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THE EFFECT OF THE REPLACEMENT OF CALCIUM BY STRONTIUM ON EXCITATION-CONTRACTION COUPLING IN FROG SKELETAL MUSCLE

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SUMMARY

1. In frog skeletal muscle strontium can replace calcium in potassium contractures for 5 hr, though it is less effective than Ca. Sr can restore the responsiveness to K after it had been lost in the presence of Mn.

2. Muscles refractory to caffeine following repeated exposure to it in the absence of Ca, recover in part following addition of Sr.

3. The uptake of ^{85}Sr was increased during mechanical activity, whereas the uptake of ^{58}Co was not changed. Resting uptake of ^{58}Co was 3–4 times greater than that of ^{85}Sr.

4. Sr fully activated the myofibrillar adenosine triphosphatase (ATP-ase), though its affinity was about 30 times less than Ca.

5. The sarcoplasmic reticulum took up Sr, though less effectively than Ca.

INTRODUCTION

In the skeletal muscle of the frog, as well as in most other contractile tissues, Ca is clearly involved in excitation–contraction coupling during K contractures. If a toe muscle of the frog is bathed for several minutes in a solution without Ca the mechanical response to elevation of K is much reduced (Frank, 1960). For periods of several hours, Ca can be replaced by a number of divalent cations, including cadmium, nickel, cobalt, strontium, magnesium and ferrous ions (Frank, 1962). The mode of action of these substitute cations is not at all apparent. The presence of these ions in the bathing solution may be necessary to maintain some properties of the membrane so that the reactions initiating contraction may occur, or, alternatively, these ions might enter the cell and thereby initiate contraction more directly.

A number of data indicate that Sr can substitute for Ca in the activation of the muscle contraction more fully than the other divalent ions mentioned. Thus Sr is the only ion which successfully substitutes for Ca in contractions of cardiac muscle (Nayler, 1965). Injection of Ca and Sr ions into crab muscle fibres produces tension, while magnesium ions are without effect, (Caldwell & Walster, 1963). Further, the ATPase activity of rabbit myofibrils, which is presumably involved in generating muscle tension, and which is readily activated by Ca, has been reported to be partially activated by Sr, whereas Cd, Co and Mn were less effective (Seidel & Gergely, 1963).

The experiments to be presented are concerned with the role of Sr and other cations in K and caffeine contractures, the movements of labelled Sr and Co, during mechanical activity and with the reactions of Sr with the ATPase and the relaxing factor system of frog muscle. The results are consistent with there being two fractions of Ca, one readily removed and replaced by the divalent cations listed above, whereas the other fraction, more difficult to remove, can be replaced only by Sr. This fraction presumably is involved in the cycle of contraction and relaxation within the muscle.

METHODS

The muscles of small (up to 25 g) frogs, *Rana pipiens*, were used for the contracture and tracer experiments. The usual bathing solution contained tris (hydroxymethyl) aminomethane or choline instead of sodium to eliminate active membrane responses. The contracture solution contained 120 mM-K and the main anion was methane sulphonate, to maintain the product of the K and Cl concentrations constant (cf. Milligan, 1965, for details).

Tension developed by the muscle in response to elevated K solutions or to caffeine (Eastman Kodak) was measured by a strain gauge and recorded with a Grass pen recorder. The contractures were compared by measuring the area under the contracture curve (Milligan, 1965).

Tracer experiments. Uptake of ^{85}Sr and ^{58}Co was measured by soaking toe muscles tied to glass rods in Tris-Cl solutions containing 30–50 μc/ml. ^{58}Co or ^{85}Sr for various periods of time. The total concentration of divalent cation was 0·3 or 3 mM.

To measure the uptake of tracer in muscles undergoing contractures, muscles were soaked in Tris-Cl solutions containing the radioactive cations for a total of 90 sec. This short soaking period was selected to minimize the uptake of tracer by the muscle. Paired muscles were used; for one the concentration of K was increased from 2·5 to 120 mM for 20 sec of the 90 sec soaking period. The resulting K contracture subsided 10–15 sec after the application of high [K]. Tracer washout from these muscles was followed for 40 min.

For counting, the muscles were tied to an uncontaminated glass rod which was in a polypropylene tube containing 1 ml. of inactive Tris-Cl solution. The tubes were placed in a standard well-type γ-scintillation detector connected to a Tracerlab '1000' scaler. Routine corrections were made for decay of the tracer and counter efficiency. The muscles were counted at 10 min intervals for 1–2 hr.

