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ACTIONS OF CALCIUM AND CERTAIN MULTIVALENT CATIONS ON POTASSIUM CONTRACTURE OF GUINEA-PIG'S TAENIA COLI

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SUMMARY

1. Using a calcium buffer system, the effect of severe calcium lack on the shape of K-contracture of the guinea-pig's taenia coli was studied. Under conditions of calcium lack, the initial phasic response was preferentially affected and it disappeared completely at concentrations below 10^{-7} M, while the ensuing tonic response persisted, though considerably diminished in size, even at the concentration of 10^{-8} M.

2. In calcium-free media, various multivalent cations, which according to Frank (1962) can support the K-contracture of a skeletal twitch muscle fibre in calcium-free solution, augmented the remaining tonic response, but did not restore the phasic response, when it was eliminated in calcium free environment.

3. When K-contractures were induced in normal calcium media, these cations produced, in contrast, an abolition of the phasic response together with a partial depression of the tonic phase. They also inhibited a part of the fully developed contracture. The last effect was no longer obtainable in calcium-free media.

4. It is concluded that the phasic response and a part of the tonic response of taenia coli depend upon the extracellular calcium for their initiation (and also for maintenance of tension in the case of the latter) and that the rest of the tonic response draws on a store of 'bound' calcium for its evolution.

INTRODUCTION

It is now generally believed that an increase in the intracellular concentration of ionized calcium following excitation in the membrane is the key factor for initiation of contraction of various types of muscles. In skeletal muscle, a continuous, membrane-limited tubular structure within

the muscle fibres, the endoplasmic reticulum, has been considered by many investigators to serve as a supply source of this ionized calcium. However, in smooth muscle, whose endoplasmic reticulum is reported to be rather poorly developed (Mark, 1956), there still exists the uncertainty whether the calcium necessary for contraction originates in similar structures as in skeletal muscle or whether it comes from outside the muscle fibres. In the present study, we attempted to elucidate the point, using calcium buffers and certain multivalent cations, which, according to Frank (1962), can support the development of potassium-induced contractures of a skeletal muscle preparation in calcium-free solution.

A preliminary report of these experiments has already been published (Imai & Takeda, 1967).

METHODS

Experiments were performed on the isolated taenia coli of the guinea-pig. The animal was killed by a blow on the head; its abdomen was opened and the colon exposed. A 15–20 mm *in situ* length of taenia coli was dissected free from the underlying tissue and suspended in a 5 ml. organ-bath, immersed in water kept at the temperature of $36 \pm 0{\cdot}3°$ C. The modified Locke solution used had the following composition (mM): NaCl, 154; KCl, 5·6; $CaCl_2$, 2·2; $NaHCO_3$, 8·0; dextrose, 5·5 and was equilibrated with a gas mixture of 95 % O_2 + 5 % CO_2. To make solutions of low stabilized concentrations of free ionized calcium, 5 mM of EGTA (Ethylene glycol bis(β-aminoethyl ether)-N, N′-tetraacetic acid) was used as a chelating agent and sodium bicarbonate was substituted by tris-maleate buffer (pH = 7·1, 5·0 mM). The solution was aerated with 100 % oxygen. The concentration of the free ionized calcium [Ca^{2+}] was calculated from the equation

$$pCa = 2pH - 7{\cdot}28 + \log\left(\frac{[\text{EGTA}]\text{ added}}{[CaCl_2]\text{ added}} - 1\right).$$

