to previous studies on Na-Ca antagonism, the effects were recorded throughout their development so that more detailed and precise information about the mechanism(s) involved was obtained. We also attempted to approximate the stoichiometry of the countertransport mechanism. Although a complete solution is not yet available, our present data indicate that Na-Ca exchange in cardiac muscle is not electrically neutral as suggested previously (Jundt et al., 1975; Benninger et al., 1976), but electrogenic, and thus, voltage dependent. Preliminary reports have appeared (Horackova and Vassort, 1976; 1978).

METHODS

The experiments were performed on frog (*Rana pipiens* and *R. esculenta*) atrial trabeculae 100–150 μm in diameter by means of the double sucrose-gap voltage-clamp technique with simultaneous recording of mechanical activity (Vassort and Rougier, 1972). Electrical and mechanical responses were recorded simultaneously, from the test "node" which was 100–150 μm long, under voltage-clamp or current-clamp conditions; experiments were performed at 18 ± 2°C. The voltage-clamp pulses, membrane currents, and contractions were displayed on an oscilloscope (Tektronix 565, Tektronix, Inc., Beaverton, Oreg.) and photographed by Grass camera (Grass Instrument Co., Quincy, Mass.), and they were also simultaneously recorded by a pen recorder (Clevite-Brush, 260, Gould Inc., Instruments Div., Cleveland, Ohio).

The normal Ringer's solution contained (mM): NaCl, 110.5; KCl, 2.4; $CaCl_2$, 1.8; $NaHCO_3$, 2.4; pH 7.2–7.4; glucose, 5. The test solutions were obtained by substituting NaCl (partially or completely) by isosmotic equivalents of LiCl or sucrose; in Na-free solutions, $NaHCO_3$ and KCl were replaced by 2.4 mM $KHCO_3$. When $LaCl_3$, $MnCl_2$, or EGTA were added, the Ringer's solutions were buffered by 10 mM (in the case of EGTA — 25 mM) Tris. A complete replacement of the perfusing solution in the test compartment was achieved within a few seconds after the introduction of the new solution; the washout of extracellular space occurred with half-time $(t_{1/2}) = 6.2 \pm 1.1$ s as indicated by the reduction of I_{Na} to a steady-state value upon application of Ringer's solution with reduced $[Na]_o = 30.4$ mM.

RESULTS

The mechanical activity was recorded under voltage-clamp conditions from frog atrial bundles held at their normal resting potential (about −70 mV) and depolarized every 4 s by pulses of 160–180 mV. At this membrane potential, i.e., close to calcium equilibrium potential, the contractile force consists mainly of tonic tension; the amount of Ca entering the cell through the Ca channels and the phasic tension initiated by this current are negligible (Vassort, 1973 a). It should be noted that each figure is representative of four to six experiments, unless otherwise stated.

Tonic Tension in Na-Free and Ca-Free Ringer's Solutions

The application of Na-free (Li) or Ca-free Ringer's solution resulted in rapid reduction of tonic tension as shown in Fig. 1. This change in the mechanical activity was accompanied by almost no change in membrane currents; occasionally, a small decrease in the steady outward current was observed. The duration of depolarizing pulse was limited to 400–600 ms to obtain >90% maximal tonic tension but to reduce at the same time a possible damage of the fibers by this high depolarization. Note that at this duration of the depolarizing pulse (400–600 ms), the tonic tension did not reach a plateau; however, with longer pulses (≧1 s) the characteristic plateau shape of tension (Vassort and Rougier, 1972) occurred. Moreover, the preparations were regularly examined at lower depolarizations (E_m ≃ −30 to 0 mV), and only experiments where the preparations did not show any change in electrical and mechanical behavior were considered. The time-course of the rapid decrease of peak tonic tension in Na-free and Ca-free Ringer's and the development of contracture in Na-free solution are shown in Fig. 2. (The phasic tension elicited by 80- to 100-mV depolarizing steps was

increased severalfold during the first 5 min in Na-free solution — unpublished results.) In some cases, upon the application of Na-free solution, the tonic tension was increased during the first two or three beats before it decreased below the control level; the amplitude of the Na-free contractures varied among
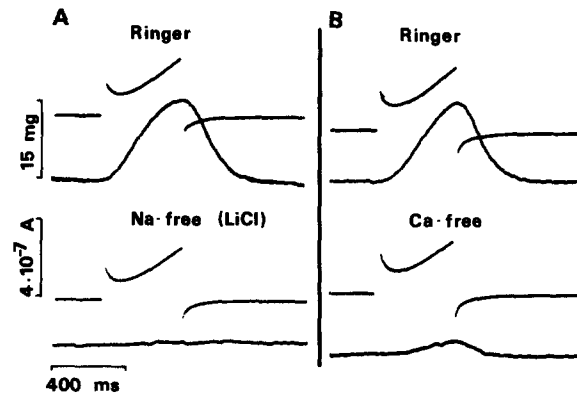

FIGURE 1. Effects of Na-free (LiCl) Ringer's solution (A) and Ca-free Ringer's solution (B) on membrane currents and tonic tension elicited by 160 mV (E_m = 90 mV) and 500-ms long depolarizing pulses under voltage-clamp conditions at a frequency of 15/min. The recordings in Na-free or Ca-free solutions were taken at 130 and 180 s, respectively, after the switch from control Ringer's solution.
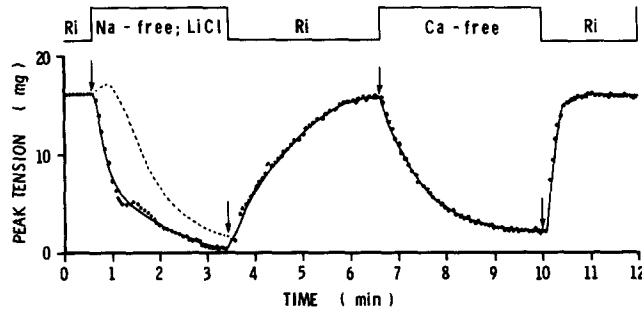

FIGURE 2. Effects of Ca- or Na-free (LiCl) Ringer's solutions on peak tonic tension recorded under voltage clamp. The solid line represents the envelope of peak amplitudes of "twitch" tonic tensions elicited by depolarizing clamp pulses of 160 mV (E_m = 90 mV) and 500 ms at a frequency of 15/min and recorded continuously by a pen recorder (see inset of Fig 3). The arrows indicate the time of application of various Ringer's solutions and the time of switching from or back to the control Ringer's solution (Ri), respectively. Application of Na-free Ringer's resulted in the development of contracture represented by the difference between the broken and solid lines.

different preparations. Similar results were obtained in a solution where Na was substituted by sucrose (not shown). The return of tonic tension to control level was faster with return to normal Ringer's solution from Ca-free medium than from Na-free medium. The solid lines in Figs. 2–8 connect the experimental

values (closed circles) of the peak tonic tensions recorded throughout the experiment by the pen recorder, as illustrated in Fig. 3.