Biochemical experiments. Treatment of glassware, choice of reagents and preparation of solutions have been described elsewhere (Weber & Winicur, 1961; Weber & Herz, 1962, 1963).

Myofibrils. The preparation differed from standard preparations of rabbit myofibrils by the shorter period of homogenization of only 40 sec. The myofibrils were collected by

centrifugation at 2000 *g*. They were washed twice with 0·1 M-KCl and 1 mM imidazole, pH 6·5, at room temperature. Afterwards they were filtered through cheesecloth and washed to remove Ca (Weber & Herz, 1963) with a solution containing 0·1 M-KCl, 1 mM imidazole, 1 mM ethylene glycol bis (*β*-aminoethylether)—N, N–tetra-acetic acid (EGTA) and 1 mM-$MgCl_2$. The myofibrils were collected by centrifugation for 5 min at 7000 *g* and were then washed 3 times with 0·1 M-KCl and 1 mM imidazole. The myofibrils contained 1·1 *μ*moles Ca/g protein. The Ca in the myofibrils and in the reticulum was analysed in a trichloroacetic acid (TCA) extract by atomic absorption spectrophotometry.

ATPase activity. Myofibrils were incubated for 30 sec in the absence of a phosphate donor system with 2·4 mM-Mg ATP. The assay was terminated by the addition of 1 ml. 20% TCA/5 ml. and the inorganic phosphate determined by the method of Taussky & Shorr (1953). The ionic strength was 0·13, pH 7·0 (20 mM imidazole) and the vessels contained 0·64 mg/ml. myofibrillar protein.

Frog reticulum. Reticulum was prepared by a modification of the method of Hasselbach & Makinose (1961) and Ebashi & Lipmann (1962). Leg muscles were removed from frogs kept at 4° C, cut up with scissors and blended in 3 times the weight of a solution containing 0·12 M-KCl and 5 mM imidazole, pH 6·5, at room temperature (about pH 7·0 at 0° C), for 40 sec.

The homogenate was centrifuged at 2000 *g* for 5 min at 0° C; the pellet was re-extracted with 100 ml. of the homogenizing solution. The first and second supernatant fractions were combined and centrifuged again at 2000 *g*. The reticulum pellet (collected at 20,000g) was washed twice and finally suspended in a concentration of 11·4 mg protein/ml. The preparation was used for 4 days. The reticulum contained 23 *μ*-moles of exchangeable Ca/g protein.

Determination of Ca and Sr uptake by reticulum. The uptakes were measured with the isotopes ^{45}Ca and ^{89}Sr. The total Sr equalled the Sr added, the total Ca was the sum of the added Ca and the Ca intrinsic in the preparation. The accumulated Ca was calculated from the Ca which had disappeared from the reticulum-free medium. The medium was obtained by Millipore filtration syringes as described by Martonosi & Feretos (1964) with one modification. Only 2 drops (about 200 *μ*l.) were allowed to pass through each filter, since filtering more than 2 drops caused the counts in the filtrate to increase. This may be due to some reticulum filtering through or to the release of Ca by reticulum accumulated on the filter. The latter explanation is more probable because the counts increased less in oxalate containing preparations where the precipitated Ca oxalate can be released less easily from the reticulum. The possibility that the reduction of counts in the first 2 drops was the result of ^{45}Ca absorption by the filter paper was ruled out by control experiments without reticulum.

The reticulum was incubated in a total volume of 5 ml. (about 0·5 mg protein/ml.) at pH 7·0 (20 mM imidazole), and ionic strength 0·08, with 2 mM creatine phosphate, 0·2 mg/ml. creatinephosphokinase, 0·4 mM-Mg-ATP and 1 mM-$MgCl_2$ for various periods of time under stirring. Various concentrations of EGTA were present as indicated.

Calculation of pCa and pSr. pCa was calculated as described previously (Weber & Herz, 1963). pSr was estimated using a binding constant for EGTA of 8·5 for log K (Chaberek & Martell, 1959). For pH 7·0 the dissociation constant (K) was calculated as $6{\cdot}04 \times 10^{-5}$. In the case of the reticulum we calculated the pSr from this constant, neglecting the binding to ATP since the ATP concentration was low and the Mg high.

