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THE IONIC DEPENDENCE OF THE STRENGTH AND SPONTANEOUS RELAXATION OF THE POTASSIUM CONTRACTURE INDUCED IN THE HEART OF THE FROG *RANA PIPIENS*

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SUMMARY

1. The tension generated by isolated frog atrial trabecules, during exposure to solutions containing a high potassium concentration, is not maintained but spontaneously relaxes. The final part of this relaxation can be fitted by a single exponential function.

2. The recovery of the tension generating mechanisms following the spontaneous relaxation of a potassium contracture depends on the preceding membrane potential and the time since the last contracture.

3. The rate of the exponential phase of the spontaneous relaxation is independent of the $[K]_o$ and hence the membrane potential, the $[Ca]_o$; and when the $[Ca]_o/[Na]_o^2$ ratio is maintained it is also independent of the $[Na]_o$. This relaxation is not influenced by atropine or pronethalol.

4. When sodium is totally excluded from the bathing medium the rate of relaxation of a later potassium contracture is much increased. It is argued that this change is due to a fall in the intracellular sodium concentration.

5. The consequences of these results are discussed, and the hypothesis that is favoured would require that contraction is induced by a transient release of calcium into the sarcoplasm, probably triggered by a potential dependent, and probably also transient, influx of calcium through the cell membrane. Relaxation is supposed to occur when this activator-calcium is then removed by an intracellular relaxing system that resembles the sarcoplasmic reticulum of other muscles. What this intracellular structure might be, is also discussed.

INTRODUCTION

The method of Hodgkin & Horowicz (1959) has already been adapted for use with isolated frog auricular trabecules, primarily to investigate the influence of $[Ca]_o$ upon the contraction of this tissue (Chapman & Tunstall, 1969, 1971; Chapman, 1971*a*). It was found that during exposure to fluids with elevated potassium concentrations trabecules develop tension rapidly. This tension then subsides slowly in a more or less exponential fashion, similar to the spontaneous relaxation of tension described for skeletal twitch fibres under similar conditions, and which led Hodgkin & Horowicz (1960) to suggest that an intracellular relaxing system should exist within these muscles. This proposal has, in fact, become subsequently well established by a variety of experiments, particularly those on muscle subcellular fragments. In cardiac muscle, where it has been proposed that much of the calcium that activates contraction enters from the bathing medium during depolarization of the sarcolemma, the role of an intracellular relaxing system has been disputed, particularly in amphibian heart where the possibility of its existence has often been totally ignored. The contracture induced by perfusion with potassium-rich Ringer and more particularly its spontaneous relaxation has been studied in this and the following paper, and the results suggest that there is, within the frog cardiac muscle cells, an energy dependent process that reduces the concentration of activator calcium in a similar way to that found in skeletal muscle fibres, although there are some interesting differences.

METHODS

The method of isolating and mounting the auricular trabecules, and the means used to record the tension they generate and the membrane potentials, has been described in detail elsewhere (Chapman & Tunstall, 1971; Chapman, 1971*a*). The muscle was stimulated electrically through platinum plates on each side of the experimental channel at 4 min^{-1} except during contractures. The experimental procedure was as described in these papers. To perform the present experiments the composition of the Ringer solutions has been varied and is shown in Table 1. Calcium was added to these solutions as a 1 M-$CaCl_2$ solution. The potassium concentration of the contracture fluids was varied by mixing solutions D and E together so as to maintain the tonicity of these fluids (although they were all hypertonic to frog serum). The sodium concentration was varied by mixing, either solutions B and F for solutions containing the normal [K], or by mixing of solutions G and D to achieve variation of the [Na] at a potassium concentration of 100 mM. The pH was maintained at 7·3, and as a general rule the buffer was not changed during an experiment. Atropine, applied as atropine sulphate, and the pronethalol, a gift from I.C.I., were added from 100 mM stock solutions. Ethyleneglycol-bis-(β-amino-ethyl ether) N,N′-tetra acetic acid (EGTA) was added as 100 mM solution brought to pH 7·3 by addition of Tris (hydroxymethyl) methylamine (Tris).

The time constants of the exponential changes in tension have been obtained by

linear regression analysis of the logarithm of the tension minus any final steady tension level, against the time since the experimental solution was altered, in much the same way as reported previously (Chapman, 1971*a*). The vast majority of the results showed high coefficients of correlation between the regression line and the experimental values and estimates of the standard error of the line rarely exceeded the size of the points used in the illustrations.

The force generated by the trabecule has been expressed as a function of the muscle wet weight, rather than the cross-sectional area because the latter is difficult to estimate in these preparations. The muscle wet weight was between 0·02 and 0·06 mg.

The experiments were performed in a cooled room which was maintained at a temperature of between 15 and 20° C with a variation of temperature during an individual experiment of less than one degree. The muscle was taken from the hearts of large healthy frogs, of the species *Rana pipiens*, first killed by pithing the central nervous system.

TABLE 1. Composition of experimental fluids (mM)

Soln.	Type	NaCl	KCl	Tris HCl	Na_2HPO_4	NaH_2PO_4	Glucose
A	Phosphate buffered Ringer	114·5	3·0	—	0·8	0·2	5·0
B	Tris HCl buffered Ringer	114·5	3·0	2·0	—	—	5·0
C	High-K phosphate buffered, hypertonic Ringer	114·5	100·0	—	0·8	0·2	5·0
D	High-K Tris HCl buffered hypertonic Ringer	114·5	100·0	2·0	—	—	5·0
E	K-free Tris HCl buffered hypertonic Ringer	114·5	—	113·9	—	—	5·0
F	Zero Na Ringer	—	3·0	129·2	—	—	5·0
G	Zero Na high-K hypertonic Ringer	—	100·0	129·2	—	—	5·0

RESULTS

The form of the potassium contracture. All preparations, when exposed to solutions containing an elevated potassium concentration and sufficient calcium, develop tension which reaches a peak after 1–5 sec and then declines slowly for about 3 min to a lower tension which is maintained indefinitely (Fig. 1*A*). In a few preparations (two in ninety-four) a second development of tension or a further onset of relaxation occurs after several minutes in the contracture medium (Fig. 1*B*, *C*). In almost all of the preparations studied, however, the tension level finally achieved during the potassium contracture was at or close to the resting tension of the muscle before the contracture had been evoked, i.e. the relaxation was more or less complete.