This is a sufficiently accurate approximation to the complete formal equation (Chabarek & Martell, 1959) in the pH range of 4·0–7·5, using $pK_{Ca} = 11{\cdot}0$, $pK_1 = 9{\cdot}43$ and $pK_2 = 8{\cdot}85$ (Schwarzenbach, 1955), provided that $[Mg^{2+}]/[Ca^{2+}]$ does not approach $K_{Mg}/K_{Ca} = 6{\cdot}3 \times 10^5$. These conditions are satisfied in the present experiments. As a model for the events occurring during a normal contraction of this muscle, potassium contracture (K-contracture) was adopted, which was produced by isotonic K_2SO_4-Locke solution ('K_2SO_4' Locke). This solution was prepared, replacing 154 mM-NaCl by 126 mM-K_2SO_4 (Evans, Schild & Thesleff, 1958). In some experiments a solution containing 126 mM-K_2SO_4 in addition to the normal ionic composition of Locke solution was used to produce K-contracture with unchanged results. Calcium sulphate was not added, since the main part of the present experiments was conducted in media with low concentrations of calcium of 10^{-6} M or less owing to the chelate formation by EGTA so that the solubility-product constant of the calcium sulphate could never be exceeded even in the presence of excess sulphate ions. Depending upon the size of the preparation, the muscle was equilibrated in the test solutions for 10–20 min before inducing K-contracture, and was allowed to recover for at least 15 min in a solution containing the normal concentration of potassium and calcium after each test. The tension developed in the contracture was recorded isometrically on a self-balancing potentiometric recorder (pen speed for full scale = 1·2 sec) with the aid of a strain-gauge transducer. The initial tension of the muscle was adjusted to 0·5–1 g. When necessary, the membrane potential of the preparation was recorded by the sucrose-gap method (Stämpfli, 1954; Burnstock & Straub, 1958).

RESULTS

Effect of calcium on the shape of K-contracture of taenia coli. As is illustrated in Fig. 1, the mechanical response of the taenia coli to 'K_2SO_4' Locke consisted of the two distinct phases: an initial rapid rise of tension (phasic response) and an ensuing tonic contraction, which lasted as long as the potassium concentration in the bathing medium remained high (tonic response). Pretreatment of the muscle with atropine sulphate (up to 10^{-5} g/ml.) or tetrodotoxin (up to 10^{-6} g/ml.) did not produce any ap-

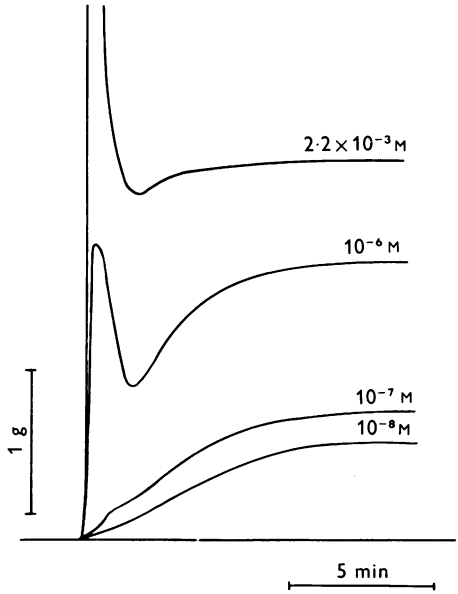

Fig. 1. Effects of low stabilized concentrations of extracellular calcium on the shape of K-contracture of the guinea-pig's taenia coli. K-contracture was induced by isotonic K_2SO_4 Locke solution ('K_2SO_4' Locke).

preciable effect on the shape of K-contracture. When the concentration of free ionized calcium in the bathing medium was reduced by addition of EGTA to the very low levels of 10^{-6}, 10^{-7} and 10^{-8} M, both phasic and tonic responses became smaller. However, the diminution of the phasic response was disproportionately greater and it disappeared completely when the concentration of the calcium was reduced to below 10^{-7} M, while the tonic response persisted, though diminished in size, even at a concentration of 10^{-8} M of calcium or less. The magnitude of the tonic phase in 10^{-8} M of calcium was on an average about one fourth of that in normal calcium media. Those in 10^{-6} and 10^{-7} M of calcium were approximately six tenths and one third, respectively. As is depicted in Fig. 2, the magnitude

of the persistent tonic response plotted against the concentrations of K_2SO_4 and hence the membrane potential gives a smooth sigmoid curve extending from the usual resting membrane potential to the potential of the full depolarization.