For the experiment with the myofibrils where a high concentration of ATP was used we calculated pSr by approximation using in addition to the EGTA constant a dissociation constant for Sr-ATP at pH 7·0 of $9{\cdot}4 \times 10^{-5}$, i.e. twice the value of the apparent Ca-ATP dissociation constant calculated for pH 7 according to Burton's data (1959). This constant was chosen on the basis of the data of Smith & Alberty (1956), who measured a ratio of approximately 2 between the two constants. We did not use their value for Sr because we

did not use their constant for Ca; the constants of Burton are preferred because the myofibrillar activity as a function of pCa gives similar values when pCa is calculated with EGTA as the main buffer and with ATP as the main buffer.

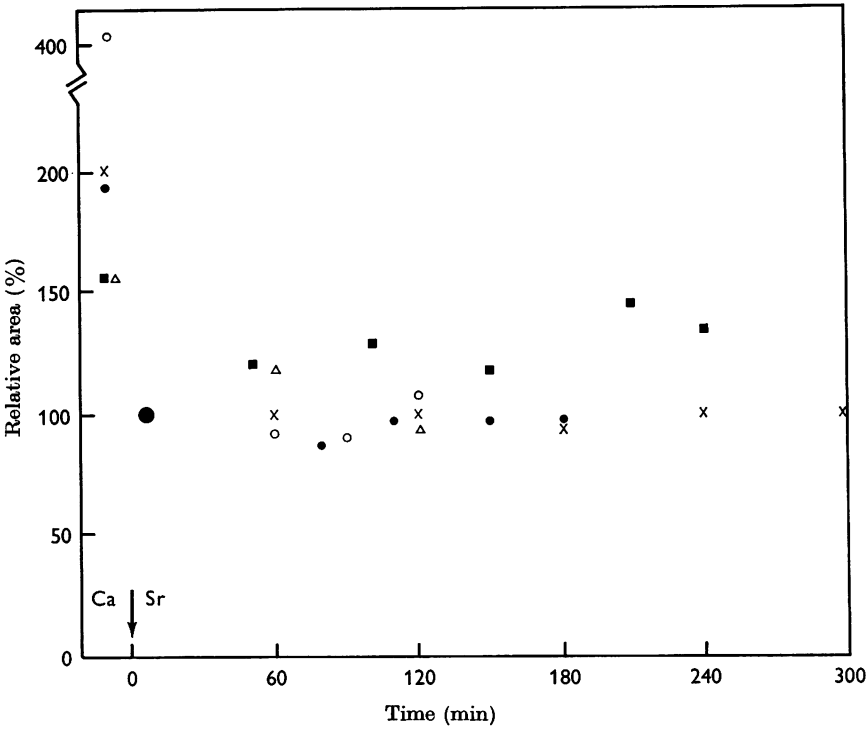

Fig. 1. Changes with time of the area under the contracture curve following replacement of Ca by Sr (all at 3 mM) in a Tris-Cl solution. The contractures were produced by 120 mM potassium methane sulphonate. The results are expressed as % of the area under the curve of the contracture obtained after bathing the muscles for 5 min in a solution containing Sr (large circle). Before the replacement of Ca by Sr a contracture was obtained, and its size is indicated by the first point shown. The different symbols refer to different muscles.

RESULTS

Contracture experiments

Divalent cations and K contractures in frog toe muscles. Following replacement of Ca by Co, Mn, Ni, Cd and Mg ions muscles will give K contractures; however, the size of the contractures progressively diminishes with time. The response to K can be restored only by re-immersing the muscle in a solution containing Ca (Frank, 1962).

If muscles were soaked in Tris-Cl solution with 3 mM-Sr but without Ca, the first K contracture was reduced in size when compared to a control K contracture in the presence of Ca (Fig. 1). However, over a period of 5 hr,

there was no change in size of the Sr contracture after the initial reduction. This ability of Sr to maintain the size of the K contracture is in contrast to the inability of Mn, Co, Ni, Cd and Mg to do so.