The tonic tension could be increased severalfold by the application of veratrine (as will be shown in Fig. 6) consequent to an increase in $[Na]_i$ (Horackova and Vassort, 1974). Fig. 3 represents results obtained in the presence of veratrine. Within 2 min (and in some preparations within <60 s), the application of Ca-free Ringer's solution abolished the major part of the tonic tension, i.e., the large positive inotropic effect induced by veratrine. The replacement of Na by Li ions resulted in an immediate decrease of tonic tension
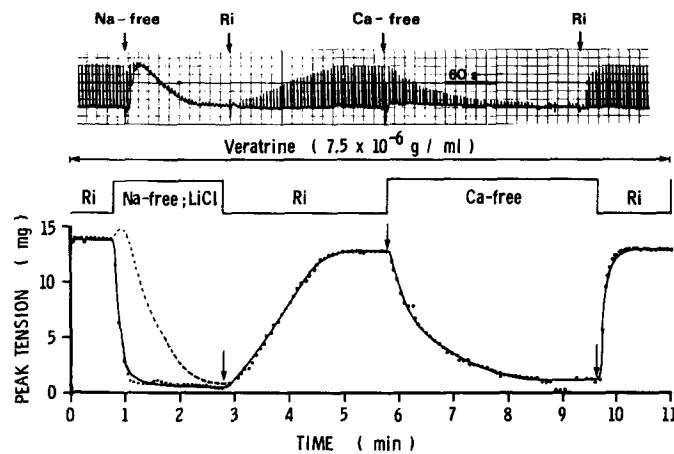

FIGURE 3. This shows the same fiber as that in Fig. 2 and a similar experiment in the presence of veratrine (7.5 × 10⁻⁶ g/ml). The drug was applied 15 min before the effects of Na-free or Ca-free solutions were studied, i.e., after the tonic tension increased to "steady-state" level (value shown in Ringer's solution). The duration of the 160-mV depolarizing pulse was only 100 ms here (as compared with 500 ms in Fig. 2); this elicited tonic tension of <1 mg before the application of veratrine, i.e., tonic tension similar to that shown in Fig. 6. Note that decrease of "veratrine-induced" tonic tension in Na-free solution is even faster than in the normal Ringer's shown in Fig. 2. The *inset* (top) shows the pen recording of the tonic contractions elicited by depolarizing pulses at a frequency of 15/min; the closed circles in the figure below represent the peak values of these contractions.

while contracture developed. Thus, both normal tonic tension elicited by a depolarization step of long duration and tonic tension induced by the application of veratrine were strongly dependent on the presence of Na and Ca ions in Ringer's solution, suggesting the importance of extracellular sites in regulating this component of mechanical activity.

Furthermore, tonic tension was also almost immediately (in 90 s) abolished by the addition of 15 mM $MnCl_2$ as shown in Fig. 4. Similarly, as in Ca-free or Na-free media, the membrane currents in this experiment were not affected significantly (Fig. 4B). However, a decrease in the steady outward current was sometimes observed.

Effect of Various [Na]$_o$ and [Ca]$_o$ on Tonic Tension

When switching to low Na Ringer's solution (30.4 mM), the peak amplitude of tonic tension transiently increased (usually without a development of contracture) and then decreased to about 40% of its original value; on return to normal Ringer's the peak tonic tension decreased further before returning to the control level (Fig. 5). The effect of low Na media was qualitatively similar when either LiCl or sucrose replaced NaCl; however, the decrease in peak tonic tension was slower in sucrose Ringer's (Fig. 5). A similar effect of low [Na]$_o$ was observed in the presence of veratrine (Fig. 6) when the tonic tension, induced by the application of the drug, decreased within 2 min to about 20%. Fig. 7
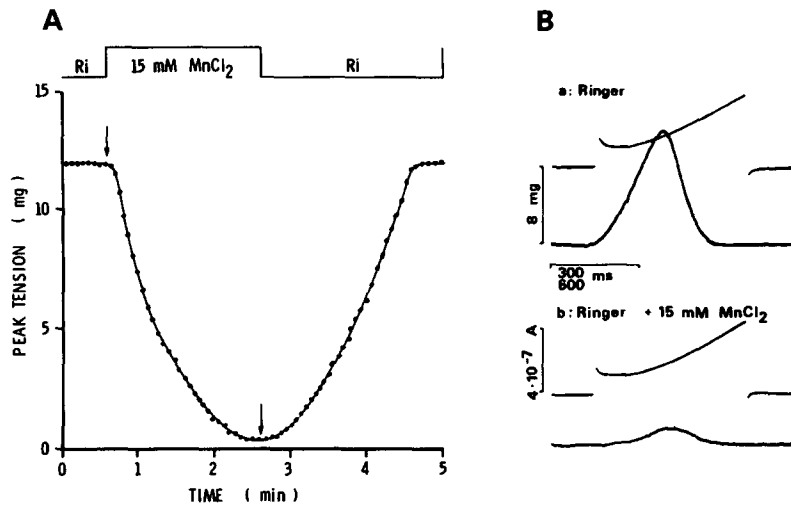

FIGURE 4. (A) Effect of 15 mM MnCl$_2$ on peak tonic tension recorded under voltage-clamp conditions. The solid line represents the envelope of peak tonic tensions elicited by a 160-mV depolarizing pulse (E_m = 90 mV) of 500-ms duration at a frequency of 15/min. The arrows indicate the application of MnCl$_2$ and the return to normal Ringer's (Ri), respectively. (B) Membrane currents (upper traces) and tonic tensions (lower traces) recorded in normal Ringer's (a) and after about 60 s of addition of MnCl$_2$ (b). (The time scales are 300 or 600 ms for the membrane currents and tensions, respectively.)

