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CALCIUM–SODIUM ANTAGONISM ON THE FROG'S HEART: A VOLTAGE-CLAMP STUDY

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SUMMARY

1. In double sucrose-gap voltage-clamped frog atrial fibres the influence of $[Ca]_o$ and $[Na]_o$ on membrane current and contraction was investigated.

2. The slow (secondary) inward current varied with $[Ca]_o$ but was almost insensitive to changes in $[Na]_o$. In contrast, the phasic (transient) contraction initiated by the slow inward current was affected by both $[Ca]_o$ and $[Na]_o$.

3. With moderate changes of $[Ca]_o$ and $[Na]_o$ from normal, the strength of phasic contraction at a given depolarization followed the $[Ca]_o/[Na]_o^2$ ratio approximately. This was best seen at membrane potentials near zero level.

4. Under the same conditions, tonic (sustained) contractions associated with prolonged depolarizations were strictly correlated to the $[Ca]_o/[Na]_o^2$ ratio at any potential. No interrelation between tonic tension and steady-state current was found.

5. With extensive changes in $[Ca]_o$ and $[Na]_o$, the sensitivity of both phasic and tonic tension to the $[Ca]_o/[Na]_o^2$ ratio declined, the negative effect of $[Na]_o$ becoming smaller than was expected from this ratio.

6. In Na-free choline-Ringer, a strong contracture developed followed by a spontaneous relaxation. Starting from the relaxed state, application of depolarizing clamps gave rise to phasic contractions with a very slow relaxation while tonic contractions were apparently lacking.

7. The results are interpreted in terms of an energy-dependent carrier mechanism exchanging one Ca for two Na ions across the cell membrane. The model implies a strong asymmetry in the rate constants governing the chemical reactions on both sides of the membrane. The system is thought to operate close to equilibrium at any potential, thereby determining the

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steady level of myoplasmic Ca. The equilibrium itself is considered to shift upon depolarization. Assuming that $[Na]_i$ is constant, the steady level of $[Ca]_i$ is expected to be proportional to the $[Ca]_o/[Na]_o^2$ ratio, the scale factor being a function of membrane potential.

8. The carrier model suggests the occurrence of a depolarization-induced inward transfer of Ca which might be involved in the generation of tonic contractions.

9. The apparent lack of tonic contractions in the absence of external Na ions may be explained by a suppression of carrier-mediated Ca influx normally occurring upon depolarization.

10. The antagonistic effects of $[Ca]_o$ and $[Na]_o$ on phasic contraction are understood as being due to alterations of the Ca pumping system rather than changes in slow inward current.

INTRODUCTION

It has long been known that force development in cardiac muscle is promoted by Ca ions and depressed by Na ions in the bathing medium (Ringer, 1883; Clark, 1913; Daly & Clark, 1921). The antagonistic effects have been quantified by the observation that the contractile strength of the frog heart, both ventricle and auricle, depends largely on the ratio of $[Ca]_o/[Na]_o^2$ (Wilbrandt & Koller, 1948; Lüttgau & Niedergerke, 1958; Lamb & McGuigan, 1966; Chapman & Tunstall, 1971). This relation holds for twitch tension initiated by an action potential as well as contractures induced by high-K solutions. In mammalian myocardium, the inotropic interplay of external Ca and Na varies considerably from one species to another and between various tissues within a single heart. Contracture tension of atrial preparations from guinea-pig, calf, and sheep hearts was found to behave much like contractures of the frog heart while ventricular preparations gave less consistent results (Scholz, 1969*a*, *b*; Gibbons & Fozzard, 1971). Twitch tension of rat ventricular strips and guinea-pig papillary muscles was reported to follow the $[Ca]_o/[Na]_o^2$ ratio in a restricted range of temperature and stimulation frequency (Reiter, 1961, 1963, 1966). The molecular basis of Ca–Na antagonism has been interpreted by assuming a carrier molecule within the cell membrane combining with either one Ca ion or two Na ions of the external fluid (Lüttgau & Niedergerke, 1958). This would result in a competition between Ca and Na inward transfer. Evidence for a competitive inhibition of Ca influx by external Na was obtained from ^{45}Ca flux measurements on amphibian and mammalian heart structures (Winegrad & Shanes, 1962; Niedergerke, 1963; Langer, 1964). Extensive flux studies on guinea-pig auricles (Reuter & Seitz, 1968; Glitsch, Reuter & Scholz, 1970) suggest the presence of a

Ca–Na counter-ion transport system responsible for active extrusion of Ca from the myoplasm. This concept implies that the competition between Ca and Na is not exclusive to the outer face of the cell membrane but also occurs at the inner face. The kinetics of this transport system resembles in many respects the complex interdependence of Ca and Na fluxes observed in squid axon (Baker, Blaustein, Hodgkin & Steinhardt, 1969).

Further information as to the nature of Ca–Na antagonism may be expected from voltage-clamp experiments on working myocardium. Synchronous measurements of membrane current and tension on various cardiac tissues have revealed some of the features of the excitation–contraction coupling process (Beeler & Reuter, 1970*a*, *b*; Goto, Kimoto & Kato, 1971; Morad & Orkand, 1971; Ochi & Trautwein, 1971; Einwächter, Haas & Kern, 1972; Vassort & Rougier, 1972). The general picture emerging from these investigations is that there exist two types of contractile response to depolarization: a phasic tension associated with the slow (secondary) inward current; and a tonic tension which relaxes only upon repolarization. The influence of Ca and Na on the two components of tension has not yet been examined in detail. The present voltage-clamp study on frog atrial fibres is an attempt to elucidate the interrelation between external Ca and Na concentrations, membrane current, and contraction at various levels of membrane potential. In these experiments antagonistic effects of the two ions on both phasic and tonic tension were observed. The results are tentatively explained in terms of an electroneutral but voltage-dependent Ca–Na exchange system governing the steady level of myoplasmic Ca.

METHODS

Solutions. Ringer fluid used as normal bathing solution had the millimolar composition: NaCl 111, KCl 5·4, $KHCO_3$ 1·8, $CaCl_2$ 2. In the test solutions $CaCl_2$ was varied between 0·04 and 4 mM and 50, 75, 90 or 100% of NaCl were substituted by osmotically equivalent quantities of choline chloride. In some experiments $MnCl_2$ at a concentration of 10 mM was added to Ringer fluid. For the sucrose-gap arrangement isosmotic KCl (121 mM) and sucrose (242 mM) solutions were used. Ringer fluid, test solutions, and KCl solution were continuously aerated by a gas mixture of 95% O_2 and 5% CO_2. The pH was adjusted between 7·0 and 7·2. All experiments were done at 4–7° C.

Experimental arrangement. Fine muscle strips (0·2–0·4 mm in diameter) dissected from bullfrog auricles were mounted in a double sucrose-gap chamber. Simultaneous measurements of membrane current and contraction were obtained from a short (about 0·1–0·2 mm) segment in the middle of the fibre bundle. The voltage-clamp circuit was essentially the same as described previously (Einwächter *et al.* 1972). With the feed-back loop taken off, membrane action potentials were recorded. Tension was measured by a force-displacement transducer (carrier frequency bridge) connected to one end of the preparation.

Possible limitations of membrane current measurement due to an imperfect voltage control at high membrane conductances were considered elsewhere (Haas, Kern, Benninger & Einwächter, 1975). Recently a detailed methodological study of the double sucrose gap technique as applied to frog atrial bundles has been presented by Tarr & Trank (1974). In order to test the quality of voltage control, these

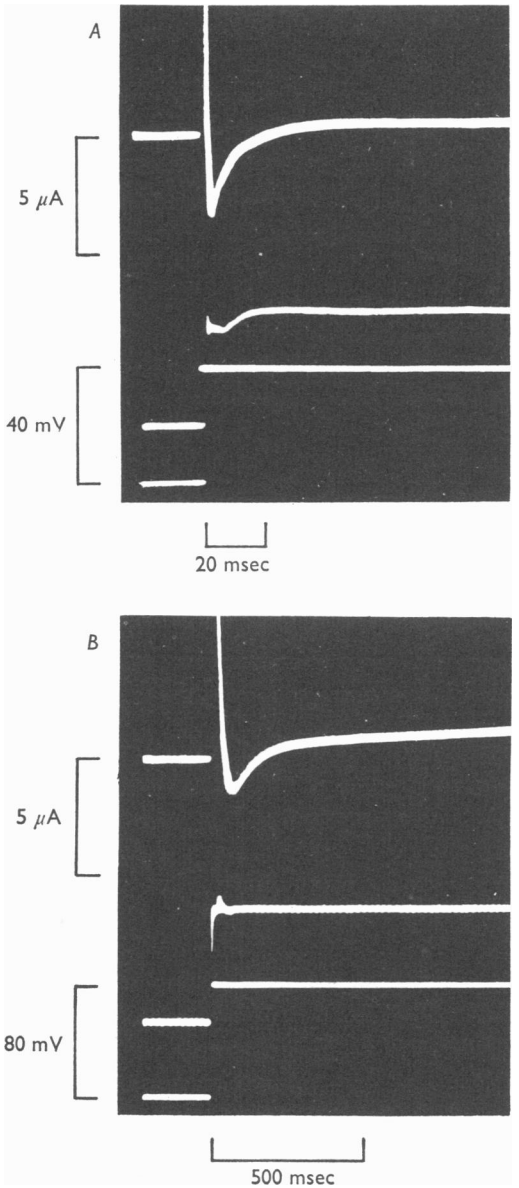

Fig. 1. For legend see facing page.