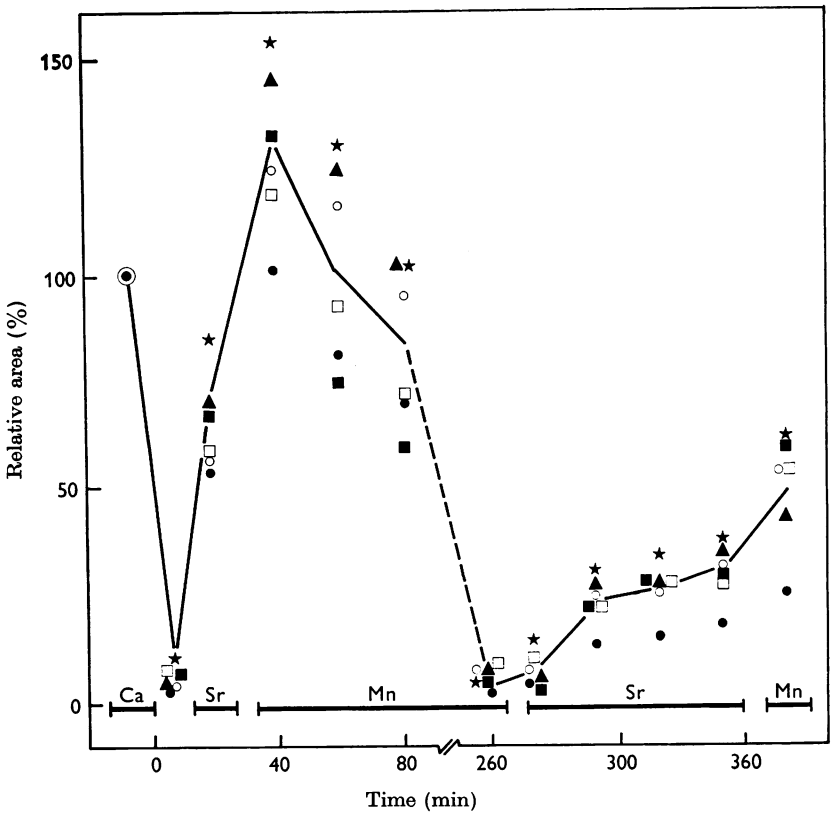

Fig. 2. Changes with time of the area under the contracture curve with Mn and Sr replacing Ca (all at 3 mM) in a choline Cl solution. The contractures were produced by 120 mM potassium methane sulphonate applied for 30–60 sec at intervals of about 5 min. The results are expressed as % of the area under the curve of the contracture obtained after bathing the muscles in Ca.

To investigate whether Sr, like Ca, can restore the responsiveness of the muscle to K after it had been lost by a prolonged treatment with a Ca-free solution containing another divalent cation, experiments were done in which a solution containing 3 mM-Sr was applied after a similar one containing 3 mM-Mn (Fig. 2). The first contracture in the presence of Mn was somewhat larger than the control contracture in Ca but the area of subsequent contractures fell to less than 10% of that of the control in Ca after about 4 hr. After the replacement of Mn by Sr the area under the contracture curve was initially unchanged; after prolonged exposure to Sr the

area increased and reached a value of about 30% of the control area in Ca (which would be equal to about 50% of the control area in Sr). Following a second replacement of Sr by Mn there was again an increase in the area under the contracture curve. This again suggests that the effect of Sr on contractures is qualitatively similar to that of Ca, though weaker.

Caffeine contractures and Sr. Muscles rendered refractory to K by washout of calcium produce contractures on the addition of caffeine (Frank, 1960). The peak tension of the first contracture initiated by caffeine is not altered by the absence of Ca from the bath solution; however, the response to subsequent applications of caffeine decreases with time. This decrease is not found in the presence of Ca or Sr (Frank, 1962).

The effect of various divalent cations on the recovery of the responsiveness to caffeine of muscles rendered refractory to 4·7 mM caffeine by repeated exposures to this drug in the absence of Ca was tested. The caffeine treated muscles were exposed to various divalent cations (1·8 mM) for 15 or 20 min. In a Ca containing solution, the tension reached 76% (average of twelve muscles, range 40–100%) of the previous control value after 15 min. In a Sr containing solution, after 15 min the tension averaged 35% (range 8–64% in five muscles) and after 2 hr reached about 60% (48–67%). There was no recovery if Mg, Ni, Mn, Co, Zn, Ba, Be or Fe were applied for 15 min. Frank (1962) reported no recovery in the presence of Sr in his experiments but the duration of the exposure was not stated.

Effect of K on ^{85}Sr uptake. It is well known that the addition of K increases the Ca uptake, as measured by tracer Ca entry, if the muscle is exposed to tracer Ca before the addition of K. The results of similar experiments with radioactive ^{85}Sr are presented in Fig. 3. Strontium uptake into muscles is significantly increased by exposing them to K in the same way which increases the calcium uptake (Bianchi & Shanes, 1959). The increased Sr uptake is apparent only if the muscles are washed for 5–10 min in solution without tracer before counting. By contrast, application of K had no effect on the ^{58}Co uptake by the muscle; similar results have been recently obtained with ^{63}Ni by Fischman & Swan (1965).