shows the time-course of changes in peak tonic tension when the [Ca]$_o$ and [Na]$_o$ were decreased simultaneously, and the ratio of [Ca]$_o$/[Na]$_o^2$ was kept constant, i.e., the same as in the control Ringer's (= 1.4 × 10^{-4} mM^{-1}). The tonic tension again increased transiently and then decreased, and, as in Fig. 5, the decrease was faster in Li-Ringer's than in sucrose-Ringer's. A further transient decrease in tonic tension occurred again upon return to normal Ringer's before the control level was reached. Regardless of the absolute values of [Na]$_o$ and [Ca]$_o$, tonic tension was transiently increased by the application of such low Na and low Ca solutions with constant [Ca]$_o$/[Na]$_o^2$ and decreased when these solutions were replaced by normal Ringer's (Fig. 8). Such transient changes of peak tonic tension were prevented (or minimized) when [Na]$_o$ was decreased less, i.e.,

when solutions with constant ratios of $[Ca]_o/[Na]_o^4$ to $[Ca]_o/[Na]_o^6$ $(1.1 \times 10^{-8}$ mM^{-3} to $8.7 \times 10^{-13} mM^{-5})$ were used as shown in Fig. 8. Note that although the absolute values of $[Na]_o$ and $[Ca]_o$ in these two representative experiments were different, the effects were qualitatively similar; the secondary decrease in peak tonic tension was dependent on $[Na]_o$. The transient changes of peak tonic tension after application of modified solutions with constant ratio $[Ca]_o/[Na]_o^2$ or $[Ca]_o/Na]_o^3$ could be the result of an unequal rate with which concentrations of
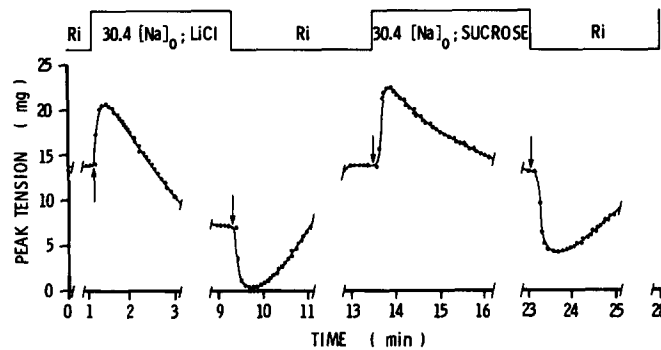

FIGURE 5. Effects of low Na Ringer's solution (LiCl or sucrose) on peak tonic tensions elicited by 400-ms and 160-mV $(E_m = 90$ mV) depolarizing pulses at a frequency of 15/min under voltage-clamp conditions. The arrows indicate the application of low Na solutions and control Ringer's (Ri).
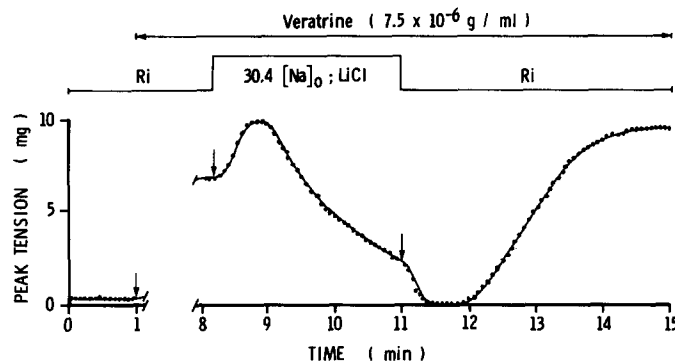

FIGURE 6. Effect of low Na (30.4 mM) Ringer's solution on the positive inotropic effect elicited by veratrine. Solid line represents the peak tension elicited by a 160-mV and 100-ms (*short*) pulse first in normal ringer's solution (Ri) and then 7 min after the application of veratrine. The arrows indicate changes in solutions as shown above the curve.

externally applied calcium and sodium ions equilibrate at the surface of the myocardial cells. To test this hypothesis, i.e., the possibility of a slower rate of diffusion equilibrium reached for Ca ions compared with that for Na ions, we performed experiments in which the $[Ca]_o$ was decreased for 20–120 s before application of solutions with various constant ratios of $[Ca]_o/[Na]_o^x$. A represent-

ative experiment is shown in Fig. 9; the low Ca–low Na solution with constant ratio $[Ca]_o/[Na]_o^2$ again resulted in a large transient increase of tonic tension above control level whether applied only 25 s (upper panel) or 2 min (lower panel) after the start of the low Ca perfusion. The application of a modified solution with constant $[Ca]_o/[Na]_o^3$ resulted in a slight transient increase of peak tonic tension, whereas solutions with constant $[Ca]_o/[Na]_o^4$ resulted in a slight decrease.

Changes in Membrane Potential during Na-Free Contracture

The following Na-free contracture experiments with simultaneous recording of membrane potential were used to demonstrate more directly the electrogenicity of the Na-Ca exchange mechanism.

As soon as a Na-free solution (Li-Ringer's) was introduced, a contracture developed that reached its maximum amplitude in about 30 s and then relaxed slowly. Simultaneously, the membrane potential became more negative, usually
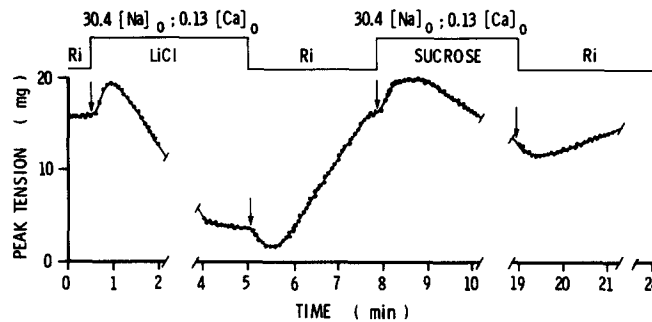

FIGURE 7. Effects of low Na_0 and low Ca_0 with a constant ratio $[Ca]_o/[Na]_o^2$ on peak tonic tension elicited by a 160-mV and 500-ms depolarizing pulse at a frequency of 15/min (solid line) under voltage-clamp conditions. The respective ionic concentrations (millimolar) and the respective replacements (LiCl or sucrose) are indicated above the arrows which show the time of application of various solutions or the control Ringer's solution (Ri).

in two phases, reaching a maximum value after about 30 s and then returning very slowly back to the original level (Fig. 10 A). Among the various preparations, there was considerable variation in the amplitude of the hyperpolarization, from a few millivolts (in the range of the technical error) to 25 mV, generally related to the development of a small or large contracture. To exclude the possibility of this change in membrane potential being an artifact due either to a change in the perfusing level (when switching to a new solution) or to the sliding of the preparation in the vaseline seals, the following experiment was performed. Membrane potential and tension were measured when the level of the fluid in the central gap of the double sucrose-gap bath was voluntarily changed (increased) or when an extra tension was applied transitorily by the strain gage. The results (Fig. 10 B) show that during a Na-free contracture neither treatment noticeably modified the time-course or amplitude of the

membrane potential; the voluntarily introduced variations of fluid level or tension were severalfold greater than ever occurred during an experiment.