Simultaneous recording of the membrane potential by the sucrose-gap method revealed that, when there was a phasic mechanical response, there were always discharges of the spike potentials superimposed on the rising phase of membrane depolarization due to 'K_2SO_4' Locke (Fig. 3). These

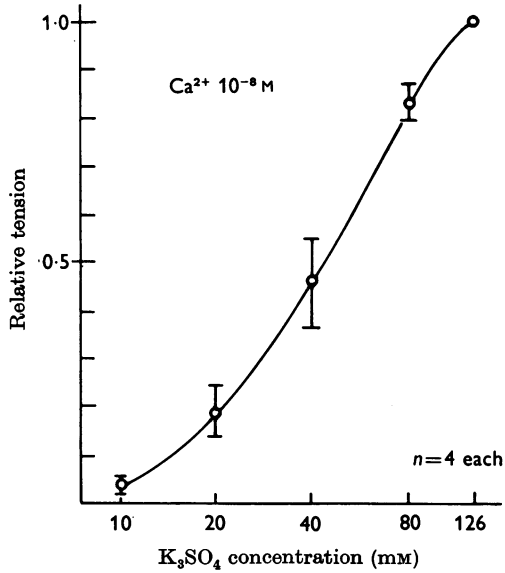

Fig. 2. Relation between the K_2SO_4 concentrations and the tonic tension in 10^{-8} M calcium media.

spike potentials were abolished in media with less than 10^{-7} M of calcium, together with the phasic response. This does not necessarily mean that all spike activities had ceased, since the spike potential recorded with the sucrose-gap method is actually the sum of the spike activities of a number of cells and a sychronized activity of a certain number of cells is needed, if the spike potentials are to be recorded. The asynchronization of the spike discharges due to a failure of conduction could be the cause of the observed quenching of the spike discharges. Anyway, it may be concluded that synchronized spike discharges were no longer obtainable when the calcium concentration was reduced to below 10^{-7} M.

It is true that introduction of taenia coli into media with very low concentrations of calcium (10^{-7} or 10^{-8} M) prepared by addition of EGTA invariably resulted in a reduction of the membrane potential, but the

depolarization was not maintained, and died away in the course of 15–20 min (Fig. 4). The similar transient depolarization following calcium deprivation was previously observed with micro-electrode in guinea-pig's taenia coli (Holman, 1958). The persistent depolarization at the steady

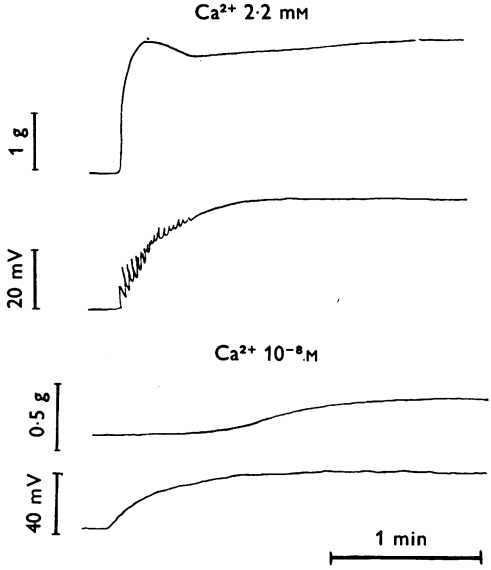

Fig. 3. Abolition of the spike potential evoked by 'K_2SO_4' Locke in media with 10^{-8} M of calcium. Sucrose-gap method. Upper curve: tension. Lower curve: Membrane potential.

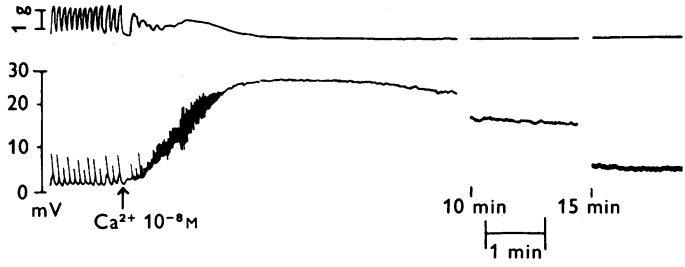

Fig. 4. Changes in the membrane potential and the tension under conditions of severe calcium lack (10^{-8} M). Sucrose-gap method.

state in the solution with 10^{-8} M of calcium directly read from the sucrose-gap records was found to be $6{\cdot}4 \pm 1{\cdot}2$ mV (mean $\pm$ S.E., $n = 16$), a figure not great enough to explain the observed quenching of the spike potentials on the basis of inactivation of the spike mechanism due to depolarization (Holman, 1958; Burnstock, Holman & Prosser, 1963). Moreover, the persistent depolarization may in fact be somewhat of an artifact, since a

change in the junction potential might suffice to explain partly the depolarization of this magnitude.