The rate of uptake of ^{85}Sr into resting muscles was 3–4 times slower than the same rate for ^{58}Co. The relation of this difference to the effect of Sr and Co on the mechanical activity is not obvious.

Biochemical experiments

Activation of ATPase. The activation of the myofibrillar ATPase by Ca is considered to be the essential step by which contraction takes place. The activating Ca ions are supposedly taken up by the sarcoplasmic reticulum leading to relaxation. To act as a substitute for Ca in the contraction–relaxation cycle Sr should be able to replace Ca in these

functions. The ATPase activity of frog myofibrils and the uptake of Sr into preparations of the sarcoplasmic reticulum were therefore investigated in the presence of Sr ions.

The activity of the myofibrillar ATPase as a function of both ionized Ca and Sr concentration is shown in Fig. 4. The ionized cation levels were calculated as described above.

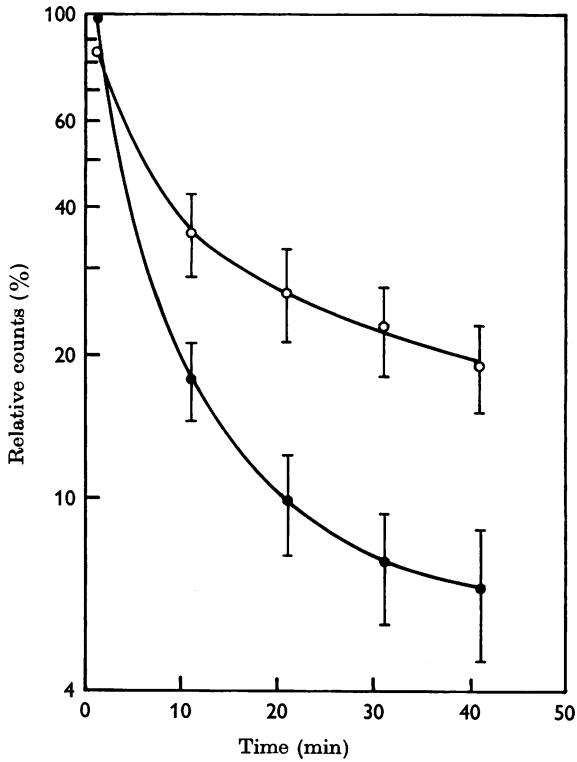

Fig. 3. Washout of ^{85}Sr into an inactive solution from muscles soaked in radioactive solutions for 90 sec. Filled circles: resting muscles; open circles: muscles to which 120 mM-K methane sulphonate was applied for 20 of the 90 sec period. Paired muscles were used. The muscles were washed for 1 min in inactive Sr containing Tris Cl solution before being counted the first time. The points are expressed as % of this value. Bars denote S.E. of mean.

The maximum rate of ATP break-down in the presence of Ca is apparently also reached with Sr, with frog myofibrils, in contrast to the finding of Seidel & Gergely (1965) in rabbit. However, Sr had a lower affinity than Ca in activating the ATPase, for at half maximum activity the requirement for ionized strontium was about 30 times that for Ca. The lower affinity of Sr for the activating site is consistent with the finding that the

binding constant of Sr to many chelating compounds is lower than that of Ca.

Although the myofibrils were not Ca free the activation of ATPase in our experiments was caused entirely by Sr because the contaminating Ca was chelated by EGTA. In a control experiment it was established that 10 μM free EGTA was sufficient to suppress the Ca activation of the myofibrillar ATPase (to 0·225 μ-mole P_i/min released per milligram protein compared to a value of 0·2 μ-mole P_i/min in the presence of 10 mM EGTA). The lowest value for free EGTA at the lowest pSr was 90 μM because of the high dissociation constant of Sr-EGTA. This separation of Ca and Sr effect was possible because EGTA binds Sr so very much less than it binds Ca.