Because La ions are known to induce a fast inhibition of Na-free contracture (Vassort, 1973 b), their effect on the membrane potential was examined. When
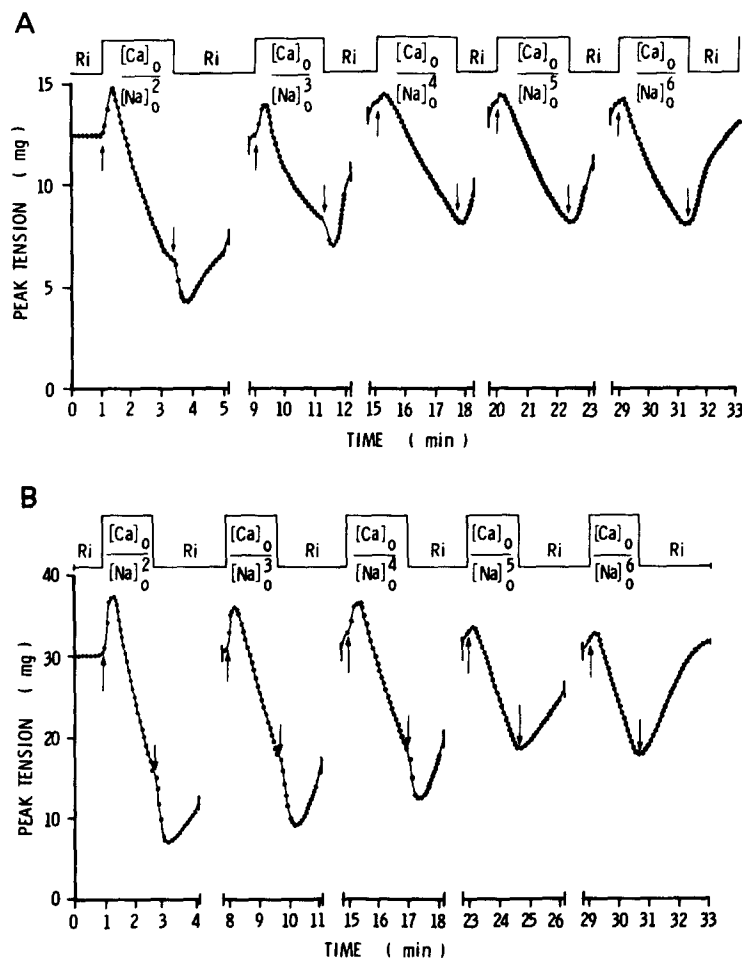

FIGURE 8. (A and B) Peak tonic tension elicited by a 400-ms and 160-mV depolarizing pulse at a frequency of 15/min under voltage-clamp conditions. Arrows indicate the time of application of normal Ringer's solution (Ri) and solutions with various constant ratios $[Ca]_o/[Na]_o^x$ (x = 2, 3, 4, 5, 6, respectively). LiCl was used as a replacement for NaCl. (A) $[Ca]_o$ = 0.5 mM and $[Na]_o$ was (mM): 59.5, 70.6, 82.0, 87.4, 89.6 for x = 2–6, respectively. (B) $[Ca]_o$ = 10^{-2} mM (buffered to this concentration with 4 mM EGTA) and $[Na]_o$ was (mM): 8.4, 19.4, 30.8, 39.9, 46.8 for x = 2–6, respectively; these solutions were buffered with 25 mM Tris to pH 7.2–7.4.

3 mM LaCl$_3$ was applied at the same time or after switching to a Na-free solution, the amplitude of the hyperpolarization was reduced (Fig. 11). Similar experiments were performed using 15 mM MnCl$_2$ (Fig. 12). The addition of these ions to Li-Ringer's prevented the development of Na-free contracture and

inhibited a part of the related hyperpolarization (Fig. 12c). Note that the addition of Mn ions to the normal Ringer's solution (Fig. 12b) also induced a small hyperpolarization.

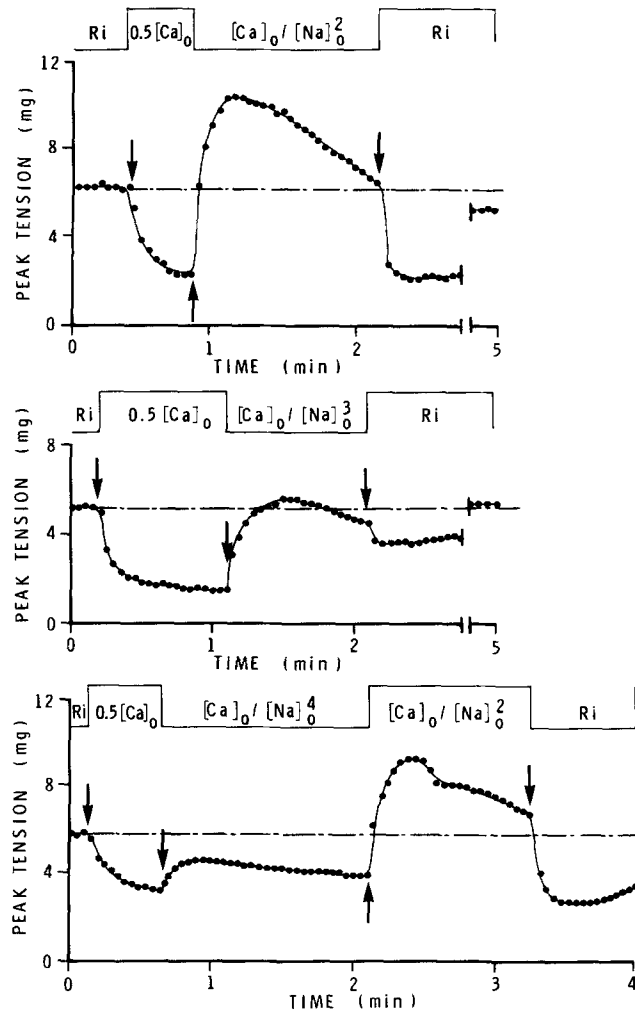To exclude the possibility that an electrogenic Na-K pump played a dominant

FIGURE 9. Peak tonic tension elicited by 140-mV depolarizing pulses at a stimulation frequency of 20/min under voltage-clamp conditions. In modified Ringer's solutions $[Ca]_o$ was 0.5 mM and $[Na]_o$ was (mM): 59.5, 70.6, and 82.0 for solutions with various constant ratios $[Ca]_o/[Na]_o^x$ with $x = 2$, 3, and 4, respectively. LiCl was used as a replacement for NaCl. The $—\cdot—\cdot—$ line represents the control (Ri) level before application of the modified solution.