authors compared the apparent clamp potential as recorded across the sucrose gap with the intracellular potential as recorded by a micro-electrode independent of the voltage-clamp circuit. They observed a severe loss of voltage control during the fast Na inward current in all preparations tested while the control during the slow inward current was adequate in some preparations and inadequate in others. Loss of control was interpreted as being due to a large series resistance located in the intercellular clefts of a multifibre bundle. An analogous study in our laboratory has not yet been completed. The results obtained so far suggest that, in suitable preparations, the quality of control may be better than in the experiments of Tarr & Trank (1974). Two examples are shown in Fig. 1. In the upper record there is almost no difference between transgap potential (V) and intracellular potential (V_i) except for an initial period of about 15 msec covering the fast inward current wave and the beginning of slow inward current. During that time V_i deviates, in the negative direction, from V by about 7 mV. A positive deviation $V_i - V$ as is expected in case of a large series resistance is not seen. Actually the current tracing suggests a rather low series resistance. From the large capacitive current at the onset of the clamp, the resistance in series to the membrane capacity (when considered as a lumped resistance) is estimated to be 50 Ω cm^2 or less (V = 40 mV; $I_c \sim 20$ μA; R_s = 2·000 Ω; membrane area in the order of 0·02 cm^2). Since the micro-electrode was inserted into a superficial fibre the individual series resistance of this fibre was probably much less than 50 Ω cm^2. Under these conditions, a negative deflexion of V_i from the command signal would seem to simply reflect a longitudinal voltage gradient due to the cable properties of the preparation (cf. Ramón, Anderson, Joyner & Moore, 1975). The lower record shows an experiment in which fast inward current was suppressed by tetrodotoxin. Again V_i equalled V during the later phases of the clamp. During the slow inward current a negative deflexion of V_i of not more than 4 mV occurred. From these and similar observations on other preparations it is concluded that an adequate voltage control is routinely afforded during the flow of small outward currents, allowing a proper measurement of tonic tension during prolonged depolarizations, and that a loss of control in the beginning of the clamp would not seriously affect the measurement of either slow inward current or phasic contraction.

Nomenclature. The following symbols are used in this paper: E, absolute membrane potential of the central segment; H.P., holding potential; V, amplitude of rectangular voltage steps, taken with respect to the holding level; I, membrane current of the central segment, specified by subscripts to different ion species.

Fig. 1. Control of the transmembrane potential of frog atrial fibres in a double sucrose-gap voltage-clamp arrangement during a 40 mV and an 80 mV step depolarization. Lower beam: clamp potential as recorded across the sucrose gap by extracellular electrodes; middle beam: membrane potential of the test segment as recorded between an intracellular micro-electrode and a nearby reference micro-electrode; upper beam: membrane current. Inward current is represented by a downward deflexion. The perfusing fluid was Ringer containing 0·5 mM-Ca in *A* and normal Ringer with 200 nM-TTX in *B*. Micro-electrode position was in the centre third of the test node. Node length of 100 μm. The resting potential recorded by the micro-electrode was equal to the -70 mV holding potential established by the voltage-clamp circuit.

RESULTS

Action potential and contraction

The typical changes in frog atrial action potential and contraction resulting from variations in $[Ca]_o$ and $[Na]_o$ are shown in Fig. 2. When $[Ca]_o$ was reduced to one quarter of the initial level, peak tension decreased by about 40% while action potential duration increased slightly (cf. Niedergerke & Orkand, 1966). A subsequent reduction of $[Na]_o$ to 50% restored the contractile strength almost completely, although action potential duration and time to peak tension were somewhat decreased.

In this and numerous similar experiments, the tension developed during an action potential was approximately determined by the $[Ca]_o/[Na]_o^2$ ratio. This is in general agreement with the findings in the frog ventricle (Lüttgau & Niedergerke, 1958). Following a sudden change in $[Ca]_o$ or $[Na]_o$ the inotropic effect reached a steady state within 2–3 min which was comparable to the estimated time required for equilibration of the extracellular space (Niedergerke, 1963; Chapman & Tunstall, 1971). Increasing $[Ca]_o$ had a positive inotropic effect which saturated between 4 and 6 mM in most preparations. Decreasing $[Na]_o$ to values lower than 50% of normal led to marked changes in action potential height and duration so that direct and indirect effects on tension became difficult to distinguish. Control of membrane potential by the voltage-clamp technique eliminated this problem.

Slow inward current and contraction

Unlike frog ventricle (Morad & Orkand, 1971; Morad & Goldman, 1973), frog atrial tissue exhibits a phasic (transient) tension in response to short-lasting depolarizing clamps (Einwächter *et al.* 1972; Vassort & Rougier, 1972). During long-lasting depolarizations the phasic tension is followed by, and partially superimposed on, the development of a tonic contraction. The phasic contraction is closely related to the slow (secondary) inward current which may activate the myofibrils either directly or indirectly by causing a release of activator-Ca from an intracellular store. The tight correlation between slow inward current and phasic contraction can easily be demonstrated by addition of Mn ions to the bathing fluid which are known to selectively block the slow channel (Rougier, Vassort, Garnier, Gargouil & Coraboeuf, 1969). Figs. 3 and 4 show the effect of Mn^{2+} on membrane current and contraction associated with clamps of 200 msec and 2 sec in duration, respectively. The suppression by Mn^{2+} of slow inward current is best seen in the 60 mV step since steady-state current is negligibly small in this record. At any level of depolarization the phasic contraction was completely depressed by Mn^{2+}. In contrast, the tonic

contraction (taken as the tension attained at the termination of the 2 sec pulses) was hardly altered.

As to the ionic nature of slow inward current, a competition of Ca and Na ions has been suggested (Rougier *et al.* 1969; Vassort & Rougier, 1972). According to Tarr (1971) the slow inward current in frog atrial muscle is

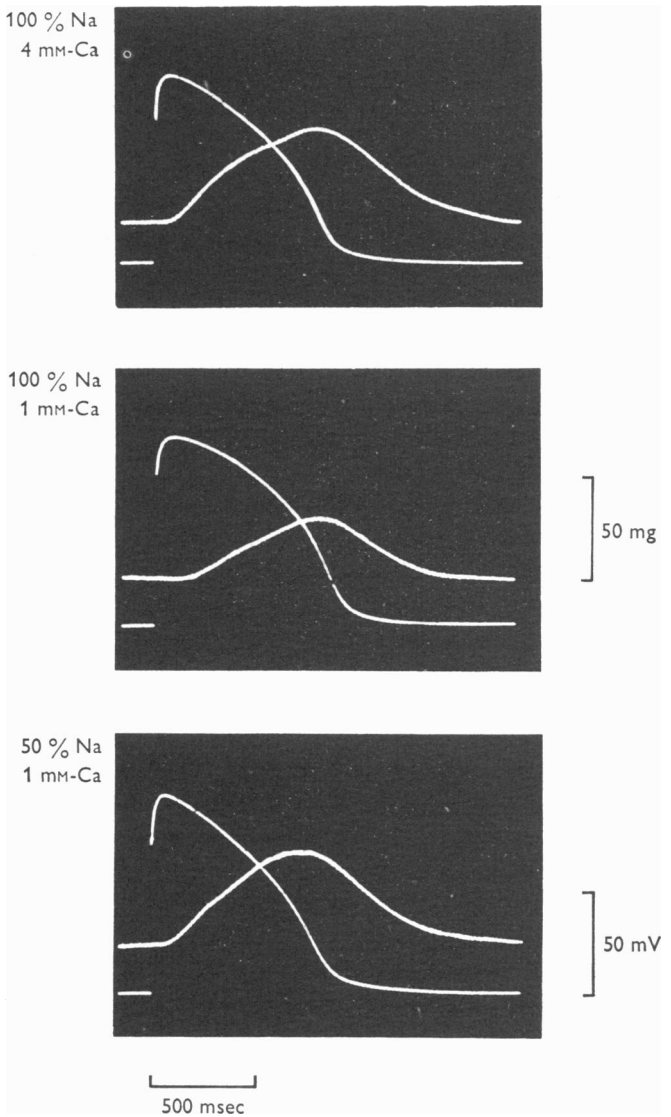

Fig. 2. Action potential and contraction of frog atrial muscle as affected by changes in the Ca and Na concentrations of the perfusing Ringer.

primarily carried by Ca ions. His main argument was the observation that slow inward current was abolished in the majority of preparations bathed with a Ca-free solution, but persisted in a Na-free solution. The influence of external Na on slow inward current is difficult to assess since fast Na current and slow inward current are usually fused to some extent and a change in slow inward current in response to a variation of $[Na]_o$ could be