In one preparation taken from an apparently moribund frog, the muscle contracted when exposed to the potassium-rich contracture fluid (high potassium) but the subsequent spontaneous relaxation of tension was only slight. Further relaxation was induced only when the original potassium concentration was established. Perfusion of this preparation with high-potassium contracture fluid containing 5 mM

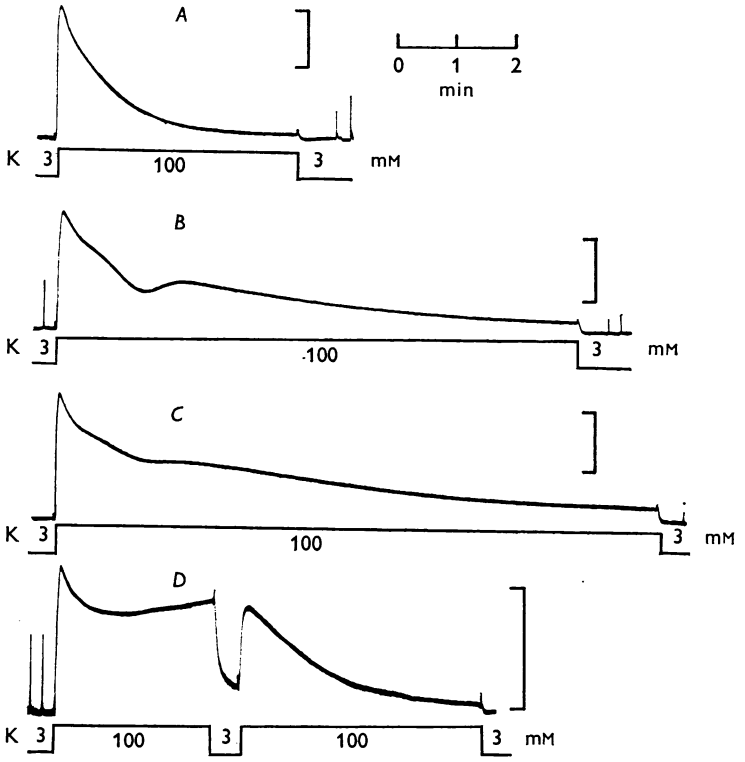

Fig. 1. The variety of the spontaneous relaxation of the potassium contractures encountered. In each record the upper trace shows the tension developed by the muscle and the lower one the timing and extent of the change in the $[K]_o$. This method of showing the changes in the constituents of the bathing solutions has been adopted in all the experimental records shown in the Figures. *A*, a typical potassium contracture with almost complete relaxation of the tension developed in the depolarizing solution, 15° C. *B*, the contracture shows a second transient development of tension after an initial phase of relaxation, 17° C. *C*, a contracture in which the relaxation is complicated by a second phase which appears after a delay, 17° C. *D*, the first potassium contracture initiated by a fluid lacking glucose shows incomplete spontaneous relaxation. The second contracture initiated by a high potassium solution, including 5 mM glucose, shows a near complete spontaneous relaxation of tension, 20° C. This preparation was taken from a frog that showed symptoms of recent death. Each experimental solution contained 1 mM calcium. The vertical bars represent a force of 10^3 N.kg^{-1}.

glucose yielded a contracture which showed an almost complete relaxation of tension in the contracture solution (Fig. 1*D*). For this reason 5 mM glucose was included in all the solutions used in the present study.

When the spontaneous relaxation of tension that occurs during a potassium contracture is analysed by plotting the logarithm of the tension, or the tension minus any final steady tension that remains, against the time in the contracture promoting fluid, then up to three sequential phases of relaxation can be recognized. These are (*a*) an initial, rapid and short-lasting fall in tension, (*b*) a second phase in which the tension falls relatively slowly (this 'plateau phase' was typical of very strong near maximal contractures and was often absent in weaker contractures), and (*c*) a final rapid phase (see Fig. 6). In all preparations showing a nearly complete relaxation of the potassium contracture tension the final phase can be fitted by a single exponential function. Because it was thought that the earlier phases might be dependent on the change of tonicity that occurs when the potassium-rich medium is applied to the muscle, and because it is likely that part of the process that leads to the activation of contraction still remains during this time, this latter phase of relaxation has received the most attention in the present work. The time constant of the exponential phase of spontaneous relaxation was $37{\cdot}8 \pm$ S.D. 15 sec at temperatures between 18 and 20° C. This value is therefore much larger than the equivalent relaxation of skeletal muscle fibres which appear to have a time constant of between 1 and 3 sec at room temperature (Hodgkin & Horowicz, 1960; Caputo, 1972).

The rate of the late exponential phase of the spontaneous relaxation did not change as the heart became hypodynamic (as recognized by the fall in amplitude of the twitch and contracture responses, cf. Chapman & Niedergerke, 1970*a*; Chapman & Tunstall, 1971), and was remarkably consistent throughout an experiment performed without variation of the temperature. The typical time constant of this exponential phase decreased from around 40 sec to the region of 15 sec during the period of this study. Although there was quite a wide variation from one experiment to the next this trend was quite distinct. The reason for this change was not seasonal because the majority of the experiments were performed at the same time of the year. It is possible that the increase of skill in dissection etc. that accrued over the years produced preparations that were less damaged and therefore relaxed more rapidly. Preparations that were purposely damaged by stretching or crushing gave contractures, but rarely showed spontaneous relaxation. The origin of this trend, therefore, remains obscure, perhaps it was due to some difference in the batches of the frogs, possibly they were obtained from different populations by the suppliers.

The redevelopment of tension following the spontaneous relaxation of the potassium contracture. The addition of millimolar quantities of caffeine, or an increase in the $[Ca]_o$, or a lowering of the $[Na]_o$ result in the redevelopment of tension, which is in each case still transient like the preceding potassium contracture (Fig. 2). This suggests that the tension

generating system of the muscle is not fatigued, and that fatigue is not therefore responsible for the spontaneous relaxation of the high-potassium contracture. If, following the spontaneous relaxation, $[K]_o$ is raised still further, a second large contraction is induced only if the initial contraction is small (i.e. the initial increase in the potassium concentration was also small) suggesting that there may be an inactivation of the contractile response which depends on the potential of the muscle membrane, similar to the phenomenon reported for skeletal muscle fibres and for mammalian heart (Hodgkin & Horowicz, 1960; Beeler & Reuter, 1970*b*).

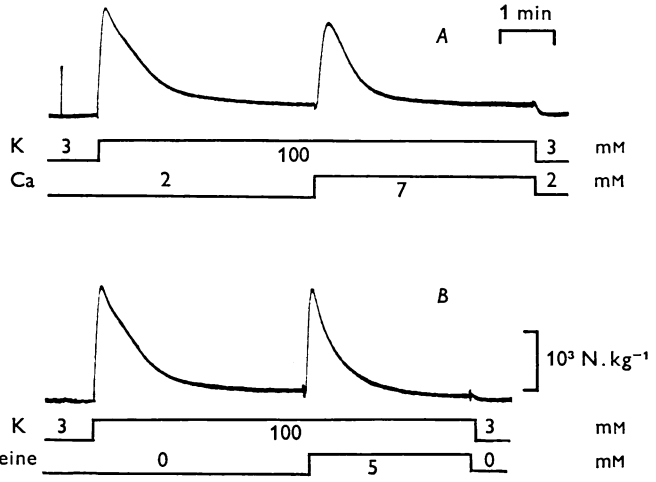

Fig. 2. Following the spontaneous relaxation of the tension induced by a 100 mM potassium contracture fluid containing 2 mM calcium, a second contracture can be evoked if *A*, the $[Ca]_o$ is raised by 5 mM, or *B*, if 5 mM caffeine is added to the contracture solution which already contained 2 mM calcium, 20° C.