role in the development of hyperpolarization, similar Na-free contractures were elicited in trabeculae that had been bathed for 3-4 h in K-free ouabain (10^{-7} M; Sigma Chemical Co., St. Louis, Mo.) containing Ringer's solution to inhibit the Na-K pump. Under these conditions the membrane resistance appeared very

low so that the current due to the electrogenicity of the exchange was no longer measured as voltage drop on the membrane resistance but directly under voltage-clamp conditions. As shown in Fig. 13 A, an outward current developed in Na-free solution simultaneously with contracture; the time-course of the current was very similar to the hyperpolarizations reported above (Figs. 11 and 12). The addition of 3 mM $LaCl_3$ to Na-free solution significantly reduced the rate of rise of the contracture and the outward current (Fig. 13 B). Similar results were obtained in two other experiments.
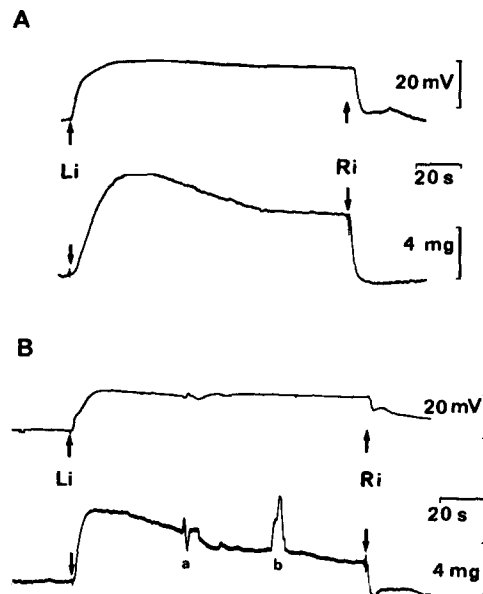

FIGURE 10. (A) Pen recordings of variations in resting membrane potential recorded in double sucrose-gap (upper trace — the *upward* deflection indicating *hyperpolarization*) and resting (diastolic) tension (lower trace — the *upward* deflection indicating *contracture*). The arrows indicate the application of Na-free LiCl Ringer's solution (Li) and the return to control Ringer's (Ri). (B) Same as in A but different fiber. Furthermore, the recordings show the effects of an induced large variation of the fluid level in the test compartment (*a*), and a sudden large tension applied to the test node of the preparation (*b*). Note that the membrane potential trace was modified only negligibly by these changes which were considerably greater than those usually ociurring during normal experimental procedure.

DISCUSSION

Our results suggest that tonic tension in cardiac muscle is regulated by a Na-Ca exchange mechanism and that this exchange is electrogenic and thus voltage dependent. These conclusions are supported by our results demonstrating: (*a*) the effects of various $[Na]_o$ and $[Ca]_o$ on tonic tension and (*b*) the effects of Na-free solutions on resting membrane potential and tension. Our results can be interpreted using a model of the Na-Ca countertransport (Fig. 14) similar to that originally suggested by Reuter and Seitz (1968) and by Glitsch et al. (1970) in cardiac muscle, and by Baker et al. (1969) in squid axon.

Model of Na-Ca Exchange Mechanism; Effect of Decreased [Na]₀ and [Ca]₀ on
Tonic Tension

It is assumed that the cardiac membrane contains a mobile carrier (R) which combines in a competitive way with one Ca ion or with X Na ions $(X \geqq 3)$ on either side of the membrane. The chemical reactions between the carrier and counter ions are assumed to be rapid, whereas, the diffusion of the carrier-cation complexes $(CaR^-$ or $Na_3R)$ across the membrane are rate limiting and influenced by electrochemical gradients for the cations; presumably, free carrier diffuses slowly. Furthermore, it is assumed that such countertransport is bidirectional, the ratio of Na-dependent Ca efflux and Na-dependent Ca influx changing with the membrane potential (i.e., Na-dependent Ca influx increasing and Na-dependent Ca efflux decreasing with depolarization). Switching from control Ringer's solution to low Na media we would anticipate that (a) the Ca
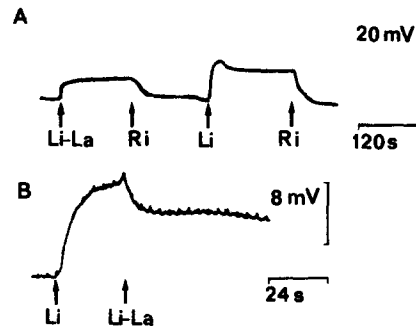

FIGURE 11. Alteration of the resting membrane potential recorded by pen recorder in double sucrose-gap in Na-free LiCl Ringer's solution (Li) with or without 3 mM LaCl₃ (La) and after return to control Ringer's (Ri). The hyperpolarization induced by Na-free solution (upward deflection) was smaller in the presence of LaCl₃ (A) and was reduced when La ions were added subsequently (B). A and B are recordings from the same fiber.

influx linked to Na efflux would be facilitated by a transient decrease in Na uphill gradient, and (b) the Ca efflux linked to Na influx would be hindered. Both changes should result in an increase in $[Ca]_i$ and thus in tonic tension.

The results presented by Figs. 5 and 6 supported our expectations; however, the increase in tonic tension was only transitory, because it quickly decreased below the control level. The rate of this decrease in tonic tension was related to the variation in $[Na]_0$. The reduction in $[Na]_0$ by about 3.5-fold (Fig. 5) decreased the tension by only about half; this implies that the explanation for this effect is indeed an inhibition of Na-Ca exchange because the tonic tension depends not only on $[Ca]_0$ but also on $[Na]_i$. The decreasing $[Na]_i$ in low Na media has been demonstrated by Na-sensitive microelectrodes in cardiac muscle (Ellis, 1977) and in crab muscle (Vaughan-Jones, 1977). Such a decrease in $[Na]_i$ would result in a reduced Na efflux and, therefore, a reduced Ca influx. This reduced Ca influx would then account for the decrease in the steady-state tonic tension observed in reduced $[Na]_0$. A similar scheme involving both internal

and external Na and Ca concentrations in tension production was proposed recently by Miller and Moisescu (1976).

The rate of the secondary decrease in the tonic tension was slower and less pronounced when sucrose (compared with LiCl) was used as a replacement of Na ions (Figs. 5 and 7). This different behavior of tonic tension in sucrose could be the result of several factors such as a slower and (or) limited diffusion of sucrose within the extracellular spaces; both these effects would result in a slower decrease of intracellular Na ions at the sites (presumably close to the membrane) affecting the rate of the Na-Ca exchange. Another factor that could be involved in this effect is the substantial reduction of concentration of Cl ions
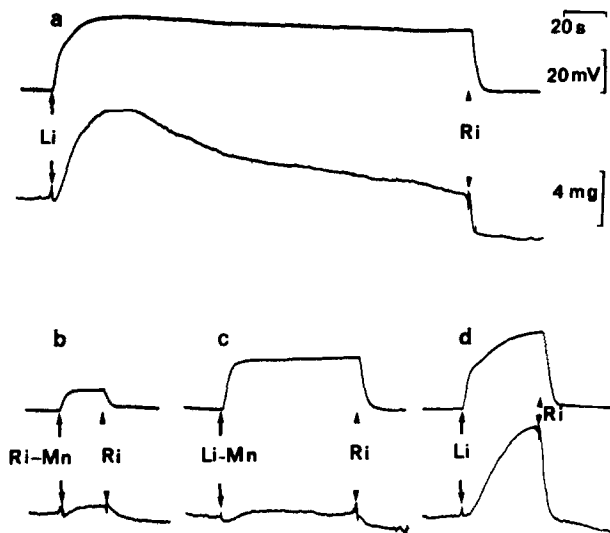