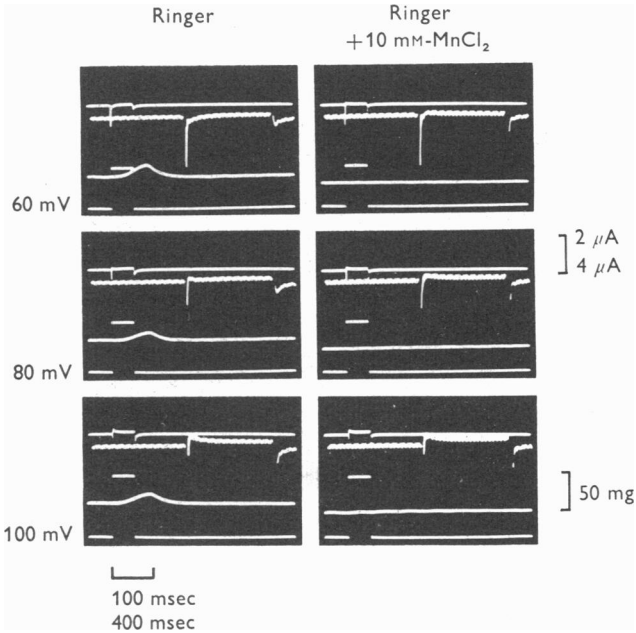

Fig. 3. Depression by Mn ions of slow inward current and phasic contraction. Membrane current (upper beam) and contraction (middle beam) associated with short-lasting depolarizations of varying amplitude (lower beam) in normal Ringer and after 6 min of exposure to Ringer + $MnCl_2$. Holding potential = −70 mV. In order to demonstrate fast and slow inward current in more detail an additional record of membrane current was taken at higher sweep speed and higher gain (100 msec and 2 μA calibration, respectively).

obscured by the concomitant change in fast Na inward current, I_{Na}. To test the Na-sensitivity of slow inward current in more detail we made use of the double pulse method as proposed by Reuter (1973) for separation of fast and slow inward current. In the experiment shown in Fig. 5 the Na system was inactivated by the first (conditioning) step depolarization so that the current initiated by the second (test) step was mainly or solely related to the slow channel. A fourfold reduction of $[Na]_o$ then caused a strong depression of peak Na current associated with the first step while

the slow inward current during the second step was almost unchanged. In eight preparations tested with the double pulse method a reduction of external Na by 75 or 90% was found to produce either no noticeable change or a slight (up to 10%) decrease of the amplitude of slow inward current. These findings suggest that a contribution of Na ions to the slow inward current, if present at all, is negligibly small. Therefore the slow

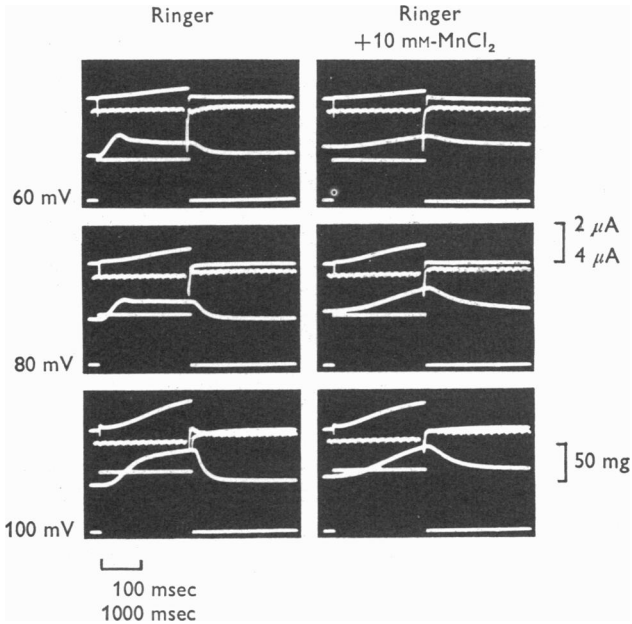

Fig. 4. Effect of Mn ions on tension development during long-lasting depolarizations. Same preparation and same arrangement as in Fig. 3. In the controls tension is the resultant of a phasic and a tonic component. In Ringer + $MnCl_2$ the phasic component is eliminated. The 100 msec time calibration and the 2 μA current calibration refer to the high speed records of membrane current.

inward current in frog atrial muscle is referred to as Ca inward current, I_{Ca}, in the following. This interpretation is in accordance with observations on cat myocardium (New & Trautwein, 1972; Weiss, Tritthart & Walter, 1974). A slight decrease of slow inward current occasionally observed upon reduction of $[Na]_o$ may be explained by a decrease of the driving force for a movement of Ca ions due to an increase of the internal Ca level rather than by depression of a Na-carried component of slow inward current.

Voltage-clamp experiments with moderate changes in $[Ca]_o$ *and* $[Na]_o$

The experiments described here were designed in order to test the sensitivity of phasic and tonic contraction to variations in $[Ca]_o$ and $[Na]_o$ and in particular to examine the influence of the $[Ca]_o/[Na]_o^2$ ratio. Fig. 6 illustrates an experiment in which depolarizing clamps of 200 msec duration were used to generate phasic contractions. Starting from 4 mM-

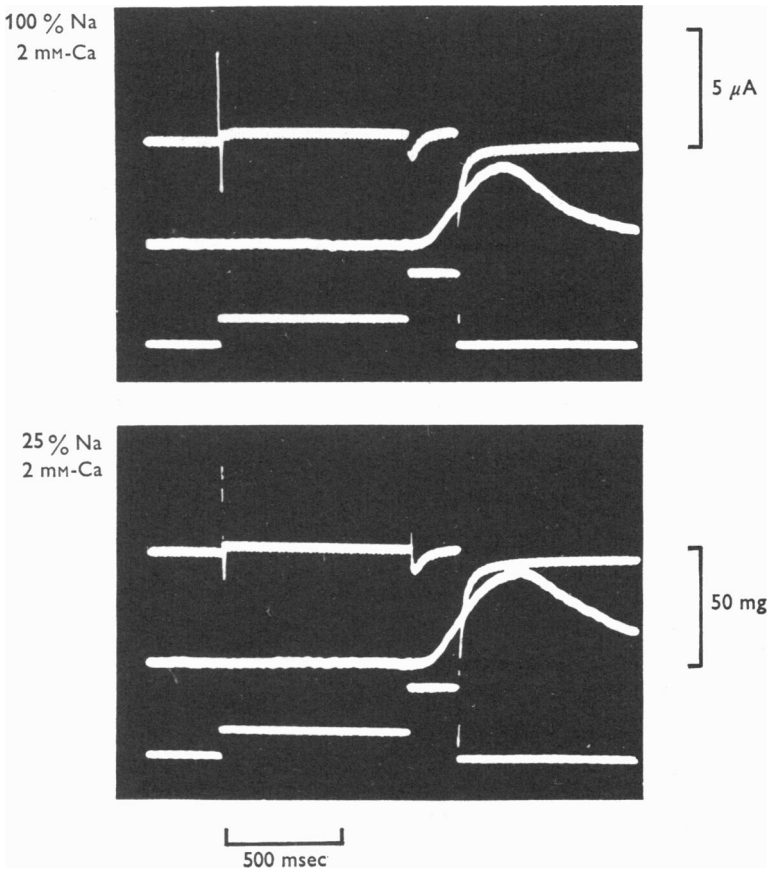

Fig. 5. Influence of external Na concentration on membrane current (upper beam) and contraction (middle beam) in a double pulse arrangement (lower beam). Holding potential = −70 mV. The membrane potential was −40 mV during the conditioning pulse and +10 mV during the test pulse. The conditioning step was chosen to give a large Na inward current without any marked slow inward current or contraction. Note that the phasic tension associated with the test pulse was markedly increased in the Na-poor solution although the underlying slow inward current appeared to be unaltered.