The contracture developed by frog auricular muscle in depolarizing solutions resembles that found in skeletal twitch muscle fibres, by showing an almost complete relaxation of tension in these solutions. However, certain differences are apparent: the rate of the exponential phase of spontaneous relaxation is at least 10 times slower than skeletal muscle; the response to caffeine following the relaxation is transient unlike skeletal muscle (Lüttgau & Oetliker, 1968); and raising the $[Ca]_o$ yields a second contracture, which emphasizes the importance of calcium ions in the bathing medium in controlling the strength of cardiac contraction.

Possible influence of substances released from the preparation by high-potassium solutions. It is conceivable that catecholamines and/or acetyl-

choline released by application of potassium chloride to heart tissue could, if they accumulated in sufficient quantities, influence contraction and relaxation. Evidence of effects of this type have been obtained in mammalian papillary muscle (Morad & Rolett, 1972). It is possible to calculate the maximum average concentration that would be established within a tissue if the amount and rate of release of the substance and its rate of loss from the tissue is known, using an equation similar to that

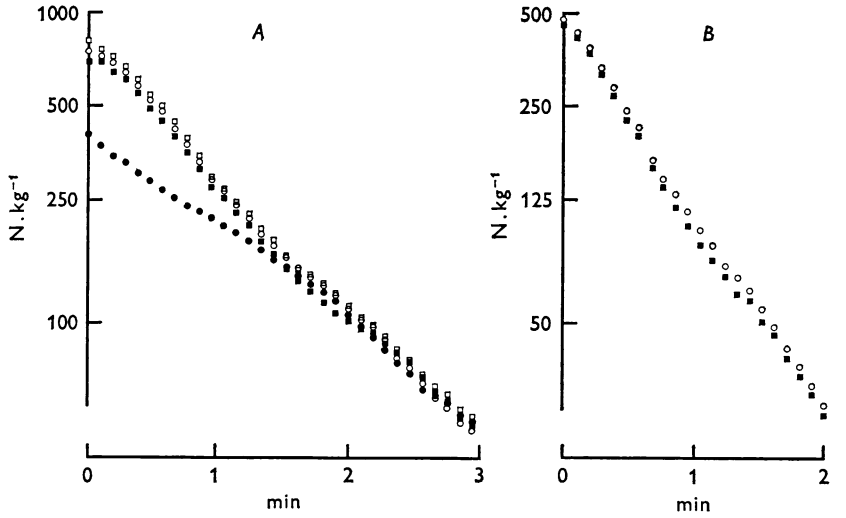

Fig. 3. The effects of pronethalol, atropine and adrenaline on the spontaneous relaxation of the potassium contracture are shown by semi-logarithmic plots of the decline of tension. *A*, ○—○, is a control contracture; ●—●, a contracture initiated in the presence of 10^{-6} M adrenaline, where the initial phase of the spontaneous relaxation is slowed; ■—■, a contracture evoked in the presence of 10^{-6} adrenaline and 10 μg ml.$^{-1}$ pronethalol. Pronethalol blocks the effects of adrenaline but when applied alone it has little or no effect on the relaxation of the contracture, □—□, 17·5° C, 1 mM calcium. *B*, from another preparation, there is no difference between the relaxation of potassium contractures evoked in the presence (○--○) or absence (■—■) at 10^{-5} M atropine. 1 mM calcium, 17·0° C.

In all cases the preparation was pretreated for 5 min before the potassium contracture was elicited.

given by Atkins (1969) for the labelling of a compartment by constant infusion. Taking the exchange time constant for the extracellular space of frog cardiac muscle (Chapman, 1971*b*; Page & Niedergerke, 1972) and assuming a similar rate of release to that found for noradrenaline in cat hearts exposed to potassium chloride (Haeusler, Thoenen, Haefely & Heurlimann, 1968) a value of 5×10^{-9} M is obtained. Experiments using catecholamines show that the amplitude of the potassium contracture and

its rate of spontaneous relaxation are reduced by these agents at concentrations above 5×10^{-7} M (Fig. 3*A*). Furthermore, pronethalol (10 g. ml^{-1}) which blocks the effects of adrenaline in frog heart (Graham & Lamb, 1968, and Fig. 3*A*), has no effect on the potassium contracture. The same is true for 10^{-5} M atropine (Fig. 3*B*). The concentrations of catecholamines and acetylcholine established during perfusion by high potassium solutions would therefore appear to be insufficient to have a significant effect on the contraction or relaxation of the atrial trabecules perfused by the technique described in this paper.

The membrane potential and spontaneous relaxation. Simultaneous measurement of the membrane potential, of a muscle cell within a trabecule, with micro-electrodes and tension produced during a potassium contracture, reveals that the potential changes rapidly when the $[K]_o$ is increased, and is maintained at that potential until the $[K]_o$ is reduced again, while the contraction shows a typical rapid rise followed by a slow spontaneous relaxation (Fig. 4). These results are different to those reported by Morad & Orkand (1971), who using a sucrose-gap voltage clamp technique found that during maintained depolarization frog ventricle muscle showed no fall in the tension it was generating, but resemble those of other workers using voltage clamp techniques who have found that relaxation does occur in spite of the sustained depolarization in mammalian as well as amphibian heart (Beeler & Reuter, 1970*b*; Léoty, Raymond & Gargouil, 1971).

The relationship between the membrane potential and the contractile strength, and the dependence of the recovery of high potassium contracture on the membrane potential. The sigmoidal relationship between the membrane potential and the strength of the associated contraction of cardiac muscle was first demonstrated by Niedergerke (1956) using potassium contractures induced in strips of frog ventricle. More recently, with the advent of the sucrose-gap technique several reports have described the relationship between the membrane potential and the tension developed by cardiac muscle. It is noticeable that two different types of relationship have been obtained; either a simple sigmoidal (Beeler & Reuter, 1970*b*; Morad & Orkand, 1971), or an N-shaped curve has been found (Ochi & Trautwien, 1971; Vassort & Rougier, 1972; Léoty *et al.* 1971; Gibbons & Fozzard, 1971*a*). In the present work, using a variation of the $[K]_o$ to alter the membrane potential, no N-shaped tension-depolarization curves have been found. It is interesting to note that other reports using the potassium contracture method also provide simple sigmoidal curves (Lamb & McGuigan, 1966; Gibbons & Fozzard, 1971*b*), although in each case the potassium contracture solution was hypertonic to the normal Ringer solution.

Frog atrial trabecules do not always show a definite potential threshold for contraction (Fig. 5), tension increasing parabolically between 3 and 25 mM-K (-75 to -40 mV resting potential) followed by a more linear increase as the membrane potential is lowered from -40 to -15 mV (25–100 mM-K). This is emphasized by the observation that a small but maintained relaxation (i.e. a reduction of the resting tension) often occurs if the $[K]_o$ is lowered from the 3 mM normally present in the Ringer solution, a feature that contrasts particularly with the response obtained in skeletal muscles (Hodgkin & Horowicz, 1960).