FIGURE 12. Effects of 15 mM MnCl$_2$ added to normal Ringer's solution (Ri-Mn) and to Na-free LiCl Ringer's (Li-Mn) on the membrane potential (upper traces) and resting (diastolic) tension (lower traces) recorded in double sucrose-gap. The traces in a–d were recorded in successive runs with about 2 min between each change. The hyperpolarization (upward deflection) in LiCl Ringer's was associated with a rise in tension (a, d), and both were decreased by Mn ions (cf. c and d). Note the addition of Mn ions to normal Ringer's induced a slight hyperpolarization (b). (The scales in a apply also to b –d.)

resulting from NaCl substitution by sucrose. The analysis of this complex phenomenon was beyond the scope of this study and further experimental evidence is needed to clarify these effects of various Na substitutes. It is worthwhile to mention here that the behavior of tonic tension with choline chloride substituting NaCl appeared to be an intermediate one between those obtained with LiCl and sucrose (unpublished results).

Tillisch and Langer (1974) observed similar transient changes in contractile force due to a variation in [Na]$_o$ in mammalian cardiac muscle and suggested that these alterations were the result of changes in [Na]$_i$.

The indication of the regulative role of Na-Ca exchange was also supported

by the evidence obtained in Na-free media (Figs 2 and 3) where the peak tonic tension (elicited by 160-mV depolarizing steps) was abolished almost completely in <120 s. A rather unexpected result, however, was the absence of the transient increase of tonic tension comparable to that observed in low Na (Figs. 5 and 7). Although we do not have a clear explanation of this behavior in Na-free solution, it could be a result of a fast depletion of Na ions at some intracellular sites regulating the Na-Ca exchange. In Ca-free media (Figs 2 and 3) or when 15 mM $MnCl_2$ was added (Fig. 4), the tonic tension rapidly declined to <10% presumably because of either the lack of Ca binding (in Ca-free) or blocking of the exchange carrier by Mn ions. Both effects would result in decreased Na-dependent Ca influx. Although the short time needed for the effects supports
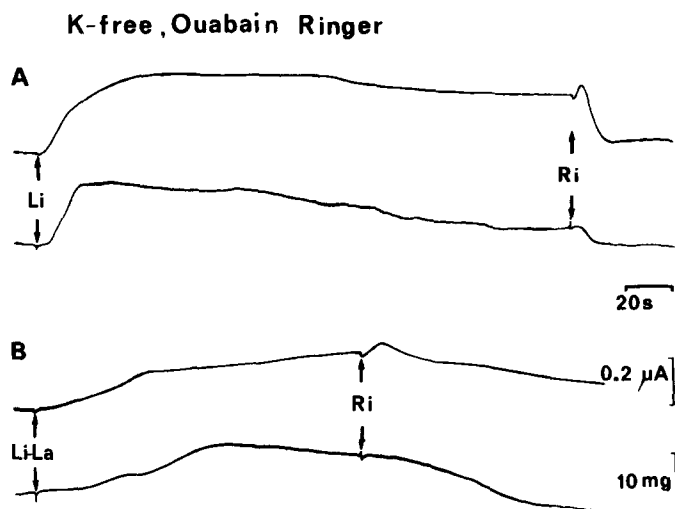
K-free,Ouabain Ringer

FIGURE 13. (A and B) The resting tension (lower traces) and development of an outward current (upper traces) recorded under voltage-clamp conditions. The fiber was bathed previously (for 3–4 h) in K-free, Ringer's solution with ouabain added (10^{-7} M); (A) after application of Na-free LiCl Ringer's (Li) and (B) after application of Na-free LiCl with 3 mM $LaCl_3$ (Li-La). The preparation was switched for 5 min to control Ringer's (Ri) between the recordings in A and B. The scales in B apply also in A.

this explanation, at present we cannot quite exclude the possibility that they originate partially intracellularly, i.e., that Mn ions may block the binding of Ca ions to the myofilaments or that the cells are depleted of Ca ions in Ca-free solution.

If, as suggested by the above results, the tonic tension depends on $[Ca]_o$ and $[Na]_i$, it appears paradoxical that the tonic tension is practically abolished within 120 s in Na-free solution, whereas there is still a residual tension after 180 s in Ca-free solution. However, it should be noted that this does not necessarily mean that $[Na]_i$ decreases faster than $[Ca]_o$. It is probable that some small residual $[Ca]_o$ and $[Na]_o$ are maintained in these Na-free or Ca-free solutions. In such circumstances the remaining $[Ca]_o$ would be more "effective" in terms

of activation of tonic tension if the stoichiometry of the exchange is indeed one Ca to three (or more) Na ions, as suggested below. Note that under this condition for a 10-fold decrease in $[Na]_o$ (and $[Na]_i$), the $[Ca]_o$ would have to decrease 1,000-fold to obtain the same effect on tonic tension. Furthermore, it is not certain whether the $[Na]_i$ in Na-free solution decreases to a final steady-state value at the time of disappearance of tonic tension or whether the tension is abolished as a result of a specific behavior of the exchange system under these Na-free conditions (Miller and Moisescu, 1976). There is a large discrepancy between the half-time of tension decrease in Na-free (Fig. 2—$t_{1/2}$ is about 20 s) and that in low Na (Fig. 4—$t_{1/2}$ is about 90 s); both of these declines of tonic tension presumably reflect the decrease in $[Na]_i$ consequent to the change of $[Na]_o$. At the present time we have no definite explanation for this discrepancy, although it could be accounted for in part by a faster decrease of $[Na]_i$ in Na-
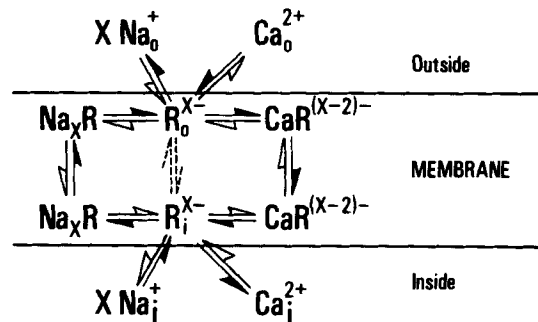

FIGURE 14. Model of electrogenic sodium-calcium exchange mechanism. The free carrier R^{x-} is assumed to be an anion (with $x \geq 3$) binding specifically with three or more Na ions and one Ca ion. (The subscripts, i and o, refer to internal or external surfaces of the membrane, respectively.) Because net charge is assumed to be transported during each exchange, the complexes are driven by the membrane potential: at steady state (resting) the direction indicated by open arrows is prevailing and with increasing depolarizations, the opposite direction (filled arrows) of the exchange would increase. (For more detail, see text.)

free solution due to the reversed Na gradient (which may facilitate the Na-K pump and the Na-Ca exchange, both of these presumably decreasing $[Na]_i$ at a faster rate). To explain this behavior more precisely, a direct measurement of $[Na]_i$ close to the membrane is needed.