Ca-Ringer, Ca and Na concentrations were successively reduced to a quarter and half, respectively. In Fig. 6*A* the records obtained with a moderate and a strong depolarization are shown. Fast and slow inward current are

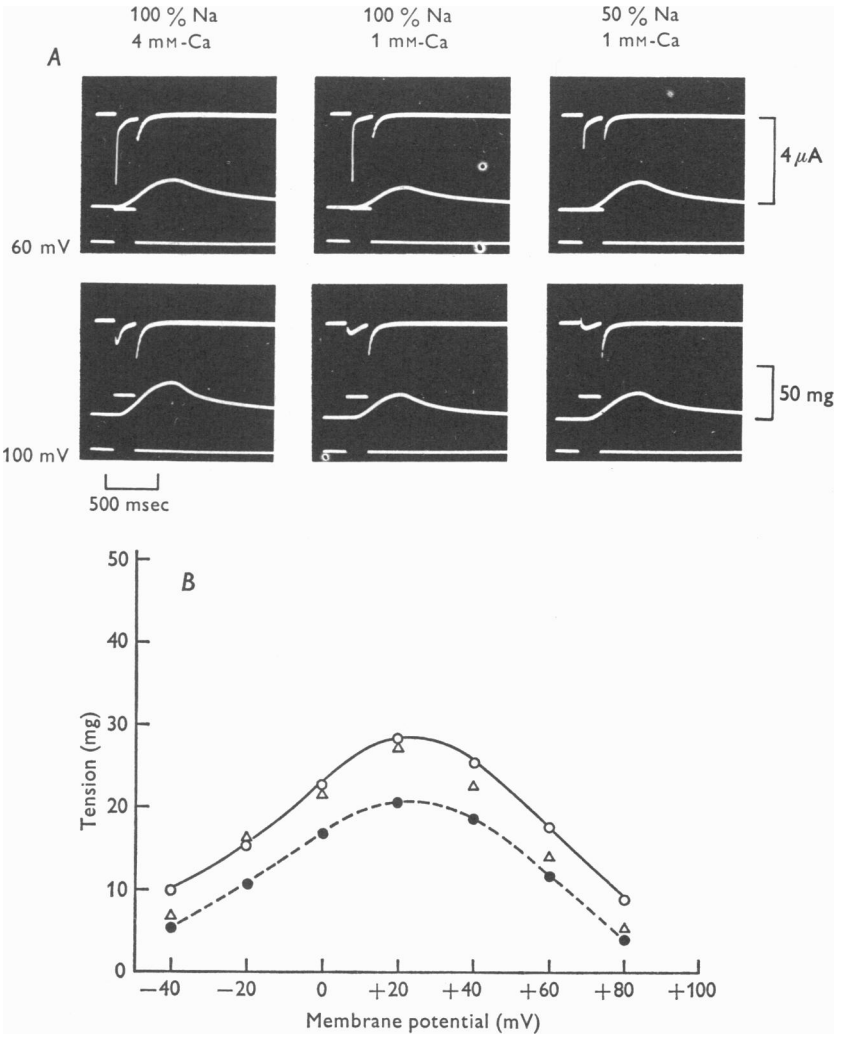

Fig. 6. Phasic contraction of frog atrial muscle as affected by moderate changes in external Ca and Na. Voltage-clamp measurements with short-lasting depolarizations of 20–140 mV amplitude. Holding potential = −60 mV. *A*, records of membrane current (top) and tension (middle) at 60 and 100 mV step depolarizations (bottom). *B*, peak tension–voltage diagram for the different perfusing fluids (○: 100 % Na, 4 mM-Ca-Ringer; ●: 100 % Na, 1 mM-Ca; △: 50 % Na + 50 % choline, 1 mM-Ca-Ringer). Time of exposure to the different fluids 5–8 min.

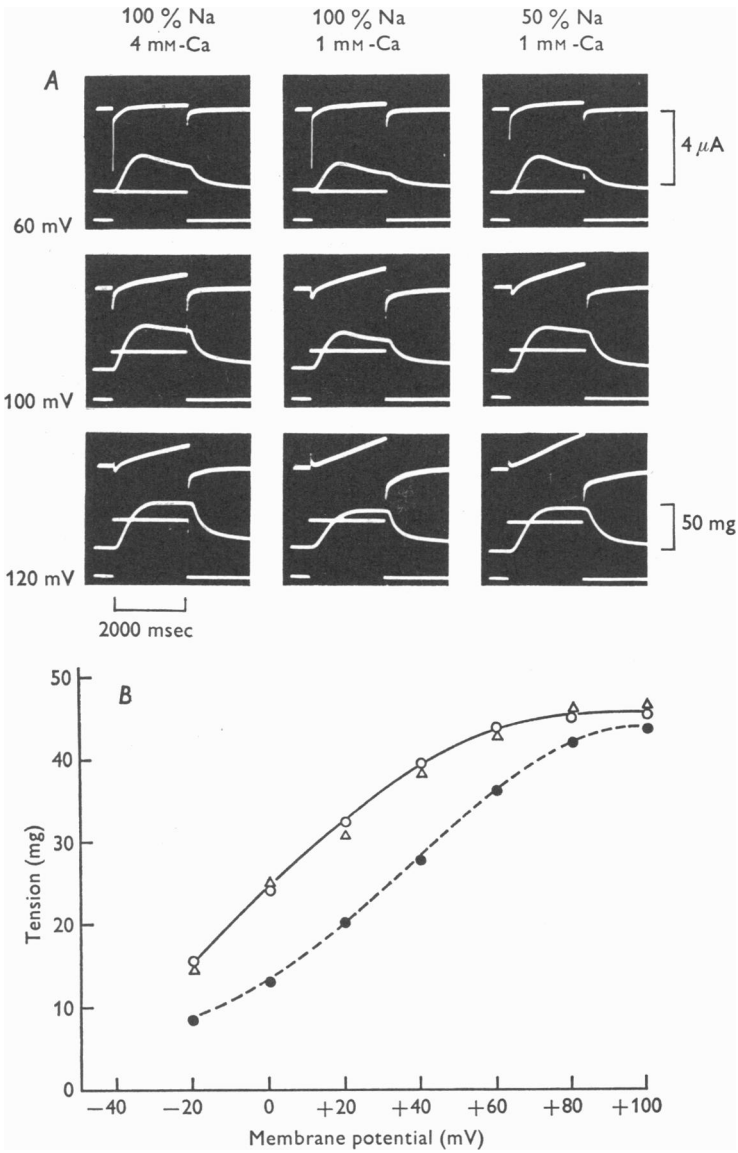

Fig. 7. Effects of changing $[Ca]_o$ and $[Na]_o$ on electrical and mechanical activity during long-lasting depolarizations ranging from 40 to 160 mV amplitude. *A*, records of membrane current and tension at 60, 100 and 120 mV clamp steps. Same arrangement as in Fig. 6 *A*. Note that there is no significant change in outward current correlating with Ca- or Na-induced changes in tonic tension. *B*, steady tension–voltage diagram for the different perfusing fluids (symbols as in Fig. 6 *B*).

partially fused but a rough estimation of I_{Ca} is possible. The magnitude of slow inward current varied with external Ca but was hardly altered by the change in external Na. In contrast, tension was distinctly affected by both ions. Upon lowering $[Ca]_o$, peak tension decreased by about 30%. This was mainly due to a decreased dP/dt but also to a slight shortening of time to peak tension. After reduction of $[Na]_o$ rising velocity and time to peak tension increased again resulting in a recovery of contraction. In Fig. 6*B* peak tension is plotted *vs.* the membrane potential during clamp. At any potential the positive inotropic effect of $[Ca]_o$ and the negative effect of $[Na]_o$ is clearly seen. At potentials between -20 and $+20$ mV, the reduction in peak tension due to the decrease in $[Ca]_o$ is almost fully compensated by lowering $[Na]_o$, i.e. peak tension is closely related to the $[Ca]_o/[Na]_o^2$ ratio. Outside this potential range the compensation is incomplete.

Fig. 7 illustrates analogous measurements on the same preparation with clamps lasting 2 sec rather than 200 msec. In Fig. 7*A* the records associated with three different levels of depolarization are shown. In such prolonged clamps a tonic tension develops which is strengthened with increasing depolarization and becomes fully activated at high positive membrane potentials. The tension attained at the break of a clamp may be taken as a measure of the steady level of tension (Einwächter *et al.* 1972). In Fig. 7*B* the tension data are summarized. The inotropic effects of external Ca and Na are evident. Following a fourfold reduction of $[Ca]_o$ steady tension decreased by 45% at $E = -20$ mV and by 8% at 80 mV, that is, the inotropic effect of Ca falls with increasing depolarization so that the curves for 1 and 4 mM-Ca-Ringer merge into a common level of maximum tension. A 50% reduction of $[Na]_o$ led to an almost complete recovery of tonic contraction at any level of depolarization, indicating a close correlation of tonic tension to the $[Ca]_o/[Na]_o^2$ ratio.

The sensitivity towards Ca, i.e. the extent to which the contractile strength varied at a given change in $[Ca]_o$, differed markedly from one preparation to another. In some preparations the positive inotropic effect of external Ca was distinctly larger than in the experiments shown in Figs. 6 and 7. One might question whether the negative effect of external Na is also enlarged in such a case. Fig. 8 illustrates the inotropic responses of a preparation with a relatively high Ca-sensitivity. Again the inotropic effect of Ca subsided at strong depolarizations. In any clamp step the depression due to the reduction of $[Ca]_o$ was fully compensated by the subsequent reduction of $[Na]_o$ so that the contracture tension was strictly determined by the $[Ca]_o/[Na]_o^2$ ratio. Thus the Ca–Na antagonism seems to be independent of the individual Ca sensitivity.

In Table 1 the mean results of eleven experiments arranged in a manner

according to Figs. 6–8 are summarized. The data indicate that (1) phasic contraction follows the $[Ca]_o/[Na]_o^2$ ratio approximately at low and moderate depolarizations and (2) tonic contraction is intimately related to the ratio at any potential.