As is illustrated in Fig. 3, the magnitude of the membrane depolarization due to 'K_2SO_4' Locke was also diminished under conditions of severe calcium lack. The following numerical data may afford an approximate idea of how large the difference was, although too much stress should not be placed on these figures, since they were those directly read from the sucrose-gap records and no allowance was made for changes in junction potentials (Bennett & Burnstock, 1966). The maximum depolarization obtained with 10^{-8} M of calcium was $33{\cdot}3 \pm 1{\cdot}5$ mV (mean $\pm$ S.E., $n = 9$), as compared with $45{\cdot}7 \pm 2{\cdot}6$ mV ($n = 12$) in normal calcium media ($P < 0{\cdot}01$).

'Reactivation' effect of multivalent cations upon the K-contracture in calcium-free media. According to Frank (1962), addition of certain multivalent cations to calcium-free solutions bathing a toe muscle of the frog could restore the K-contracture of this muscle, after it had been eliminated by removal of the extracellular calcium ions. Using the sartorius muscle of the frog, we could confirm his findings (unpublished observation). Therefore, it was of interest to see whether the same phenomenon could be observed in the smooth muscles or not. To deprive the extracellular space of calcium, calcium-free Locke solution was used in this experiment, since the introduction of EGTA would result in chelate formation with these cations. Precautions were taken to ensure that the Ca-free Locke solution contained minimal calcium due to contamination. The water used for making calcium-free solution passed through an ion-exchange column and was glass-distilled afterwards. All glassware was rinsed with the deionized water having a specific resistance of less than 2×10^{-7} mho. Analysis of the calcium-free solution by Perkin-Elmer Model 303 Atomic Absorption Spectrophotometer showed that the calcium concentration was around 10^{-6} M. The initial phasic response of the K-contracture of taenia coli was abolished after soaking for $\frac{1}{2}$–1 hr in calcium free-media with repeated washings. This contradicts the findings obtained with the calcium buffer system. Presumably, the long-term exposure to calcium-deficient medium exerted some adverse effect upon the spike mechanism leading to the elimination of the spike potential. An alternative explanation would be that the membrane can bind calcium sufficiently powerfully to remove it from the EGTA complex, and that this calcium is somehow involved in the spike production. The ensuing tonic phase was rather resistant to calcium deprivation; even after soaking for 2 hr in calcium-free media with repeated washings, a definite contraction could still be elicited, in harmony with the findings obtained with EGTA-calcium buffers. Addition of certain multivalent cations under these conditions resulted in an augmentation

of the remnant tonic contraction, although the tension attained was never so great as it was in normal calcium media. Figure 5 illustrates the effect of 5 mM of cadmium ion given 15 min before inducing K-contracture. In addition to cadmium ions, cobalt, nickel, manganese and magnesium ions, in ranges of 1·0–10·0 mM, were tested and were found to be effective in restoring the tonic contractile response, although the potencies were variable with different ions ($Cd^{2+} > Co^{2+} > Ni^{2+} > Mg^{2+}$). It is true that manganese ions could restore the original level of the tonic phase, but the tonic phase of K-contracture continued to increase after manganese ion during the observation period of almost an hour, without attaining a steady state. Moreover, even after repeated washings with normal Locke