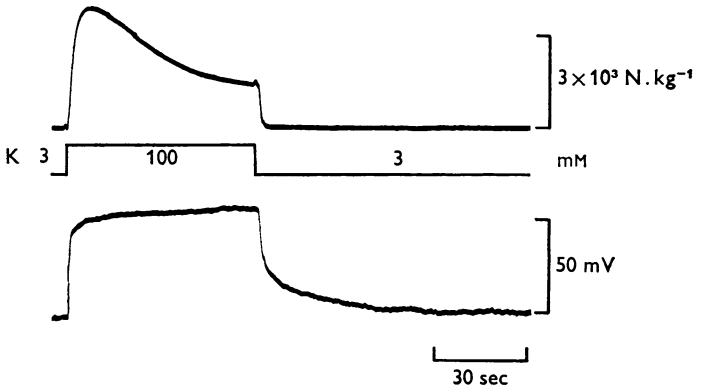

Fig. 4. The simultaneous change in the membrane potential and the development of a contractile response of an atrial trabecule when the $[K]_o$ is raised from 3 to 100 mM in the presence of 1 mM calcium (middle trace). The contracture shows a typical transient form (upper trace) while the change in membrane potential is sustained so long as the $[K]_o$ remains elevated (lower trace). 19° C.

To study the recovery of the contracture response, following the full relaxation of the contracture, the heart was first exposed to solution B which contained 3 mM-K and 1 mM-Ca, the potassium concentration was then increased to 100 mM (solution C) and the resulting contracture was allowed to build up and then spontaneously relax while still being perfused by the high potassium fluid. When the tension had fully subsided the perfusing solution was altered to one containing a lower potassium concentration, but at a constant tonicity (by mixing solution D and E) for a period of between 3 and 8 min, made standard for each particular experiment. After this time the 100 mM-K fluid was readmitted to the experimental channel and the second contracture so elicited was allowed to relax as befcre, then another solution containing a different potassium concentration was perfused over the muscle for the same fixed period. This procedure was repeated for several concentraticns of potassium and the whole sequence was then performed in reverse order to recognize any slow unidirectional changes in contractility which might result from the lack of stimulation in low potassium solutions. As the muscle cannot be electrically stimulated in higher $[K]_o$, the preparation was not stimulated at all during this type of experiment. This might account for the slow fall of the values of contracture tension that were recorded as the experiment continued.

Experiments of this type show that restoration of depolarization-induced contraction is dependent on the membrane potential which follows a contracture, the most marked restoration occurring in low $[K]_o$ and hence at more negative membrane potentials. In skeletal and mammalian cardiac muscle it depends on the time between the contractures and the membrane potential during that time (Hodgkin & Horowicz, 1960;

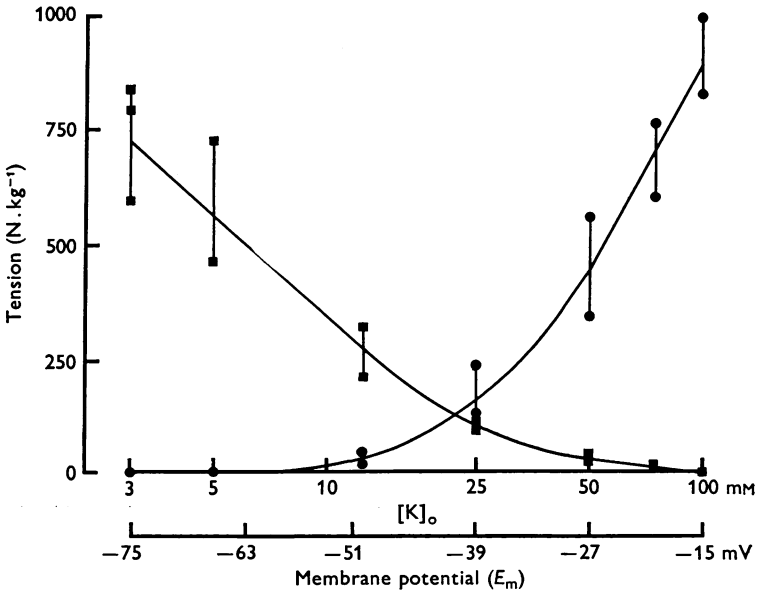

Fig. 5. The relationship between the peak contracture tension and the $[K]_o$ or the membrane potential (●—●), after the muscle had been perfused by 3 mM potassium Ringer solutions for 10 min before each contracture was elicited. The relationship between the peak tension evoked by 100 mM potassium contracture fluid and the $[K]_o$ or membrane potential that was established for the 10 min that preceded each contracture is also shown for the same preparation (■—■). This second curve corresponds to the restoration curves obtained by Hodgkin & Horowicz (1960). The lines were drawn by eye. All solutions contained 1 mM calcium. 19·5° C.

Beeler & Reuter, 1970*b*). Similar results are obtained from frog atrial muscle, as is illustrated in Fig. 5. However, this similarity is not complete because the curve relating the degree of the restoration of the contracture to the membrane potential is less steep in frog heart.

The recovery of the high potassium contracture is also dependent on the time since the last contracture was initiated. In 3 mM-K Ringer (membrane potential −80 mV) the half time for this recovery of contractility was 65 sec ± S.D. 15 sec, which is significantly slower than the time reported for recovery in skeletal muscle fibres (Hodgkin &

Horowicz, 1960). The time course of the spontaneous relaxation is independent of the size of these contractures and the time between contractures and is, therefore, also independent of the degree of restoration that has taken place.

The after-effects of contractures. The experimental procedure of contractures elicited by high-potassium solutions, preceded and followed by regular electrically induced twitches, does not lend itself well to an investigation of the after effects of contractures, because following even short high-potassium contractures the twitches often undergo complex changes in their time to peak. These changes are not eliminated by pronethalol or atropine and are, therefore, not due to release of adrenaline or acetylcholine, but probably reflect changes in the action potential. In some 5% of experiments, however, the changes in the time to the peak of the twitches were small. In these experiments the changes in twitch strength, following a potassium contracture, were relatively simple. Contractures that were terminated soon after the peak of their response, by lowering the [K], were followed by twitches whose amplitude was greater than those that had preceded the contracture. The twitches then declined more or less exponentially to the original level with a time constant in the region of 20 sec at room temperature, a result similar to those described by Wood, Heppner & Weidmann (1969) for mammalian mycardium. On the other hand, twitches that followed the full spontaneous relaxation of a high potassium contracture were reduced in size and built up again in an exponential way to the precontracture level, with a time constant similar to that of the fall in twitch tension that follows a short duration potassium contracture. This time constant is similar to that of the faster phase of the staircase response in frog heart (Chapman & Niedergerke, 1970*b*), and is likewise very sensitive to the temperature of the bathing medium (Chapman, 1971*a*). One tentative conclusion is that short duration high potassium contractures are followed by the same changes in the contractile state of the muscle that follow periods of elevated heart rate. On the other hand, after a fully relaxed high potassium contracture, conditions resemble those following a period of reduced heart rate.