In this respect it is of interest to note that the decrease of tonic tension in Na-free solution is even faster in the presence of veratrine (Fig. 3 compared with Fig. 2). This effect is not surprising, however, if we consider that the decrease in $[Na]_i$ in this situation is consequent not only upon the removal of external Na (as in the absence of veratrine) but also upon the superimposed effect of the lacking Na influx with each depolarization. This Na influx is essential for the activation of the tonic tension (the positive inotropy) in veratrine. We have demonstrated previously (Horackova and Vassort, 1974) that when stimulation is stopped, the tonic tension in the presence of veratrine returns to the control

level with half-time about 30 s. Note that this effect alone (i.e., the lack of Na influx) almost accounts for the decrease of tonic tension in Na-free solution in the presence of veratrine; the decreased level of tonic tension for this short pulse of 100 ms (reached in Na-free solution with a $t_{1/2}$ of 20 s — Fig. 3) is about the same as before the application of veratrine (Fig. 6).

Finally, the time-courses of tonic tension related to Ca removal and reapplication should be considered. The decrease in tonic tension consequent upon Ca removal was usually slower than the return of tension in control Ringer's (Figs. 2 and 3). This probably is due to the large affinity of some extracellular binding sites for Ca ions that somewhat delays part of the Ca washout. This asymmetry of time-courses during washout and return to control Ringer's was observed with Ca-free solution, but not with low Ca Ringer's (e.g., $[Ca]_o = 0.5$ or 0.13 mM), where both time-courses were symmetrical (unpublished results).

Stoichiometry and Electrogenicity of Na-Ca Exchange

Lüttgau and Niedergerke (1958), in the study investigating the antagonism between Na and Ca in regulating cardiac contraction, suggested that changes in $[Na]_o$ (sucrose substituted) and $[Ca]_o$ did not affect the contractile force in frog myocardium when the ratio $[Ca]_o/[Na]_o^2$ was kept constant; this implied in their model that the carrier is binding either one Ca or two Na ions. Later studies of Reuter and Seitz (1968) and Glitsch et al. (1970) on cardiac muscle, and Baker et al. (1969) on squid axon indicated that Na and Ca ions compete for the carrier not only at the outside but also at the inside of the membrane and that their transport across the membrane is linked. These authors suggested that the exchange ratio for these ions is two Na for one Ca, i.e., that the transport mechanism is electroneutral; however, Baker et al. (1969) indicated that this ratio could be higher under certain experimental conditions. Finally, Mullins and Brinley (1975) provided the first evidence for an electrogenic, voltage-dependent Na-Ca exchange in squid axon.

Our results supported the hypothesis of the Na-Ca exchange and also indicated that this exchange may not be electroneutral. If it were electroneutral (i.e., exchanging two Na ions for one Ca ion), using low Na and low Ca media and keeping $[Ca]_o/[Na]_o^2$ constant (i.e., the same as in Ringer's solution), we should not have observed the transient changes in tonic tension because the competition of external Ca and Na ions for the carrier's binding sites should remain unchanged and the original decrease in Na gradient which would facilitate Na extrusion should be compensated for by less tendency for Ca entry. However, regardless of the absolute values of decreased $[Na]_o$ and $[Ca]_o$, the applications of these low Na, low Ca solutions where the ratio $[Ca]_o/[Na]_o^2$ was kept constant always induced diphasic changes when these solutions were introduced and replaced by normal Ringer's solution, respectively (Figs 7 and 8). One could argue that the transient changes in tension in these various solutions with constant ratio $[Ca]_o/[Na]_o^2$ are the result of different rates of diffusion equilibria for Na and Ca ions reached within the preparation after the changes in $[Na]_o$ and $[Ca]_o$. However, the rate of perfusion under our experimental conditions is fast; the solutions are almost completely exchanged within a few seconds. To obtain a more direct estimate, in six trabeculae we compared

the rate of decrease in the tonic tension when only $[Ca]_o$ was lowered (0.5 mM) and the rate of increase in the tonic tension when only $[Na]_o$ was lowered (59.5 mM). The rates were not significantly different; the peak tonic tension decreased with $t_{1/2}$ of 7.2 ± 3.2 s upon application of Ca-poor solution and transiently increased in Na-poor solution with $t_{1/2}$ of 6.5 ± 2.5 s (unpublished results). These values are close to values estimated by other authors (Chapman and Niedergerke, 1970, Page and Niedergerke, 1972) for the equilibration of these ions within the aqueous spaces immediately adjacent to the myocardial cells in frog heart. Furthermore, our results indicate that the transient changes of tonic tension in various solutions with constant ratio of $[Ca]_o/[Na]_o^2$ could not be explained by a slower rate of diffusion equilibrium for Ca ions compared with Na ions because the transient increase in tension occurred without a significant change in amplitude even with Ca preequilibration, i.e., when low Ca solution was applied 25-120 s before the low Na, low Ca solution (Fig. 9).

The transient changes of tonic tension were suppressed when $[Na]_o$ was decreased less, i.e., using solutions with a constant ratio between $[Ca]_o/[Na]_o^4$ and $[Ca]_o/[Na]_o^8$. This effect was independent of the absolute values of $[Ca]_o$ and $[Na]_o$ (Fig. 8). Although these results are only a crude approximation of the real exchange ratio, they indicated stoichiometry of the exchange of three (or more) Na ions for one Ca ion. If this is correct, then either other cation(s) accompany the Ca ion, *or* anion(s) accompany the Na ion, *or* the exchange involves a net transfer of charge (a flow of current) which would make the transport mechanism electrogenic and thus voltage dependent. Although we could not exclude the first two possibilities, our data on changes of membrane potential obtained in Na-free solution favor the electrogenic nature and thus the voltage-dependence of the Na-Ca exchange.

It was suggested earlier (Vassort, 1973 *a*; Chapman, 1974) that the Na-free contracture is consequent upon alteration of Na-Ca exchange. Furthermore, studies of Goto et al. (1972) and Chapman (1974) demonstrated the occurrence of transitory hyperpolarization within the first few minutes after Na withdrawal, and this was confirmed in the present study (Figs. 10–13). Until now, no clear interpretation existed to account for this hyperpolarization. We considered three factors that may be involved in the development of this effect: (*a*) an increased negative diffusional potential (as predicted by the constant-field equation); (*b*) a stimulation of an electrogenic Na-Ca exchange that would inhibit the Na-dependent Ca efflux and accelerate the Na-dependent Ca influx, resulting in decreased steady inward current and/or increased outward current generated by the Na-Ca exchange; and (*c*) a stimulation of an electrogenic Na-K pump. Thus, we attempted to determine the participation of factors (*b*) and (*c*) in the hyperpolarization after Na withdrawal.