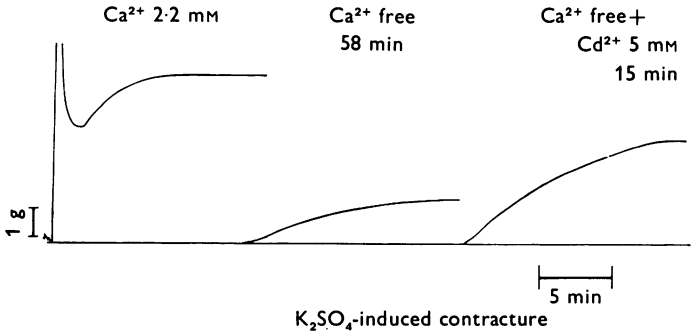

Fig. 5. 'Reactivation' effect of cadmium ion on the residual tonic mechanical response in calcium-free media.

solution, relaxation could hardly ever be obtained, indicating the participation of certain irreversible processes other than the 'reactivation' process in the action of this ion. The initial phasic response, which was abolished in calcium-free medium, was not restored by this procedure.

Effect of multivalent cations upon the K-contracture. In addition to the above described actions, these multivalent cations, when added to media with the normal concentration of calcium, produced characteristic changes in the shape of K-contracture induced thereupon, namely, the suppression of the phasic response and a partial inhibition of the ensuing tonic phase, in association with the abolition of the spike potentials evoked by 'K_2SO_4' Locke. Figure 6 depicts the action of 5 mM of cadmium ions. In this respect, magnesium and manganese ions differ a little from other ions. Magnesium ions, in doses between 1 and 10 mM, produced a slight potentiation of the tonic phase, with almost no inhibitory effect upon the initial phasic response. Manganese ions did suppress the phasic response, but it rather augmented the tonic phase (Fig. 7). Here again, some irreversible processes provoked by manganese ions seem to be playing a role, for the

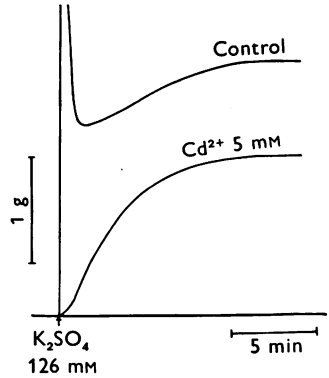

Fig. 6

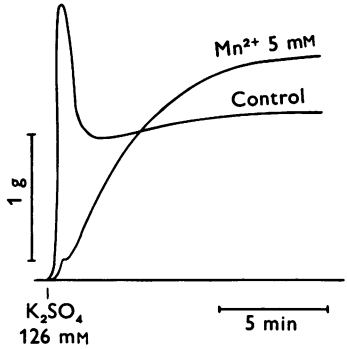

Fig. 7

Fig. 6. Effect of cadmium ion on the shape of K-contracture in normal calcium media.

Fig. 7. Effect of manganese ion on K-contracture.

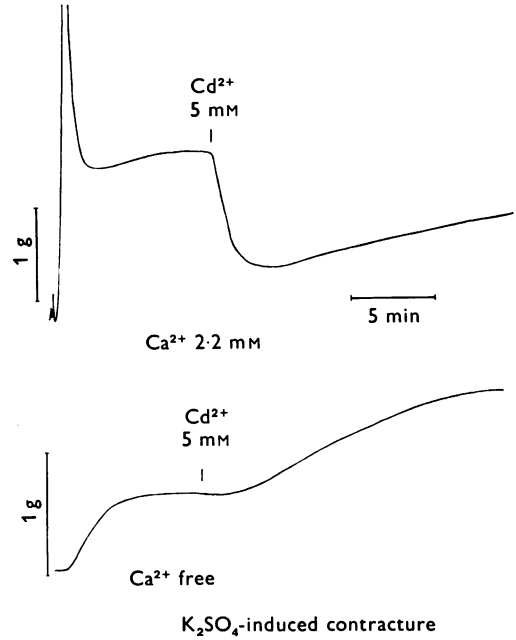

Fig. 8. Actions of cadmium ion on the fully developed K-contracture. Upper curve: In normal calcium media. Lower curve: In calcium-free media.

tonic phase grew bigger without reaching a steady level under the action of manganese ions.