$[Ca]_o$ *and the high potassium contracture.* The marked dependence of the strength of the potassium contracture on the $[Ca]_o$ has already been reported in some detail for frog atrial trabecules (Chapman & Tunstall, 1971; Chapman, 1971*a*). No depolarization induced contraction is developed when the $[Ca]_o$ bathing the heart has been reduced to less than 10^{-8} M by addition of 1 mM-EGTA to the nominally calcium-free solutions, after as little as 10 sec no high potassium contracture can be evoked. In fact, removal of much of the bathing calcium during a high potassium contracture results in an immediate full relaxation of the muscle. Vassort &

Rougier (1972) have obtained quite contrary results; however, the difference between the results may be due to the different methods that have been used. In the experiments reported here, the strip of muscle is exposed to homogeneous solutions, while in the case of the double sucrose-gap method the muscle is exposed to three solutions at five different places along the muscle (apart from the Vaseline seals in between them). One might easily believe that the tension recorded by the transducer in the central part of the trabecule is generated by regions of the muscle not exposed to the EGTA. It would be interesting to know if tension can be promoted by depolarization of the muscle in the central compartment when the solutions in all five compartments contain EGTA.

The reduction of the twitch responses and potassium contractures following a fully spontaneously relaxed high potassium contracture suggest that the activator-calcium that is removed during this time is not available for contraction when the membrane is subsequently depolarized. It is possible that, under these conditions of membrane depolarization, calcium is expelled from the sarcoplasm into the external fluid, because a marked reduction of the $[Ca]_o$ results in a rapid relaxation of the tension developed during a potassium contracture (see Fig. 9 in Chapman & Tunstall, 1971). If such a mechanism existed, during spontaneous relaxation the expulsion of calcium ions would be against a considerable concentration gradient, and therefore would presumably be an active process. Changes in the concentration gradient might be expected to alter the rate of calcium expulsion and hence the rate of spontaneous relaxation. Contractures have been elicited by exposing trabecules to 100 mM-K Ringer containing a wide range of $[Ca]_o$ (from 0·1 to 8·0 mM), and although there are small variations in the spontaneous relaxation, the rate of the exponential phase shows no consistent relationship to the $[Ca]_o$, while the earlier part of the contracture is sometimes slowed in high $[Ca]_o$. The absence of an influence of $[Ca]_o$ suggests that the expulsion of calcium through the sarcolemma is not the rate limiting step in the process that brings about this form of relaxation.

$[K]_o$ *and spontaneous relaxation.* When the potassium concentration in the contracture fluid was varied by mixing solutions D and E so that the relative tonicity was not altered, although the contracture fluids themselves were hypertonic to the normal Ringers solution, the exponential part of the relaxation showed only a small variation in rate not consistently related to the $[K]_o$ and hence the membrane potential of the muscle fibres (Fig. 6). Strong contractures initiated by high potassium sometimes showed a prolonged early slower phase of relaxation, while weak contractures, in low potassium, generally had an initial more rapid phase of relaxation (Fig. 6*A*). These results differ from those found in skeletal muscle, for the

rate of the relaxation of the potassium contracture of frog toe muscle has been shown by Foulks & Perry (1966) to depend upon the potassium concentration and hence the membrane potential, the rate of the slower phase of relaxation (presumably equivalent to the relaxation described in the present experiments) becoming greater at higher $[K]_o$.

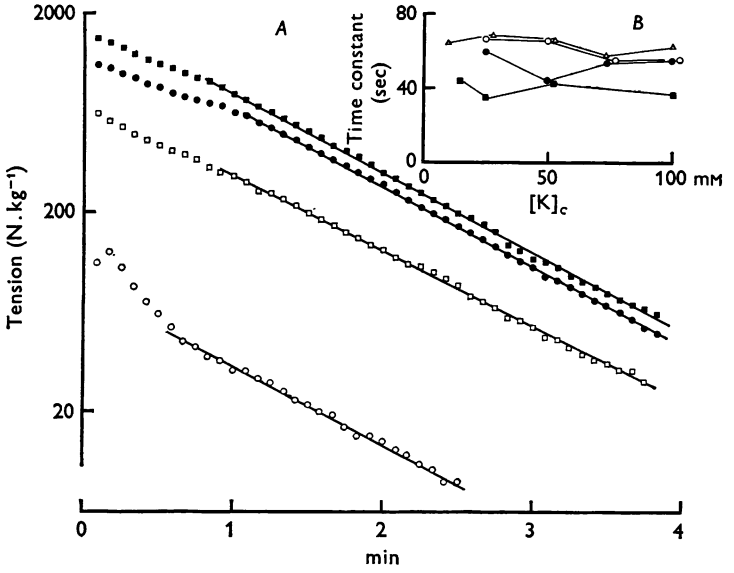

Fig. 6. *A*, semilogarithmic plots of the time course of the fall in tension that occurs during perfusion of the muscle with solutions containing various potassium concentrations: ■—■, 100 mM potassium; ●—●, 50 mM potassium; □—□, 25 mM potassium; and ○—○, 10 mM potassium. All contracture fluids were of the same tonicity and contained 1 mM calcium. 15° C. The continuous lines are regression lines. *B*, the time constant of the exponential phase of the spontaneous relaxation is plotted against the $[K]_o$ for four typical experiments. Although there is some variation in the time constant there is no consistent relationship between it and the $[K]_o$ in the contracture fluids and hence also the membrane potential.

Effects of anions. Experiments, in which the chloride concentration of the Ringer and contracture fluids was varied using either cyanate or methylsulphate to replace the chloride, showed that there was little short term effect of varying the bathing anion. There are certainly more profound long term effects that will not, however, influence the results presented in the present paper.