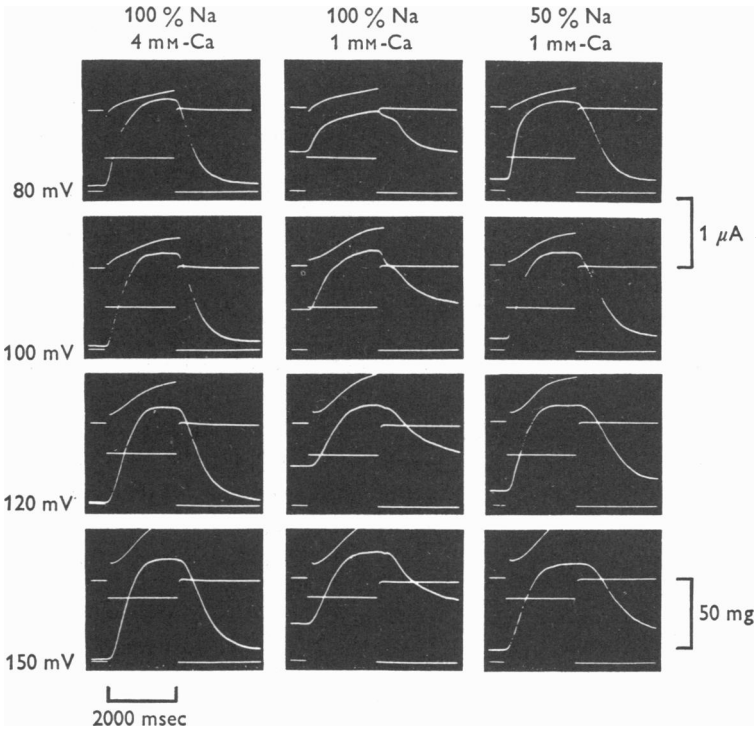

Fig. 8. Antagonistic effects of external Ca and Na on tonic tension. Voltage-clamp measurements analogous to those in Fig. 7 on a preparation which was very sensitive to changes in $[Ca]_o$. The Ca-sensitivity was previously tested by the twitch tension developed during an action potential. A shift in the base line for tension recording (middle column compared with left and right column) does not mean a change in resting tension.

Effects of more extensive alterations of $[Ca]_o$ *and* $[Na]_o$

In further voltage-clamp experiments the Ca–Na antagonism was tested by combining a sixteen- or hundred-fold reduction of $[Ca]_o$ with a four- or tenfold reduction of $[Na]_o$, respectively, starting from 4 mM-Ca-Ringer. Ca depletion induced a strong depression of both phasic and tonic contraction. Subsequent reduction in $[Na]_o$ caused the tension to increase again but, as a rule, recovery was incomplete. A typical experiment is depicted in Figs. 9 and 10. Phasic contraction generated by short-lasting depolarizations (Fig. 9) decreased to 30% or less of the control values

TABLE 1. Contractility of frog atrial muscle as affected by changes in $[Ca]_o$ and $[Na]_o$. The data are the mean values ± S.E. of the means of peak tension of phasic contractions initiated by clamps of 200 msec duration and of the steady level of tonic contraction during clamps of 2 sec duration. Reference solution was Ringer fluid containing 100% Na and 4 mM-Ca. Time of exposure to the test solutions was 7–15 min. Of eleven preparations used for the experiments with 4 and 1 mM-Ca six preparations were chosen for the experiments with 0·25 mM-Ca and five preparations for the experiments with 0·04 mM-Ca. Tension was normalized by setting maximum tonic contraction = 100 in the controls. *E*: membrane potential during clamp. The holding potential was between −60 and −80 mV

	Peak tension			Steady tension						
E (mV)	100% Na 4 mM-Ca	100% Na 1 mM-Ca	50% Na 1 mM-Ca	100% Na 4 mM-Ca	100% Na 1 mM-Ca	50% Na 1 mM-Ca	100% Na 0·25 mM-Ca	25% Na 0·25 mM-Ca	100% Na 0·04 mM-Ca	10% Na 0·04 mM-Ca
−40	18 ± 3·8	8 ± 2·8	14 ± 3·6	16 ± 4·0	5 ± 2·8	18 ± 4·0	—	12 ± 3·4	—	—
−20	36 ± 5·5	22 ± 4·0	37 ± 5·2	42 ± 7·5	10 ± 4·8	44 ± 6·8	—	33 ± 5·4	—	19 ± 4·4
0	52 ± 8·2	35 ± 5·1	53 ± 8·0	57 ± 8·3	28 ± 4·7	55 ± 8·4	12 ± 3·8	40 ± 6·8	—	29 ± 5·2
20	55 ± 7·9	38 ± 5·0	52 ± 7·8	75 ± 10·5	44 ± 6·8	73 ± 11·3	18 ± 4·5	50 ± 7·8	—	41 ± 5·4
40	49 ± 6·7	31 ± 5·4	44 ± 6·2	95 ± 9·3	68 ± 8·5	91 ± 8·8	30 ± 5·2	63 ± 9·1	9 ± 3·0	45 ± 5·8
60	33 ± 5·5	22 ± 4·3	29 ± 5·2	98 ± 10·6	86 ± 7·2	94 ± 8·5	58 ± 8·3	78 ± 8·7	13 ± 3·7	48 ± 4·9
80	14 ± 3·2	7 ± 3·0	10 ± 3·3	100	92 ± 8·5	96 ± 7·3	67 ± 8·5	82 ± 8·3	17 ± 4·3	53 ± 6·3
100	—	—	—	100	97 ± 9·1	98 ± 8·4	72 ± 9·3	87 ± 8·1	22 ± 4·7	57 ± 7·1

when $[Ca]_o$ was changed from 4 to 0·25 mM. Recovery of peak tension, after reduction of $[Na]_o$ to 25% of normal, was almost complete in the 40 mV step but became incomplete with increasing depolarization. In the Ca-poor, Na-rich solution (middle column) time to peak tension was shorter than in the two other solutions by about 25%. Of major interest are the records of membrane current because fast and slow inward current

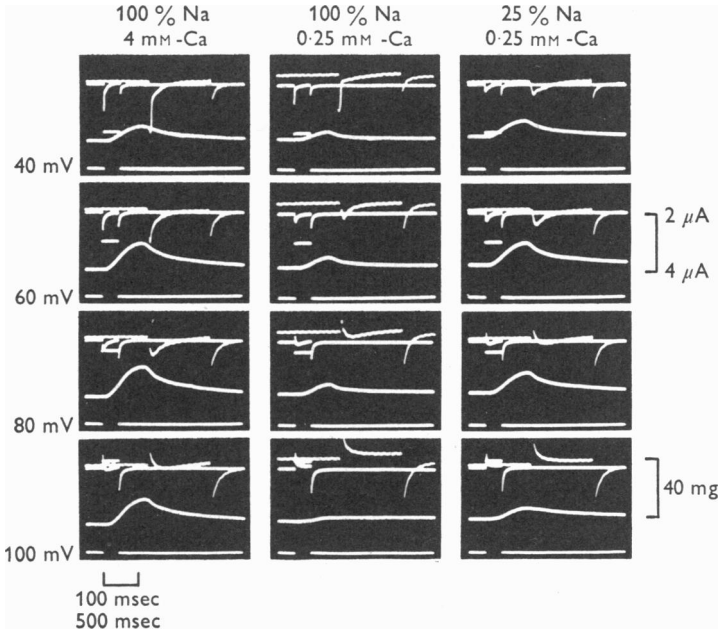

Fig. 9. Phasic contraction of frog atrial muscle as affected by Ca- and Na-depletion. Family of membrane current and tension records obtained with clamps of 200 msec duration and 40–100 mV amplitude. Holding potential = −70 mV. Time of exposure to the test solutions 6–8 min. Membrane current (top) was recorded at low and high sweep speed, tension (middle) and voltage (bottom) at low speed only. The fast records of membrane current (displayed at a doubled gain) were adjusted to show the details of inward current during depolarization. Note that the apparent rate of inactivation of slow inward current decreases with increasing depolarization (cf. Beeler & Reuter, 1970*a*).

were rather well distinguishable in this particular preparation. A closer examination of the high-speed current tracings suggests that lowering $[Ca]_o$ markedly decreased the magnitude of slow inward current at any potential while the subsequent reduction of $[Na]_o$ had little or no effect. This finding supports the view that slow inward current is mainly carried by Ca ions and is not affected much by external Na. Thus the slow inward

current seems unlikely to form the basis for the antagonistic effects of $[Ca]_o$ and $[Na]_o$ on phasic contraction.