Further, these cations, when administered to the muscle after the full development of the K-contracture, partially abolished the tonic response (Fig. 8). As is illustrated in Fig. 9, with 5 mM of manganese ions this re-

laxation was associated with only a slight increase in the membrane potential. However, the inhibitory action was not maintained and the tension started to rise again after the lapse of several minutes to reach a new steady level, which was usually a little lower than the initial level (Fig. 8). This secondary increase in tension was not accompanied by a change in the membrane potential. It was interesting to note that this relaxant effect was no longer obtainable if K-contracture was induced in calcium-free media; instead, we saw only the gradual rise of tension, the counterpart of the one which was seen in normal calcium media after the initial transient relaxation (Fig. 8).

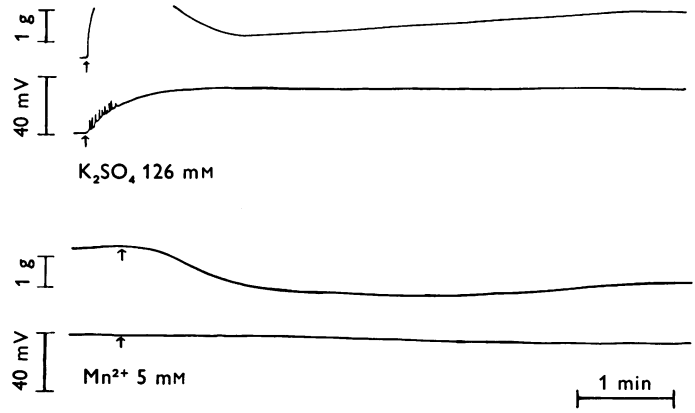

Fig. 9. The effect of manganese ion on the fully developed membrane depolarization due to 'K_2SO_4' Locke. Simultaneous recording of the membrane potential and tension. The lower two curves are continuous from the upper two.

DISCUSSION

As was first indicated by West, Hadden & Farah in 1951 and later confirmed by several investigators, the mechanical response of intestinal smooth muscle to high potassium medium consists of two distinct phases: an initial rapid contraction ('spike phase' or 'phasic response') and an resulting tonic contraction ('tonic phase'). When the concentration of calcium in the bathing medium was reduced to below 10^{-7} M, the initial phasic response was abolished, while the ensuing phase could still be elicited. Calcium is the only physiologically occurring cation which would cause shortening of the muscle when injected into the skeletal muscle fibres in low concentrations and it is now generally believed that a sudden rise of the intracellular calcium ion concentration is a key factor for initiation of contraction of the various types of muscles. According to recent studies with rabbit myofibrils, purified actomyosin of rabbits,

glycerinated skeletal and smooth muscles of the hog, intact leg muscle of a crab and the single muscle fibres of the barnacle, the minimum concentration of intracellular calcium ions needed for this activation lies slightly below 10^{-6} M (see Portzehl, Caldwell & Rüegg, 1964; Filo, Bohr & Rüegg, 1965; Hagiwara & Nakajima, 1966*b*). Therefore, to explain the resulting state of increased tone, which persisted in a bathing medium with lower than this threshold concentration of calcium, one must postulate the presence of a store of 'bound' calcium within the muscle fibre. By releasing calcium ions from this binding site and raising their intracellular concentration to an effective level, the smooth muscle can respond to high potassium medium with sustained increase in tension even under conditions of severe calcium lack. This hypothesis is in agreement with previous work indicating that 'bound' calcium is involved in the contraction of uterine smooth muscle (Edman & Schild, 1962; Daniel, 1963). Since the magnitude of the tonic phase, which persisted in the medium with 10^{-8} M calcium and hence was considered to make use of only the 'bound' calcium, was graded by the magnitudes of the membrane potential, ranging from the normal resting level to the potential of the complete depolarization, it may be inferred that the 'bound' calcium constitutes the basis of the so-called resting 'tonus' (Bozler, 1948) of the smooth muscle, which is considered to vary with the membrane potential. Incidentally, the preferential inhibition of the tonic phase was reported under many conditions to be possibly related with the inhibition of metabolism, such as glucose-deprivation, low temperature, and substitution of Na by lithium (Pfaffman, Urakawa & Holland, 1965). It seems, therefore, that the availability of 'bound' calcium is somehow regulated by metabolism.