$[Na]_o$ *and spontaneous relaxation of the potassium contracture.* Baker, Blaustein, Hodgkin & Steinhardt (1969) on squid giant axon and Reuter & Seitz (1968) on guinea-pig auricles, have shown the dependence of

calcium efflux on the external sodium concentration, and suggest that an inward movement of sodium might be energetically coupled to the process that extrudes calcium from the cells. Reduction of the $[Na]_o$ induces the low sodium contracture which may have an effect upon the form of the following high potassium contracture. For this reason, the influence of sodium reduction on spontaneous relaxation was studied in two series of experiments, one in which the bathing sodium concentration was varied at a constant $[Ca]_o$ and the other at a constant ratio of $[Ca]_o/[Na]_o^2$. The

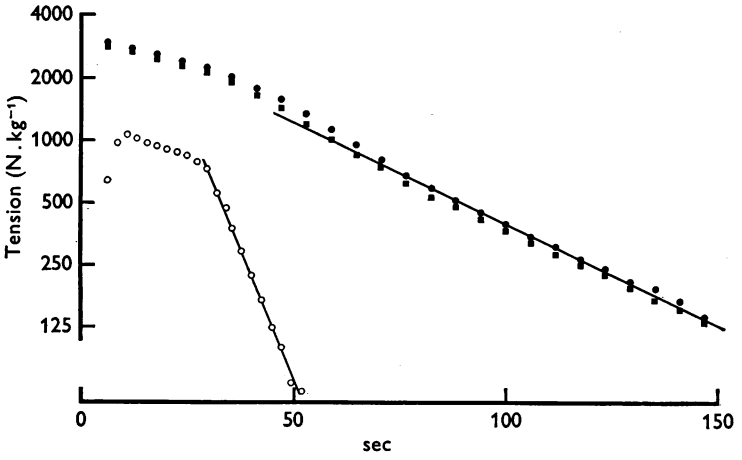

Fig. 7. Semilogarithmic plots of the spontaneous relaxation of 100 mM potassium contracture in the presence of 114·5 mM sodium before (●—●) and after (■—■) a potassium contracture evoked in the absence of external sodium ions (○—○). Before each contracture the trabecule had been exposed to solutions containing 3 mM potassium and the same [Na] as the contracture fluid for 7 min. The $[Ca]_o$ throughout was 1 mM and the temperature was 18° C.

variation of the sodium in the normal potassium Ringer was achieved by mixing solutions B and F, while the same variation in the high potassium contracture fluid was made by mixing solutions D and G.

The spontaneous relaxation of a contracture, induced by raising the potassium to 100 mM in the total absence of external sodium, was considerably faster than the relaxation found with the normal sodium concentration in the bathing solution (Fig. 7). The mean values for ten experiments, at room temperature, being 26·6 ± S.E. 4·1 sec in 114·5 mM sodium and 8·0 ± S.E. 1·3 sec in nominally sodium-free solutions (actual sodium concentration was 6×10^{-6} M). This result occurred when sucrose, lithium chloride, or Tris HCl were used to replace the sodium chloride. Fig. 8 shows the variation of the time constant of the exponential phase

of the spontaneous relaxation, for a 100 mM potassium contracture evoked in reduced $[Na]_o$ at a constant $[Ca]_o/[Na]_o^2$ ratio in two preparations. The $[Na]_o$ had been reduced for at least 5 min before the potassium-rich solution was admitted to the experimental channel. Over quite a large range of $[Na]_o$ there is little or no change in the time constant of relaxation. However, in zero-sodium solutions the rate of relaxation is increased, but in this case the $[Ca]_o/[Na]_o^2$ ratio had not been maintained in the sodium-

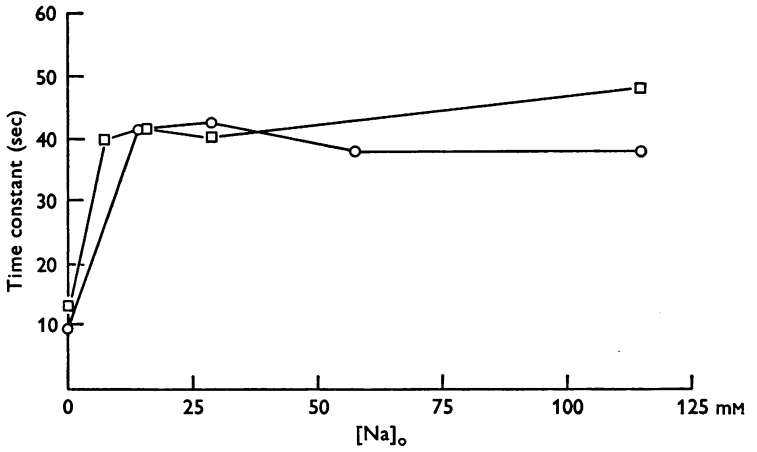

Fig. 8. The time constant of the exponential phase of the spontaneous relaxation of contractures evoked by 100 mM potassium solutions, over a range of $[Na]_o$ at which the ratio of the $[Ca]_o/[Na]_o^2$ was maintained, show little change in the experiments illustrated. In the zero-sodium medium where the $[Ca]_o/[Na]_o^2$ is not maintained, the rate of relaxation is enhanced as in Fig. 9. In each experiment the muscle was exposed to the low-sodium solutions for at least 6 min before the potassium contracture was elicited. The points represent the means of up to five determinations.

free medium, so that this effect could be directly due to the absence of external sodium ions, or due to the change in the $[Ca]_o/[Na]_o^2$ ratio. When the sodium is varied at constant $[Ca]_o/[Na]_o^2$ or at a constant $[Ca]_o$, it is found that the time constant for the exponential phase of the spontaneous relaxation is always smaller when the sodium concentration is reduced without any accompanying reduction of the calcium concentration (Fig. 9). This difference in the rate of relaxation was generally small and exceeded 15% in only one of ten preparations, and was always smaller than the reduction that was achieved in nominally sodium-free solution. It therefore becomes likely that this change in the rate of the relaxation is due to the reduction of the bathing sodium concentration rather than an increase in the ratio of $[Ca]_o/[Na]_o^2$. This conclusion is supported by the

observed failure of quite considerable changes in the $[Ca]_o$ to alter the rate of this relaxation in normal $[Na]_o$.

Reduction of the $[Na]_o$ to a low value would bring about a reduction of the intracellular sodium, as a result of the activity of the sodium pump and by the movement of the sodium down its concentration gradient, possibly by the exchange process proposed by Reuter & Seitz (1968). When the $[Ca]/[Na]^2$ ratio in the bathing medium is maintained, any exchange through

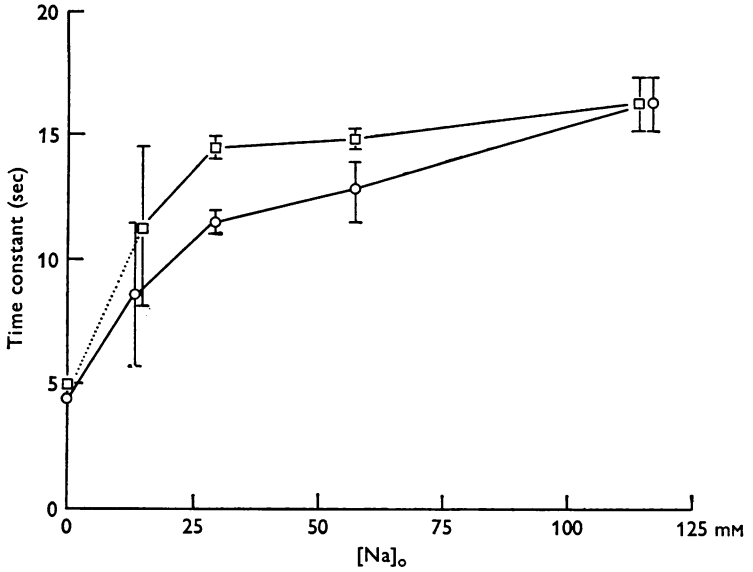

Fig. 9. The rate of the spontaneous relaxation of 100 mM potassium contractures initiated in various $[Na]_o$ when the $[Ca]_o/[Na]_o^2$ had been kept constant in all but the sodium-free solutions as signified by the dashed line (□—□), is compared with the rates of relaxation achieved when the $[Na]_o$ was reduced without a compensating change in the $[Ca]_o$ which was 1·6 mM (○—○). 18° C. The points represent the means and the vertical bars the range, of up to five determinations.

a pathway of the type proposed by Reuter & Seitz should be reduced, if it is calcium that is exchanging for sodium and if there is no electrical inequality established by the exchange. Intracellular sodium ions could influence the rate of relaxation by directly controlling the release of the calcium ions from the sarcolemma, or they could compete with calcium ions at the site which brings about relaxation. Indeed, Palmer & Posey (1967) have found that sodium ions inhibit the uptake of calcium by isolated cardiac sarcoplasmic reticulum.