The effects on tonic contraction during long-lasting clamps (Fig. 10) were similar to those shown in Figs. 7 and 8. Upon lowering $[Ca]_o$ tension decreased by about 80% at low depolarizations and by 55% at V = 100 mV. At strong depolarizations (not shown in Fig. 10) the % change

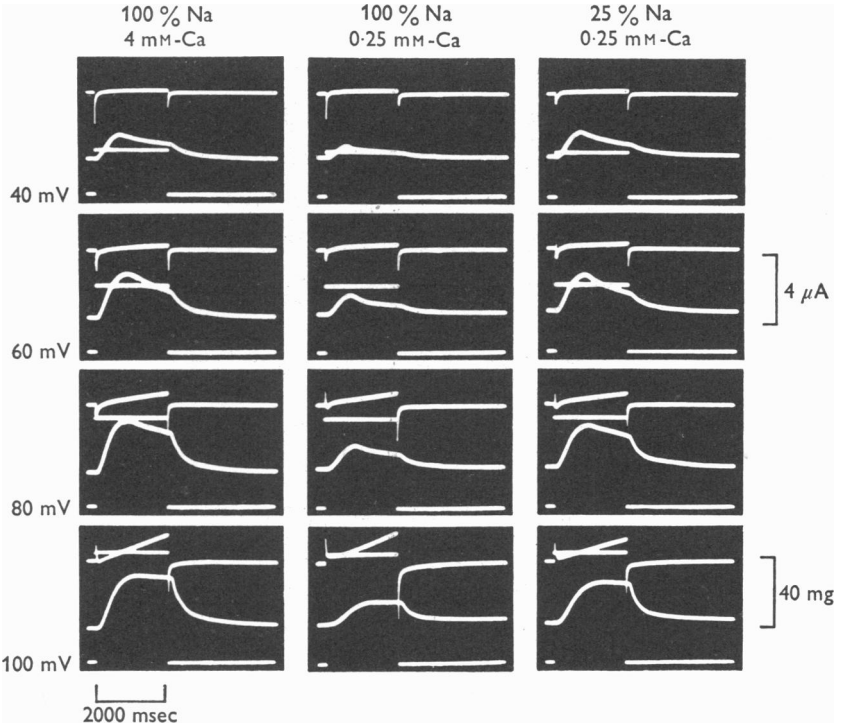

Fig. 10. Effects of Ca- and Na-depletion on tonic tension. Same preparation and same arrangement of the records as in Fig. 9.

was smaller, finally tending to zero. Upon lowering $[Na]_o$ steady tension increased again but failed to reach the control values. In contrast to the tonic contraction, slow outward current was almost unaffected by the changes in $[Ca]_o$ and $[Na]_o$.

The mean values of the inotropic effects obtained with a sixteen- or hundred-fold reduction of $[Ca]_o$ and corresponding reductions of $[Na]_o$ are listed in Table 1. Because of a large variability of the phasic responses in different preparations the compilation was restricted to the measurements of tonic tension.

Experiments with Na-free solutions

When the perfusing fluid was switched from normal Ringer to a Na-free fluid containing 2 mM-Ca (with choline chloride as substitute for NaCl) a strong contracture developed which was followed by a spontaneous relaxation. Within 2–3 min tension stabilized at about 10% of the peak value (cf. Chapman, 1974). The resting potential was not markedly shifted.

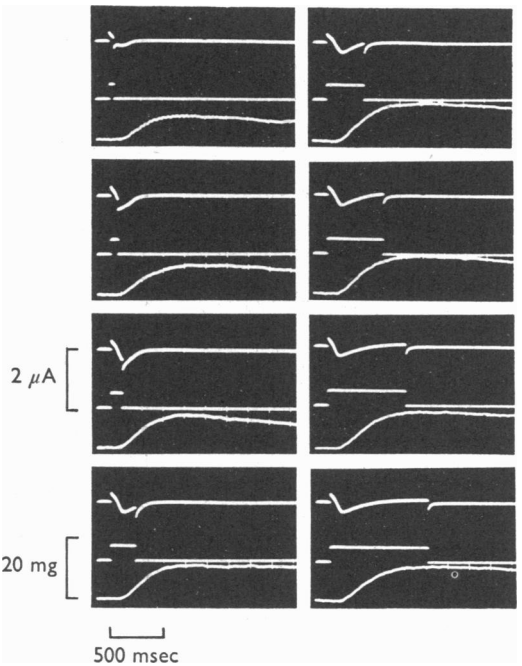

Fig. 11. Influence of time of depolarization on mechanical responses in Na-free solution. Tension development (lower beam) and membrane current (upper beam) associated with 30 mV step depolarizations of varying duration (middle beam). Holding potential = −70 mV. Clamp duration ranged from 40 to 900 msec. Time of exposure to choline-Ringer was 6–8 min.

Voltage-clamp steps were applied after contracture tension had subsided to a steady level. Upon depolarization there occurred slow inward current waves similar to those seen in normal Ringer. However, the contractile responses which superimposed on the contracture residue (Figs. 11 and 12) differed from normal in several respects. (1) The rising time of tension was prolonged and relaxation was drastically slowed down in choline-Ringer. (2) The minimum time of depolarization required to elicit noticeable

tension was significantly reduced. Clamps of not more than 20 msec in duration were sufficient to initiate tension compared with 40–70 msec in normal Ringer. (3) At a given depolarization maximum tension was reached when the clamp duration equalled the apparent full period of slow inward current. Further prolongation of the clamp failed to affect tension. (4) Regardless of clamp duration the potential dependency of tension was that of a phasic contraction as observed in normal Ringer: Tension was

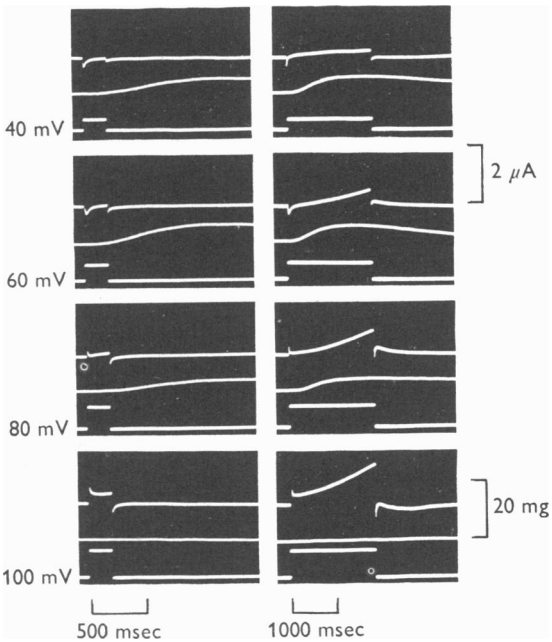

Fig. 12. Influence of membrane potential on contractile responses in Na-free solution. Membrane current (upper beam) and tension (middle beam) associated with depolarizing clamps of varying amplitude (lower beam). Holding potential = −60 mV. Clamp duration was 200 msec (left column) and 2 sec (right column). Time of exposure to choline-Ringer was 7–10 min.

maximal for depolarizations to a level near zero potential and decreased at strong depolarizations.

The above results are in qualitative agreement with those reported by Vassort (1973) using LiCl as substitute for NaCl.

DISCUSSION

Under ordinary conditions, the contractile status of muscle fibre is mainly controlled by the level of free Ca in the myoplasm, $[Ca]_i$, which determines the quantity of Ca bound to the myofilaments (Hellam &

Podolsky, 1969; Katz, 1970; Winegrad, 1971). Thus the steady tension may be considered as a function of $[Ca]_i$. On the other hand, the present voltage-clamp data suggest that, at a given membrane potential E and with moderate changes in $[Ca]_o$ and $[Na]_o$, steady tension is determined by the $[Ca]_o/[Na]_o^2$ ratio. Combining these two statements leads to the idea of $[Ca]_i$ as a function of E and $[Ca]_o/[Na]_o^2$. In the following an attempt is made to explain this relation on the basis of transmembrane Ca–Na countertransport.

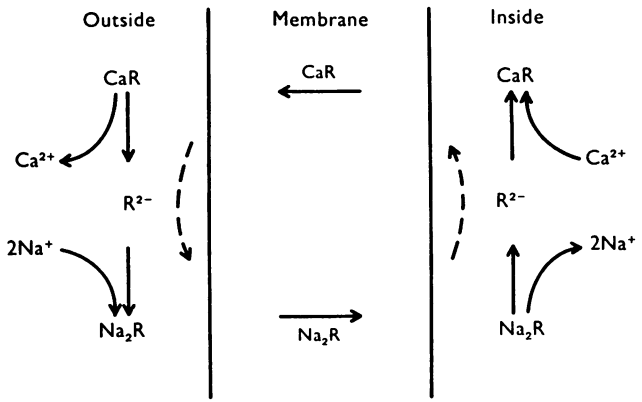

Fig. 13. Schematic diagram of a transmembrane Ca–Na exchange mechanism. In a steady state the flows of CaR and Na_2R are equal and opposite, resulting in a fixed exchange ratio of 1 Ca:2 Na. Thus no net charge is transported whatever the electric charge of the carrier molecule. In order for a Ca outward transport to occur an input of energy is required, e.g. by coupling of exergonic reactions (interrupted arrows) to carrier–substrate reactions.