It has been suggested that in squid axon La ions inhibit the ouabain-insensitive Na efflux (Baker et al., 1969) and Mn ions inhibit Na-dependent Ca efflux (Blaustein, 1977). However, La and Mn ions could not be considered highly specific inhibitors of the Na-Ca exchange mechanism because these ions also affect kinetic parameters of various membrane currents (Kass and Tsien, 1975). In cardiac muscle, these ions also antagonize the development of Na-free contracture (Chapman and Ochi, 1972; Vassort, 1973 *b*). It is worthwhile to note

that in the present study, both Mn and La ions simultaneously inhibited the development of the hyperpolarization and the contracture induced by Na-free solution (Figs. 11 and 12). An outward current elicited by Na replacement with Li was recorded under voltage clamp (unpublished results) also when the preparation was kept at holding potential 15 mV negative to E_r, i.e., close to or below E_k (E_m = -85 mV); E_k was determined as described by Ojeda and Rougier (1974). These experiments excluded the possible effects of $[Ca]_r$-activated potassium permeability (an increased outward I_k) as a source of the hyperpolarization and the underlying outward current occurring during the Na-free contractures. Moreover, a similar inhibitory effect of La ions on the outward current and contracture both elicited by Na-free solution was also observed when the Na-K pump was inhibited (by bathing the preparations in K-free media with ouabain added; Fig. 13). This result ruled out Na-K pump activity as the only source of electrogenicity and supported (with the reservations mentioned above) the electrogenic nature of Na-Ca exchange. Such an electrogenic mechanism could account (at least partially) for the development of hyperpolarization occurring in normal Na-containing solution in the presence of Mn ions (Ochi, 1970; Takeya and Reiter, 1972; present study, Fig. 12 b). This could be explained by the effect of $MnCl_2$ decreasing the steady inward current generated by the Na influx linked to Ca efflux; this inward current is, according to purely thermodynamic considerations (see Mullins, 1976), the predominating current of the exchange at the resting membrane potential (assuming $[Na]_i$ = 11 mM and $[Ca]_i$ = 10^{-7} M, see below).

If we assume that the regulatory calcium concentration, $[Ca]_i$, is determined by

$$\frac{[Ca]_o}{[Ca]_i} = \frac{[Na]_o^3}{[Na]_i^3} e^{-E_m F/RT},$$

or by

$$[Ca]_i = [Ca]_o \frac{Na_i^3}{[Na]_o^3} e^{E_m F/RT},$$

where E_m is the membrane potential, F and R are the Faraday and Universal gas constant, respectively, and T is temperature (Baker, 1972; Blaustein, 1976), one can see that such a voltage-dependent mechanism would account for a $[Ca]_i$ of 10^{-7} M at E_m = -70 mV (resting membrane potential) and of 2.7 × 10^{-5} M at E_m = 70 mV in normal Ringer's with $[Na]_i$ = 11 mM. It should be noted that a $[Ca]_i$ of 1.3 × 10^{-8} M is obtained with $[Na]_i$ equal to 5.5 mM; i.e., a twofold change in $[Na]_i$ might induce a nearly 10-fold change in the $[Ca]_i$ and in the current generated by the exchange (I_{ex}).

To date we have been unable to demonstrate changes in I_{ex} at high depolarizations (E_m = 90 mV) in situations where the activity of the exchange system is altered as evidenced by changing tonic tension, e.g., upon application of 15 mM $MnCl_2$ (or Na-free solution). Although in Mn-Ringer's solution a decrease of the outward current was sometimes observed, we could not determine whether this effect was due to inhibition of the outward I_{ex} (the predominating exchange

current at high depolarization) or to a decrease in potassium permeability caused by diminished $[Ca]_i$. To solve this problem and to distinguish between changes in I_k and I_{ex}, a more specific inhibitor of one of these currents is needed.

Our results disagree with the conclusions of Jundt et al. (1975), suggesting that the Na-Ca exchange in cardiac muscle is voltage independent and thus electroneutral (2:1). Their conclusion was based on a finding that the application of a hypertonic solution with high $[K]_o$ did not significantly affect the total Ca efflux. However, this result was obtained under an experimental situation involving several simultaneous alterations besides the change in membrane potential. Thus, other effects (e.g., the direct effect of high $[K]_o$) cannot be excluded. Furthermore, it is not quite clear whether simultaneous changes in Ca influx were not affecting the measured Ca efflux. It appears that more direct experimental evidence is needed to substantiate their conclusion.

On the other hand, Benninger et al. (1976) in a recent voltage-clamp study on the Na-Ca antagonism in frog heart also suggested an electroneutral 2:1 Na-Ca countertransport; however, they proposed a voltage-dependent system, assuming a voltage-dependent affinity of the carrier to account for the origin of tonic tension. They concluded that the exchange was electroneutral, because in experiments using low Na, low Ca solutions with constant ratio $[Ca]_o/[Na]_o^2$, they observed no changes in contractile force when measured in solutions with small variations of $[Na]_o$ and $[Ca]_o$. However, when the reduction of $[Na]_o$ and $[Ca]_o$ was comparable to the reduction in our experiments (Fig. 7), the tonic tension was decreased below the control level. The authors ascribed this decrease to the reduction in $[Na]_i$ and suggested that the tension is related to $[Ca]_o/[Na]_o^2$ under conditions when $[Na]_i$ is virtually unaltered. Furthermore, the large transient increase in Na-dependent Ca influx (and decrease in Na-dependent Ca efflux) immediately after the change in $[Na]_o$ and resulting in transient increase in tonic tension (Figs. 7 and 9) is over within a couple of minutes and therefore does not enter their analysis because they measured the tension 5–8 min after the change.

Although support for our model in cardiac muscle is limited to the present data, recent findings in squid axon and barnacle muscle (Blaustein et al., 1974; Mullins and Brinley, 1975; Blaustein, 1976; Baker and McNaughton, 1976; Mullins, 1977) suggested electrogenicity and voltage dependence of the Na-Ca exchange.

In conclusion, an electrogenic and voltage-dependent Na-Ca exchange is a novel hypothesis in cardiac muscle that might account for the origin on tonic tension and for various previously reported observations. Although the regulative effects on contractile force may be more pronounced in amphibian heart as a result of the lack of other regulatory mechanisms (SR system) and the larger surface-volume ratio of the cells, the exchange mechanism may also play an important role in regulating contractility in mammalian heart. This role could be especially pronounced when $[Na]_i$ is changing (e.g., under the effect of veratrine, cardiac glycosides, etc.). Furthermore, the electrogenicity of the Na-Ca exchange may contribute also to the maintenance of the cardiac membrane potential.

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