When the Ringer solution bathing the muscle was changed to one containing a reduced sodium and a reduced calcium in such a way that the ratio of the $[Ca]_o/[Na]_o^2$ was kept constant, in all cases, irrespective of the

actual concentrations, the amplitude of the twitch response declined, probably due to the reduction of the overshoot of the action potential that occurs in these solutions (Niedergerke & Orkand, 1966), for the amplitude of the high-potassium contractures was not similarly reduced (Fig. 10). When the sodium concentration was reduced still further (with the calcium concentration also being reduced) a strong transient contracture developed although it was generally weaker than the contracture that

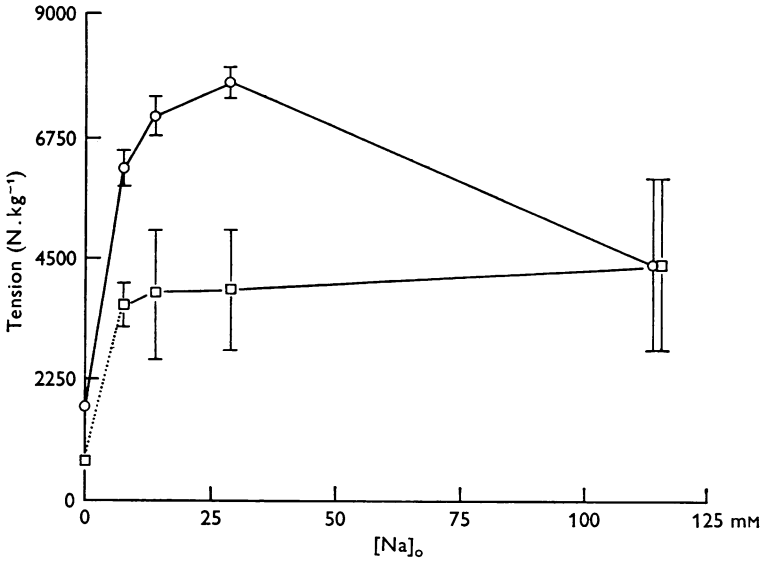

Fig. 10. The amplitude of the 100 mM potassium contracture is unchanged when the $[Na]_o$ is reduced so long as the $[Ca]_o$ is also reduced so as to keep the ratio of $[Ca]_o/[Na]_o^2$ constant (□—□). When the $[Na]_o$ is reduced at a constant $[Ca]_o$, then there is an increase in the potassium contracture amplitude as the $[Na]_o$ is reduced (○—○). In low $[Na]_o$, particularly sodium-free solutions, the amplitude of the potassium contracture is depressed to quite a low value in the presence of high (1·6 mM for the open circles) and low (6·25 μM for the open squares) calcium concentrations when the $[Na]_o$ has been reduced for 10 min before the potassium contracture is elicited by raising the $[K]_o$ from 3 to 100 mM. 18° C. The points represent the means and the vertical bars the range of up to five determinations.

developed when the calcium concentration was not simultaneously reduced. This low-sodium contracture was not due to the contamination level of calcium in the experimental solutions making a significant contribution to the free calcium, because atomic absorption spectroscopy revealed a total contamination of calcium of 6×10^{-6} M, and a low-sodium contracture was recorded when the $[Ca]_o$ was as high as 10^{-4} M and the sodium was 28·7 mM. These results suggest that although the strength of

the high-potassium contracture depends on the $[Ca]_o/[Na]_o^2$ ratio, there is probably also some other effect, perhaps due to the reduction of the $[Na]_o$ alone which is important in determining the strength of the low-sodium contracture.

The strength of the potassium contracture in sodium-free solutions was always reduced in amplitude even when the $[Ca]_o$ had been maintained (Fig. 10). The depression of this contracture showed some dependence on the time the heart had been in sodium-free media and on the calcium concentration. At a $[Ca]_o$ of 10^{-5} M, perfusion by a high-potassium solution failed to initiate a contracture after 7–10 min, while in 1 mM calcium potassium-rich solution could still induce a small contracture after over 1 hr in the sodium-free perfusate. In a few preparations a twitch response could be elicited by electrical stimulation. Twitch-like responses of this sort occurring in sodium-free solutions have been reported in frog heart by Bozler (1971) and by Busselen, Verdonck & Carmeliet (1972). The relatively large size of these twitch responses made it unlikely that the reduction of the strength of the high-potassium contracture is due to the fatigue of some process involved in the generation of tension, moreover, a maximal tension can be evoked, in zero sodium, by addition of low concentrations of caffeine (Chapman & Miller, 1972). This means that the relationship between the membrane potential and the tension generated is much steeper than that found when the muscle is bathed by the normal sodium concentration, and resembles skeletal muscle; this similarity is extended to the rapid rate of spontaneous relaxation of the potassium contracture observed under these conditions. These results suggest that in zero $[Na]_o$ the contraction of frog heart is dependent on the availability and release of intracellular calcium. The twitch response relaxed at the same rate as the potassium contracture. This may mean that repolarization induced relaxation and spontaneous relaxation are due to the same process in sodium-free medium. The amplitude of the sodium-free contracture and its relaxation have been found, in the present work, to be almost independent of the $[Ca]_o$ so long as this is above 10^{-5} M, although the following high-potassium contracture is dependent on this $[Ca]_o$, which again illustrates the difference between the two types of contractures and strengthens the idea suggested by Chapman & Ochi (1972) that there are important differences in the processes that underlie the development of these two types of contracture.