Model of a Ca–Na exchange mechanism. The model used is similar to the concepts proposed by Lüttgau & Niedergerke (1958) and by Reuter and his colleagues (Reuter & Seitz, 1968; Glitsch *et al.* 1970; Jundt, Porzig, Reuter & Stucki, 1975). It is assumed that the cardiac cell membrane contains a mobile carrier (or some thermodynamically equivalent mechanism) which combines reversibly with one Ca ion and in competition with two Na ions on either side of the membrane. The loaded carrier (CaR or Na_2R) is able to cross the membrane by diffusion while the free carrier (R) cannot pass the membrane. Substrate–carrier reactions and carrier diffusion are interconnected in a cyclic pathway (Fig. 13). The transport capacity (maximal transfer rate) of the system is thought to be high relative to any other mechanism involved in Ca entry into, or sequestration of Ca from, the myoplasm. This assumption means that the system

operates close to equilibrium. Thus the chemical reactions can be written

$$\frac{[Ca]_o[R]_o}{[CaR]_o} = K_1; \qquad \frac{[Na]_o^2[R]_o}{[Na_2R]_o} = K_2;$$
$$\frac{[Ca]_i[R]_i}{[CaR]_i} = K_3; \qquad \frac{[Na]_i^2[R]_i}{[Na_2R]_i} = K_4; \tag{1}$$

where the subscripts i and o refer to the two sides of the membrane and the K are the respective Michaelis constants. Elimination of $[R]_o$ and $[R]_i$ leads to

$$\frac{[CaR]_o}{[Na_2R]_o} = \frac{[Ca]_o K_2}{[Na]_o^2 K_1} \quad ; \quad \frac{[CaR]_i}{[Na_2R]_i} = \frac{[Ca]_i K_4}{[Na]_i^2 K_3}. \tag{2}$$

The equilibrium distribution of carrier within the membrane is determined by Boltzmann's principle

$$\frac{[CaR]_o}{[CaR]_i} = \frac{[Na_2R]_o}{[Na_2R]_i} = \exp\left\{\frac{zFE}{RT}\right\}, \tag{3}$$

where z is the valency of the loaded carrier. For the sake of simplicity z may be taken as zero so that

$$\frac{[CaR]_o}{[CaR]_i} = \frac{[Na_2R]_o}{[Na_2R]_i} = 1. \tag{3a}$$

In any case the left sides of the two eqns. (2) are numerically equal and by combining the terms we obtain

$$\frac{[Ca]_o[Na]_i^2}{[Na]_o^2[Ca]_i} = \frac{K_1 K_4}{K_2 K_3}. \tag{4}$$

The term K_1K_4/K_2K_3 may be called the equilibrium constant K_{eq} of the system. Eqn. (4) says that the level of internal Ca established by the Ca–Na exchange mechanism is linearly related to the $[Ca]_o/[Na]_o^2$ ratio provided that $[Na]_i$ is kept constant by some other mechanism, e.g. the Na–K exchange pump. It should be noted that eqn. (4) is independent of any assumption as to the electric charge of the carrier.

The numerical value of the equilibrium constant K_{eq} can be estimated from the actual Ca and Na concentrations. In resting fibres of frog atrial tissue $[Na]_i$ is about 22 mM/l. fibre water (Haas, Glitsch & Kern, 1966) and $[Ca]_i$ may be taken as 10^{-4} mM (Katz, 1970; Winegrad, 1971). With $[Na]_o = 111$ and $[Ca]_o = 2$ mM the ratio $[Ca]_o[Na]_i^2/[Na]_o^2[Ca]_i$ becomes

$$2 \times (22)^2/(111)^2 \times 10^{-4} \sim 800, \quad \text{that is,} \quad K_{eq} \gg 1.$$

This means a strong asymmetry of the Michaelis constants governing the chemical reactions on both sides of the membrane: $K_4/K_3 \gg K_2/K_1$. Since the Michaelis constants are an inverse measure of the respective affinities it is

concluded that the affinity of the carrier for Ca relative to that for Na is much higher at the inner face than at the outer face of the membrane.

The magnitude of K_{eq} is intimately related to the energetics of the Ca–Na exchange system. As was suggested by Reuter & Seitz (1968) the downhill (inward) movement of Na could provide part, or perhaps all, of the energy required for the uphill (outward) transport of Ca. In our model the free energy change associated with reversible extrusion of 1 mole Ca^{2+} is

$$\Delta F = RT \ln [Ca]_o/[Ca]_i + 2RT \ln [Na]_i/[Na]_o = RT \ln K_{eq}. \quad (5)$$

With the Ca and Na concentrations of frog atrial muscle stated above the energetic terms are

$$RT \ln [Ca]_o/[Ca]_i = 5{\cdot}9 \quad \text{and} \quad 2RT \ln [Na]_i/[Na]_o = -1{\cdot}9 \text{ kcal},$$

that is, the energy derived from Na inward transfer is much too small to account for Ca outward transport. Thus an input of energy from external sources seems indispensable. An energy requirement of about 4 kcal per cycle could be covered, e.g. by hydrolysis of 1 mole ATP at an ATP:ADP ratio in the order of 1:10. Theoretically, an energy input could be coupled to the formation of CaR at the inner face or the break-down of CaR at the outer face of the membrane.

The idea of an ATP-dependent Ca–Na exchange system is supported by the observations that Na-dependent extrusion of Ca from squid giant axons is influenced by the metabolic state of the cell (Baker, 1972; Baker & Glitsch, 1973) and Ca efflux from dialysed squid axons is enhanced by an addition of ATP to the perfusing fluid (DiPolo, 1974; Mullins & Brinley, 1975). This effect was interpreted as being due to an alteration in the affinity of the Ca transport system for external cations induced by ATP.

Tonic contraction and Ca–Na exchange. The exact nature of initiation of tonic contraction in cardiac muscle is unknown. A simple transmembrane inflow of Ca ions upon depolarization seems unlikely since tonic tension is maximal at membrane potentials positive to the Ca equilibrium potential, E_{Ca}. According to Morad & Goldman (1973) an influx of Ca ions into the myoplasm might be coupled to an efflux of K ions across the membrane. The present data do not favour this hypothesis since marked changes in tonic tension induced by alterations of $[Ca]_o$ or $[Na]_o$ were not accompanied by corresponding changes in slow outward current (cf. Fig. 10).

Whatever the nature of excitation–contraction coupling, the maintenance of a steady tension at a given depolarization suggests a balance between inflow of Ca ions into, and extrusion out of, the myoplasm at an increased level of $[Ca]_i$. A rise of $[Ca]_i$ could be related to an augmented steady inflow of Ca, a decreased pumping activity, or both. The first explanation would imply that the Ca pump (represented by the Ca–Na

exchange mechanism) is unaffected in its physico-chemical parameters but is no longer at a quasi-equilibrium; the second one, that the pump is still operating near equilibrium but the equilibrium itself is shifted. The latter hypothesis is preferred since it results in simple stoichiometric relations similar to those underlying the Ca–Na antagonism. Thus the following assumptions are made.

(1) Upon depolarization the state of the Ca–Na exchange mechanism is altered in a fashion that the affinity ratios K_2/K_1 and K_4/K_3 approach each other and the equilibrium constant K_{eq} decreases; that is, the chemical asymmetry of the system is diminished and its depressing effect on $[Ca]_i$ is attenuated. This assumption seems consistent with the sensitivity of Ca efflux to membrane potential observed by Mullins & Brinley (1975) on dialysed squid axons.

(2) Regardless of a change of the Michaelis constants, the maximal transfer rate is thought to be large compared with the actual rate of Ca transport.

From this viewpoint the question as to the molecular mechanism of transmembrane Ca inflow would seem to be of minor interest. Possibly a specific mechanism responsible for a steady inflow of Ca does not exist at all since Ca inward movement might be brought about by the Ca–Na exchange mechanism itself. If the equilibrium constant of the system were to undergo a rapid decrease in response to depolarization the system would produce an *inward* transfer of Ca persisting until the new equilibrium between external and internal Ca is reached.

Clearly the postulation of a quasi-equilibrium of the Ca–Na exchange mechanism during depolarization implies that external and internal Ca and Na concentrations are still interconnected by eqn. (4), with an appropriate change in the numerical value of K_{eq} compared with the resting state. Generally speaking, K_{eq} is considered as a function of membrane potential which has a value $\gg 1$ at the resting level and declines with depolarization. These assumptions allow a simple interpretation of the antagonistic effects of $[Ca]_o$ and $[Na]_o$ on tonic tension. With moderate changes of $[Na]_o$ from normal, the internal Na level as controlled by the Na pump is expected to remain approximately constant. The steady level of $[Ca]_i$, then, will be proportional to $[Ca]_o$ and inversely related to $[Na]_o^2$, the scale factor being $[Na]_i^2/K_{eq}$. Thus the contractile force is expected to follow the $[Ca]_o/[Na]_o^2$ ratio quantitatively at any potential. This is consistent with the complete recovery of tonic tension observed after a fourfold reduction of $[Ca]_o$ and a 50% reduction of $[Na]_o$. With more extensive changes in $[Na]_o$, the internal Na level is probably no longer constant. Thus the term $[Na]_i^2/K_{eq}$ will decrease upon a strong reduction of $[Na]_o$ even when K_{eq} is unchanged. This would explain our observation

that the negative inotropic effect due to a sixteen- or hundredfold reduction of $[Ca]_o$ was not fully compensated by a four- or tenfold reduction in $[Na]_o$.