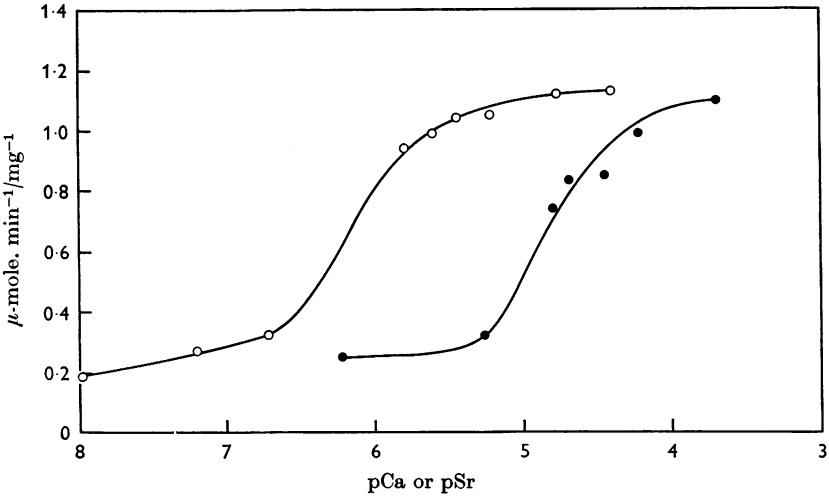

Fig. 4. Dependence of ATPase activity on Ca and Sr concentration. Myofibrils: 0·64 mg protein/ml. μ (ionic strength) 0·13, 20 mM imidazole buffer, pH 7, and 2·4 mM-Mg-ATP Ca free. Ca, Sr and EGTA added in suitable amounts.

Uptake of ions by the sarcoplasmic reticulum. The equilibrium filling of sarcoplasmic reticulum from frog with Sr and Ca as a function of ion concentration is shown in Fig. 5. The calcium data are from unpublished observations of A. Weber & R. Herz. Different preparations were used, so the difference in amount taken up does not necessarily indicate a difference in capacity. The reticulum fraction used in the Sr experiments took up 82 p-mole Sr/mg in addition to 23 p-mole Ca/mg intrinsically present at 6 μM Sr. However, this Sr^{2+} may not be saturating and the maximal uptake of Sr may be higher.

The ion concentration in the medium required for maximal filling of the reticulum is higher for Sr than for Ca; half maximal filling with Ca is reached at about 2×10^{-8} M-Ca^{2+} and with Sr at about 3×10^{-7} M-Sr^{2+}. In other words, the reticulum is not capable of lowering the Sr^{2+} concentration of the medium to the same extent that it can lower the Ca^{2+} concentration.

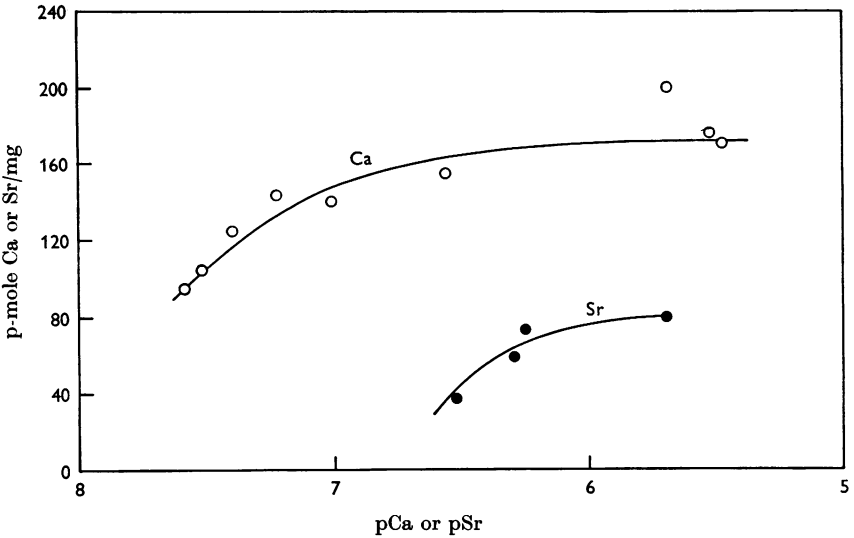

Fig. 5. Equilibrium filling of fragmented sarcoplasmic reticulum with Ca and Sr as a function of concentration. Conditions for Sr experiment: 0·46 mg protein/ml., μ = 0·08, 20mM imidazole buffer, pH 7, creatine phosphate 2 mM, and Sr and EGTA added in appropriate amounts. Frog reticulum collected at 2000–20,000 *g*. Intrinsic Ca 53 p-mole or 23 p-mole/mg. This preparation bound 130 p-mole Ca/mg at Ca^{2+} = 0·096 μM. Ca data from unpublished results of A. Weber & R. Herz; frog reticulum collected at 8000–20,000 *g*.