DISCUSSION

Contractures, evoked by raising the potassium concentration in the perfusing solution, are similar in frog auricular trabecules and twitch skeletal muscle fibres. After an initial period, the relaxation has been fitted by a single exponential function in the present work and by Hodgkin & Horowicz (1960) and by Caputo (1972) in frog twitch fibres, and in both muscles recovery of this contracture shows a strong dependence on the membrane potential and the time between contractures. There are, however, certain important differences; for in frog heart (*a*) the rate of spontaneous relaxation is independent of the membrane potential, (*b*) in the presence of sodium ions it is independent of $[Ca]_o$, (*c*) this relaxation persists in solutions containing caffeine, and (*d*) contraction is markedly dependent on the $[Ca]_o$. These differences suggest some connexion between the relaxing system and the surface membrane in skeletal muscle that is not present in frog myocardium. A likely candidate is therefore the T-system which is absent in frog heart (Staley & Benson, 1968; Chapman, 1971*b*). The restoration of the potassium contracture depends on the membrane potential in both types of muscle which may mean that this process does not require the existence of a T-system.

At least part of the ionic calcium that activates contraction in frog heart comes directly from the bathing medium when the membrane is depolarized. This calcium has been supposed to act as a trigger for further release of calcium from intracellular sites as well as contributing to activation of the contractile apparatus itself (Chapman & Tunstall, 1971; Chapman, 1971*a*), or it has been thought to be sufficient to activate contraction in its own right (Morad & Orkand, 1971). In either case, if when the membrane is depolarized the release of calcium ions into the sarcoplasm commences and continues throughout the period of depolarization, it will be the influx from the outside medium that will eventually contribute the major part of the activator calcium, because the intracellular stores will have a finite capacity. Under such conditions, the sarcoplasmic calcium concentration will increase throughout the period of membrane depolarization, and presumably the tension generated by the muscle will also continue to increase. This is essentially the model proposed by Morad & Orkand (1971), but it is difficult to visualize how relaxation can be brought about on repolarization of the membrane unless the intracellular calcium then passes out into the medium surrounding the muscle fibres. This possibility cannot be totally excluded because Niedergerke (1963) has described a large increase in the efflux of radioactive calcium after relaxation of contractures in frog ventricles. In the present experiments relaxation occurred even while the membrane is depolarized, a time when

Niedergerke found no loss of cellular calcium. Furthermore, the lack of dependence of the rate of spontaneous relaxation on $[Ca]_c$ is not consistent with the idea of a calcium pump in the cell membrane being rate limiting on the process of relaxation and therefore the first stage in the removal of calcium ions from the sarcoplasm. There is also a marked difference between the temperature dependencies of the calcium efflux and the spontaneous relaxation of heart muscle (Chapman, 1973). One seems obliged to assume that relaxation is brought about by some intracellular agency, and that the release of activator calcium is not sustained, i.e. a hypothesis similar to that devised by Hodgkin & Horowicz (1960) to account for similar results obtained from skeletal muscle. The transient release of activator calcium is probably due to some property of the surface membrane, because restoration of the high-potassium contracture depends on the membrane potential and there is probably no T-system in frog heart. This release of calcium would involve an increase in the calcium permeability of the surface membrane when the latter is depolarized, which then becomes inactivated, depending on the membrane potential and the time this potential has been maintained. Indeed, such a potential dependent 'calcium current', which shows a potential and time dependent inactivation, has been described in mammalian and amphibian heart (Beeler & Reuter, 1970*a*; Rougier, Vassort, Garnier, Gargouil & Coraboeuf, 1969).

It has generally been concluded that the intensity of contraction is related by a first order equation to the $[Ca]_i$ (Ebashi, Endo & Ohtsuki, 1969; Fuchs & Briggs, 1968) although others have entertained the possibility that several calcium ions might be required at the unit level of contraction (Ashley, 1970; Chapman & Tunstall, 1971; Julian, 1971). It is necessary to assume that relaxation is brought about by a reduction of the intracellular calcium concentration, and that the tension developed at any time is some relatively simple function of this concentration. If several calcium ions are involved then the observed time constants are not the ones that relate to the underlying process that removes calcium from the sarcoplasm but they should be in a constant proportion to them, depending on the exact relationship there is between the $[Ca]_i$ and the degree of activation of the contractile apparatus.

The intracellular relaxing system that has been proposed in the above discussion requires some identification, with two possibilities seeming worthy of consideration. These are, the sarcoplasmic reticulum, and the mitochondria. Caffeine can only initiate contractures in frog heart when the muscles have already developed tension in either a high-potassium or low-sodium contracture (Chapman & Miller, 1971), which suggests that the activator calcium, that is removed from the sarcoplasm during this

relaxation, is released by application of caffeine. This supports the existence of an intracellular site of calcium uptake in frog heart that is in some ways very similar to that found in skeletal muscle, for caffeine has been found to promote release of calcium ions from isolated sarcoplasmic reticulum (Weber & Herz, 1968), but not from isolated mitochondria (Weber, 1968; Carafoli, Patriarca & Rossi, 1969). Although mitochondria can take up significant amounts of calcium, as yet there is no way known by which this calcium can be released, apart from the removal of substrate supplies directly or by exposure to respiratory inhibitors (Drahota Carafoli, Rossi, Gamble & Lehninger, 1965) and even then the rate of release would be too slow to account for the rate of rise of $[Ca]_i$ during contraction. This means that in the intact muscle the mitochondrion will be a blind alley to cellular calcium. It would appear that the sarcoplasmic reticulum is the most suitable candidate but one must question if there are sufficient quantities of this structure in frog heart to bring about the relaxation of frog heart muscle. The electron microscope investigations describe varying amounts and organizations of sarcoplasmic reticulum, although they all agree that there is less than in mammalian heart (Staley & Benson, 1968; Sommer & Johnson, 1969; Page & Niedergerke, 1972). The amount of sarcoplasmic reticulum has been estimated at 0·5% of the non-protein muscle cell volume in frog ventricle (Page & Niedergerke, 1972) and at 4–8% in frog twitch fibres (Page, 1964), a ratio of something less than 20:1 in favour of the twitch fibres. It may be significant that the ratio of the rate of spontaneous relaxation in the two muscle types is between 10:1 and 30:1 with the twitch fibres relaxing faster. If the sarcoplasmic reticulum in frog heart is able to establish a concentration gradient between it and the sarcoplasm, similar to that found across the surface membrane, then it could remove about 10^{-5} moles calcium/kg tissue water from the sarcoplasm. This quantity is in the region of the amount required to saturate the calcium-binding sites of the cardiac troponin-tropomyosin complex (Katz, 1970). Chapman & Miller (1972) have obtained evidence that the intracellular sites within frog heart can, at certain times, release sufficient calcium to promote a maximal contracture, even in the virtual absence of extracellular calcium. This means that it is necessary for the cardiac sarcoplasmic reticulum to be able to sequester, at least temporarily, quite large amounts of calcium. The transience of this caffeine contracture could mean that the sarcoplasmic reticulum can reabsorb calcium ions in the presence of caffeine as found by Thorpe & Seeman (1971) or, as suggested by Chapman & Miller (1971), that there are two relaxing systems in frog heart or that there are two stages in the removal of calcium ions from the sarcoplasm, with the cell membrane as the second stage providing a back-up system

for the sarcoplasmic reticulum, rather than the mitochondria as suggested previously (Nayler & Hasker, 1966; Chance, 1965; Carafoli *et al.* 1969).

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