The problem as to how a depolarization could reduce the magnitude of K_{eq} may be considered from an energetical viewpoint. Eqn. (5) suggests that an input of energy causes K_{eq} to exceed unity. A decrease of energy input will then lead to a decreased value of K_{eq}. At present no direct evidence is available for such an effect. An attractive hypothesis is that the ATP potential might decrease due to a change in the ATP:ADP ratio during depolarization.

Phasic contraction and Ca–Na exchange. The present experiments indicate that the slow inward current which initiates the phasic contraction is responsive to changes in $[Ca]_o$ but rather insensitive to changes in $[Na]_o$ while the contraction itself depends on both $[Ca]_o$ and $[Na]_o$. In general, peak tension does not strictly follow the $[Ca]_o/[Na]_o^2$ ratio: a decrease in tension caused by a r-fold reduction of $[Ca]_o$ is only partially compensated by a $\sqrt{r}$-fold decrease in $[Na]_o$.

The positive effect of external Ca on phasic contraction is likely to result from a combined action of several factors. One factor is the slow inward current. The higher the external Ca level is, the larger the Ca inward transfer at a given depolarization. The effect of an augmented Ca influx on tension development is strengthened by an increase of the resting level of internal Ca as is expected to occur upon a rise of $[Ca]_o$. A further factor to be considered is the rate of Ca extrusion from the fibre. The observation that time to peak tension decreases with decreasing $[Ca]_o$ suggests that Ca extrusion is slower in a Ca-rich solution than in a Ca-poor solution. Clearly, a slowed Ca extrusion will result in a higher peak value of $[Ca]_i$. An inverse relation between $[Ca]_o$ and Ca extrusion is easily understood in terms of our model of a Ca–Na exchange mechanism. Accumulation of internal Ca due to a slow inward current wave means a disturbance of the quasi-equilibrium state established at rest. After repolarization the Ca pump tends to restore the low resting level of $[Ca]_i$. The first step is the formation of Ca-loaded carrier at the inner face of the membrane in excess of the resting state. Assuming that carrier diffusion is the rate-limiting step in the cyclic carrier mechanism and using the shorthand notation

$$Ca + Na_2R \rightarrow CaR + 2Na \tag{6}$$

for the formation of the Ca complex, the rate of Ca extrusion from the myoplasm is given by

$$d[Ca]_i/dt = -k[Ca]_i[Na_2R]_i + k'[CaR]_i[Na]_i^2, \tag{7}$$

where k and k' are the respective rate constants for the forward and the

reverse reaction (with $k'/k = K_3/K_4$). At a given level of $[Na]_1$, the rate of the net reaction will be increased by an increase of $[Na_2R]_1$ or a decrease of $[CaR]_1$. The initial values of $[Na_2R]_1$ and $[CaR]_1$, however, are thought to be almost equal to $[Na_2R]_0$ and $[CaR]_0$, respectively. With increasing external Ca concentration, $[CaR]_0$ will rise while $[Na_2R]_0$ is expected to fall. Thus an increase in $[Ca]_0$ would result in a delayed extrusion of Ca from the fibre.

The negative inotropic effect of external Na can be given an analogous interpretation. Like an increase of $[Ca]_0$, a reduction of $[Na]_0$ will induce a rise of the resting level of internal Ca and a depression of the Ca pumping rate compared with normal (eqn. (7)). Assuming that a given quantity of Ca ions enters the fibre during depolarization, a retardation of Ca extrusion will result in an increased dP/dt as well as a prolongation of time to peak tension. This would account for the observation that a reduction of $[Na]_0$ causes peak tension to increase although the slow inward current which initiates phasic tension does not undergo any marked change (Figs. 5, 6, 9).

Theoretically, the rate of Ca extrusion by the Ca–Na exchange mechanism will be unchanged if both $[Ca]_0$ and $[Na]_0$ are varied in a fashion that the $[Ca]_0/[Na]_0^2$ ratio remains constant. Accordingly, a close correlation between peak tension and the $[Ca]_0/[Na]_0^2$ ratio would be expected. However, this simple concept is complicated by the behaviour of slow inward current. Since this current contributes to the positive effect of Ca on phasic contraction but not to the negative effect of Na, an 'imperfect' Ca–Na antagonism is awaited, that is, the negative effect of external Na on phasic tension is smaller than predicted by the $[Ca]_0/[Na]_0^2$ ratio. With strong reductions of $[Na]_0$, the concomitant decrease in $[Na]_1$ will further attentuate the relation of phasic tension to the $[Ca]_0/[Na]_0^2$ ratio.

Contraction in a Na-free medium. After removal of external Na, development of reversible contractures without any marked depolarization has been observed in frog ventricular and atrial preparations (Lüttgau & Niedergerke, 1958; Lamb & McGuigan, 1966; Vassort, 1973; Chapman, 1974), guinea-pig auricles and atrial trabeculae of calf hearts (Scholz, 1969*a*, *b*) and ventricular strips of the goldfish heart (Busselen & Carmeliet, 1973). The simplest interpretation of a Na-lack contracture is that transmembrane exchange of internal Ca for external Na is inhibited in a Na-free medium and the control of the internal Ca level is left to some other mechanism which, under normal conditions, is inferior to the pumping mechanism of the sarcolemma. In this connexion the mitochondria would seem to be of particular importance (Baker, 1972; Winegrad, 1973; Lehninger, 1974).

According to Lüttgau & Niedergerke (1958) and Scholz (1969*a*) a

Na-lack contracture is hardly affected by elevation of the external K concentration – that is, the strength of contracture does not increase with depolarization. The same feature seems to apply to the present voltage clamp experiments with choline-Ringer. In these experiments the small residual tension of the resting preparation which persisted after spontaneous relaxation of the initial contracture was taken as base line for tension recording. An analysis of the data suggests that no further tonic tension was induced by depolarization. Rather the sustained contractions evoked by depolarizing clamps are understood as phasic contractions with a very slow relaxation.

At first sight the apparent absence of a tonic response in depolarized fibres exposed to Na-free Ringer seems to conflict with the positive inotropic stimulus provided by a moderate reduction of $[Na]_o$ from normal. This phenomenon sheds light on the nature of the mechanism underlying the generation of tonic tension. If initiation of a tonic contraction were based on some mechanism independent of external Na, e.g. a release of Ca from internal stores directly controlled by the membrane potential or a transmembrane Ca–K exchange as proposed by Morad & Goldman (1973), large tonic contractions should be expected even in a Na-free medium. Our results do not favour such a hypothesis. According to Vassort (1973), development of tonic tension might be correlated with a competition of Ca and Na for some intracellular binding sites the affinity of which varies with the membrane potential. With normal values of internal Na, a depolarization would result in a displacement of Ca by Na ions and an increase of $[Ca]_i$. In the absence of external Na, the internal Na level is thought to undergo a substantial fall so that a release of Ca ions during depolarization is inhibited. A related but simpler interpretation is the following.

As was mentioned previously, initiation of a tonic contraction might be explained by alteration of the Ca–Na exchange mechanism, namely, a decrease of the equilibrium constant $K_{eq} = K_1K_4/K_2K_3$ during depolarization resulting in a transient carrier-mediated Ca influx. In detail, Ca influx could be related to a rise of $[CaR]_o$ following a decrease of K_1/K_2 and/or a fall of $[CaR]_i$ due to an increase of K_3/K_4. In Na-free solutions, $[CaR]_o$ will be maximal irrespective of the numerical value of K_1/K_2. It can be visualized that, with a strong reduction of $[Na]_i$, $[CaR]_i$ is also maximal and is not reduced by a decrease in K_3/K_4. In this case a depolarization will fail to induce an inward transfer of Ca.

In conclusion, it can be said that a simple model of a transmembrane exchange of one Ca ion for two Na ions does largely account for the inotropic effects of external Ca and Na on frog atrial fibres, in particular, the close correlation between tonic tension and the $[Ca]_o/[Na]_o^2$ ratio. The

assumption that $[Ca]_i$ is controlled by a mechanism located in the surface membrane seems consistent with the rather simple ultrastructure of frog heart cells (Sommer & Johnson, 1969; Winegrad, 1971). It seems conceivable that a similar mechanism of Ca–Na exchange is inherent in all cardiac structures. However, the functional significance of Ca–Na exchange might be less in tissues of a more complex ultrastructure. In mammalian heart fibres with a well-developed T-tubular and sarcotubular system, a transmembrane Ca–Na exchange may be inferior to intracellular processes of Ca storage and release. This could explain why Ca–Na antagonism is more pronounced in the frog heart than in mammalian myocardium.

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