DISCUSSION

The evidence presented above shows that Sr can substitute for Ca in a number of functions connected with mechanical activation, contraction and relaxation. For example, K contractures can be supported without decay for a long time in the presence of Sr, the ability of the muscle to respond to K and to caffeine can be restored by Sr in conditions in which no other ion except Ca is effective, Sr is accumulated by the sarcoplasmic reticulum and is capable of activating the myofibrillar ATPase. Further, R. J. Podolsky (personal communication) found that in isolated muscle fibres without sarcolemma, contraction is initiated by application of Ca and Sr whereas Ni has only a weak effect and Mg is without effect.

These results are pertinent to defining the role of Ca and Sr on one hand and the rest of the divalent ions on the other hand in excitation–contraction coupling in frog skeletal muscle. It appears that there is a non-specific role which can be performed by any of the divalent ions when present in the extracellular space. Since Ni is more effective than Sr when applied externally to the intact fibre (Frank, 1962) and the opposite holds for the skinned fibre, it seems likely that cations present outside are not

directly involved in contraction. Thus the role of the outside cations seems to consist in maintaining some property of the cell membrane (Jenden & Reger, 1963).

Another role or set of roles for which more specific requirements exist apparently can be met only by Ca and Sr. The ability of Ca and Sr to restore the responsiveness of the muscle to K or caffeine after it had been lost in the presence of Mn, Ni or Co parallels the ability of Ca and Sr to activate contraction when injected intracellularly, to activate skinned fibres and the myofibrillar ATPase and the property of these ions of being accumulated and released by the sarcoplasmic reticulum. This correlation helps to identify the specific roles of Ca and Sr mentioned and it is in keeping with the proposed role of the ATPase activation and with the accumulation and release of Ca from the sarcoplasmic reticulum in the mechanical activation of muscle.

Frank (1962) has suggested that K contractures in presence of substitute cations are initiated by the release of Ca from some binding site in or on the muscle. A similar mechanism has been proposed for the action of caffeine by Bianchi (1961) who showed that the rate of ^{45}Ca efflux from ^{45}Ca loaded muscles is increased by this agent. It has also been found that caffeine initiates a release of ^{45}Ca from the vesicles of reticulum prepared from frog muscle (Herz & Weber, 1965). Sr has been shown by Frank (1962) to be able to replace Ca in maintaining the caffeine contracture in a Ca depleted muscle. Since Sr is taken up by the isolated sarcoplasmic reticulum it is probable that caffeine contractures in the presence of Sr are initiated by a release of the Sr previously accumulated in the sarcoplasmic reticulum.

It might be remarked that the lower affinity of the sarcoplasmic reticulum for Sr as compared to Ca may be expected to manifest itself in the mechanical behaviour of the muscle. However, a muscle may relax following Sr activation in spite of the fact that the reticulum is not capable of reducing the Sr concentration as much as the Ca level because the myofibrillar ATPase has a lower affinity for Sr. In a muscle whose ability to contract in response to caffeine had been restored by Sr the contracture may be smaller even though Sr can activate the ATPase to the same extent as does Ca because 30 times more Sr than Ca is required to saturate the myofibrils. Thus, if similar amounts of Sr and Ca are released the Sr would have less effect. In addition, the capacity of the reticulum for Sr might be less than for Ca.

Bianchi & Shanes (1959) have shown that a larger amount of ^{45}Ca is retained by muscle if a short period of bathing in a solution containing tracer ^{45}Ca is followed by depolarization with potassium. An increase in the amount of ^{85}Sr retained by the muscle under similar conditions has been

found but not of ^{58}Co. However, the amounts of ^{45}Ca or ^{85}Sr retained by the muscles do not seem to be sufficient for a maximum activation of the myofibrils (cf. Bianchi & Shanes, 1959). The meaning of these results therefore is not clear. The impossibility to demonstrate, with the technique used, an increased entry of ^{58}Co during K contractures does not necessarily mean that the enhanced entry of divalent ions other than Ca or Sr does not occur during potassium contractures. The uptake of Sr and Ca can be demonstrated due to their binding by the sarcoplasmic reticulum; however, since Co is not taken up by the reticulum it might have been eliminated from the muscle in the course of washout of tracer from the extracellular space.

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