The initial phasic response was abolished in bathing media with less than 10^{-7} M calcium. This phase of the K-contracture rightly named 'spike' phase by West *et al.* (1951), was found to be inseparably associated with synchronized spike activity, suggesting that the spike potential was operating as the trigger of this phase. Based on the inhibitory action of calcium on the phasic contraction of vascular smooth muscle, Bohr (1963) also concluded that the rate-limiting factor of this phase is membrane excitation. Recently, Nonomura, Hotta and Ohashi (1966*a*) reported on the inhibitory action of manganese ion upon the spike potential of the guinea-pig's taenia coli. According to them, tetrodotoxin did not produce any inhibitory effect at all. Later, Nonomura, Hotta, Tsukui & Ohashi (1966*b*) extended the previous findings and concluded that various multivalent cations belonging to the transition elements group all share in common the same inhibitory action on the spike potential. In the present study, we observed the abolition of the spike discharge induced by 'K_2SO_4' Locke, after addition of various multivalent cations. Since the calcium-spike of the

barnacle muscle fibres was found to be competitively inhibited by manganese ions, but not by tetrodotoxin (Hagiwara & Nakajima, 1966*a*), the data just cited suggest that the spike potential in taenia coli is calcium-linked, instead of sodium-linked as in other excitable tissues. If something like this is correct, the severe reduction of the concentration of the extracellular calcium would naturally result in an abolition of the spike potential, which, in turn, would lead to the elimination of the phasic contractile response, without any intervening processes. This may possibly be the situation we observed in media with less than 10^{-7} M of calcium, although the present data obtained with the sucrose-gap method cannot necessarily give a conclusive evidence (for discussion see p. 157). As for the mechanism linking the spike potential with the mechanical response, it may be postulated either that the 'bound' calcium is somehow released under the influence of the depolarization due to the spike potential or that the calcium-influx during the spike potential serves by itself as a trigger for tension development. The fact that the phasic mechanical response could still be elicited even at a concentration of calcium of 10^{-7} M is in favour of the first alternative, inasmuch as the threshold concentration of calcium for the manifest tension development has been shown to be close to 10^{-6} M, although this does not rule out the second alternative. The work by Urakawa & Holland (1964) also favours the first view. They studied the calcium movement during K-contracture of the isolated taenia coli of the guinea-pig and found that both the phasic and the tonic responses were associated with an increased rate of ^{45}Ca entry. As they could observe a rise of tissue calcium content only during the tonic phase, they concluded that in the phasic response calcium was released from a cellular site, whereas in the tonic response calcium crossed the membrane to maintain contraction. Anyway, the phasic contractile response is dependent upon the calcium-influx and hence upon the presence of the adequate amount of calcium in the extracellular space, for the spike potential is indispensable for its initiation.

Though persistent in low calcium media, the magnitude of the remaining tonic phase became progressively smaller as the extracellular concentration of calcium was reduced. This may be partly due to the less effective depolarizing effect of 'K_2SO_4' Locke in calcium deficient media. However, in view of the considerable reduction of the tonic contractile response, it is apparent that we should elaborate, in addition, some other explanations.

Based on Hodgkin & Horowicz (1960), Lüttgau (1963) explained the phasic nature of the mechanical response of skeletal twitch muscle fibres to high potassium medium, using the same terminology as Hodgkin & Huxley (1952) used to explain the transient increase in sodium permeability

upon excitation. According to Lüttgau, the contraction-activating system of the skeletal twitch muscle fibres becomes 'inactivated' in a characteristic manner depending upon the magnitude of the membrane potential, and calcium ions produce a shift of this inactivation curve in such a direction that more polarization is needed in low calcium media to maintain the contractility at the same level as it is in normal calcium media. In other words, at the same membrane potential, greater portion of the contraction-activating system becomes 'inactivated', when the calcium concentration in the bathing fluid is reduced. If the same holds true for smooth muscles, the decreased tonic response may be explained by assuming the development of a similar 'inactivation' process. Recently, Frank (1962) demonstrated that a large number of multivalent cations could restore K-contracture of the skeletal twitch muscle fibres when it was abolished in calcium-free media. Some of the most effective of these were: Cd^{2+}, Be^{2+}, Mg^{2+} and Sr^{2+}. Frank's interpretation of this phenomenon was that these cations could release calcium from a binding site on the muscle fibres and the released calcium then took the place of the extracellular calcium ions lost by diffusion into the calcium-free bathing solution. However, in the light of Lüttgau's interpretation just cited, more plausible explanation for this phenomenon would be that these cations removed the 'inactivation' process which had developed under conditions of severe calcium lack. Whichever interpretation we may take, it is apparent that the reduction of the mechanical response in low calcium media does not necessarily mean that the passive transfer of the calcium ion is involved. That is the reason why we examined the effect of the multivalent cations in the present experiment. Addition of these cations to calcium-free media bathing the taenia coli resulted in a partial recovery of the reduced tonic phase, indicating the presence of a similar 'inactivation' process of the contraction-activating system in this smooth muscle preparation as that of the skeletal twitch muscle fibres. However, the 'reactivation' was never complete. Different from the skeletal twitch muscle fibres, the tonic phase of K-contracture in taenia coli persisted without any decline, as long as the potassium concentration in the bathing solution remained high. The longest observation we made extended for almost an hour. Thus, the magnitude of the membrane polarization seems to exert no influence on the 'inactivation' process of this smooth muscle preparation. Only the presence of an adequate amount of calcium was essential. This disparity of the contraction-activating system may explain, at least in part, why the 'reactivation' by multivalent cations was not complete. However, when we think of the following two facts, another interpretation seems to be more convincing. First, the pretreatment of the muscle with certain multivalent cations resulted in a partial inhibition of

the tonic response to 'K_2SO_4' Locke, as well as a complete abolition of the initial phasic response. Second, these cations, when given to the muscle after the full development of K-contracture, produced a partial abolition of the tonic contractile tension. The inhibition was not maintained, but was followed after the lapse of time by a gradual recovery. When calcium was removed from the extracellular space, the initial transient relaxant effect was abolished, while the ensuing gradual tension increase remained. It has been pointed out in the earlier part of this discussion that multivalent cations may inhibit the calcium-influx during the spike potential. Nonomura *et al.* (1966*a*) referred briefly to their finding that the contractile response of the fully depolarized taenia coli to acetylcholine was also abolished by manganese ion. The same action was found in our laboratory with several multivalent cations other than manganese (unpublished observations). Since the first report by Evans *et al.* (1958) on the feasibility of contraction in the fully depolarized smooth muscle, not a few investigators have demonstrated that, associated with the contraction of depolarized muscle there was an inward movement of calcium across the muscle fibre membranes and that this calcium-influx might constitute the basis of the ability to contract, present in fully depolarized muscle. If this assumption is right, the data of Nonomura *et al.* (1966*a*) may be taken as evidence indicating that multivalent cations possess an inhibitory action on the calcium-influx not associated with the spike activity, in addition to their inhibitory action on the calcium-influx during the spike potential. Thus, the multivalent cations are capable of depressing the calcium-influx in general, and those portions of K-contracture which were inhibited by these cations may be attributed to an inward movement of calcium from outside the muscle fibres. As already mentioned (p. 164), Urakawa & Holland (1964) also reached the same conclusion, based on their observations of the movement of the radioactive calcium. The fact that the relaxant effect of these cations on the maintained phase of K-contracture was abolished in calcium-free media is compatible with this interpretation, for the calcium-influx was no more obtained in calcium-free media. Under these conditions, only the 'reactivation' effect of these cations came on to the stage, which we saw as a gradual rise of tension.

To summarize, the K-contracture of taenia coli could consistently be explained in the following way: The initial phasic response is inseparably associated with the spike potential and hence with the calcium-influx, which functions as the trigger and somehow liberates the 'bound' calcium into the muscle fibres. A portion of the ensuing tonic phase is dependent upon the inward movement of calcium from the extracellular space, while the rest is brought about by the release of 'bound' calcium due to the sustained membrane depolarization.

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