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THE EFFECT OF CAFFEINE ON CALCIUM EFFLUX AND CALCIUM TRANSLOCATION IN SKELETAL AND VISCERAL MUSCLE

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SUMMARY

1. KCl-induced depolarization resulted in a large stimulation of the ^{45}Ca efflux from both cockroach skeletal muscle and rat ileal smooth muscle.

2. Caffeine (10 mM) induced a large stimulation of ^{45}Ca efflux from skeletal muscle, but a fall in the efflux from ileal muscle, especially if the efflux was previously stimulated by KCl depolarization.

3. Caffeine inhibited calcium uptake by skeletal muscle mitochondria and sarcoplasmic reticulum, was without effect on ileal muscle mitochondria, but significantly increased calcium binding by ileal muscle membrane vesicular preparations.

4. The induction of contractures and stimulation of ^{45}Ca efflux in skeletal muscle by caffeine are clearly related to inhibition of intracellular calcium binding by the sarcoplasmic reticulum and mitochondria.

5. The relaxation of ileal muscle by caffeine and the inhibition of fibre calcium efflux correlate well with caffeine enhancement of intracellular calcium binding. These experiments suggest that the membrane vesicular compartment may be the main agency centrally involved in fibre calcium regulation in this muscle during the contraction–relaxation cycle.

INTRODUCTION

In vertebrate skeletal muscle there is ample evidence that contraction is associated with an efflux of calcium from the fibres (Bianchi & Shanes, 1959; Frank, 1962; Weiss, 1966; Isaacson & Sandow, 1967*a*; Isaacson, 1969; Chen *et al.* 1972), this efflux being related to calcium translocation within the fibres. Alkaloid drugs such as caffeine and quinine, which induce contractures in vertebrate skeletal muscle, have been shown to stimulate fibre calcium efflux, indicating that their contracture effect is related to a steep rise in myoplasmic free calcium (Bianchi, 1961; Frank, 1962; Isaacson & Sandow, 1967*a*, *b*; Isaacson, 1969; Chen *et al.* 1972). Very little is known about calcium movements in invertebrate skeletal muscle, either in normal or drug-treated conditions, although several reports have now shown that alkaloids do induce contractures (Huddart, 1969*a*, *b*, 1971*a*; Bittar *et al.* 1974).

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In visceral muscle, great confusion surrounds the actual action of alkaloid drugs. In some cases caffeine induces contractures (Ito & Kuriyama, 1971; Siegman & Gordon, 1972) while in other cases caffeine has been clearly shown to depress activity (McFarland & Pfaffman, 1972; Sakai & Iizuka, 1972). There have, as yet, been no studies on the effect of alkaloids on calcium efflux from visceral muscles, although the effect of KCl-induced depolarization on the efflux has been studied (Sperelakis, 1962; Briggs, 1962; Chujo & Holland, 1963; Urakawa & Holland, 1964). In this paper we report the results of experiments on both invertebrate skeletal muscle and vertebrate smooth muscle, where the relationship between the contractile actions of caffeine and its actual effect on calcium efflux from the fibres has been investigated and compared.

The efflux of calcium from muscle fibres is of significance in that it is a measure of the fibre free calcium concentration and the extent of calcium translocation between the various fibre intracellular compartments. In order to detect more precisely the loci of caffeine action within the individual muscle fibres, we have examined the effects of caffeine on calcium uptake and release by the main fibre compartments thought to be responsible for calcium translocation during the contraction–relaxation cycle. Very little is known about the centres of calcium storage involved in contraction activation in smooth muscle, and it is hoped that this approach may cast light on the actual mechanism of calcium translocation during the excitation–contraction coupling cycle.

MATERIALS AND METHODS

Efflux experiments

The smooth muscle preparation used was from the longitudinal muscle of the ileum of Wistar strain albino rats. The longitudinal smooth muscle layer of the ileum was removed from mesentery-free 2 cm strips by gently dissecting this layer away from the underlying circular muscle layer. Isolated longitudinal strips were maintained in Krebs solution containing (in mM) NaCl 120·7, KCl 5·9, $CaCl_2$ 2·5, $MgCl_2$ 1·2, Na_2HPO_4 1·2, $NaHCO_3$ 15·5, glucose 11·5. This solution was maintained at 37 °C throughout the dissection and subsequent treatment and was aerated with 95 % O_2 and 5 % CO_2.

Small strips of isolated longitudinal smooth muscle were loaded for 1 h in ^{45}Ca incubation media which consisted of normal Krebs saline containing ^{45}Ca (4μ Ci/ml). After incubation, the preparations were quickly washed free from surface contamination by three rapid rinses in Krebs saline, blotted on moist filter paper, and each preparation was placed in an Eppendorf pipette tip connected to a continuous flow pump which delivered Krebs saline from a reservoir at a rate of 5·5 ml/min. The saline passing over the muscles was collected at 10 min intervals (5 min for the first two fractions) on a conventional fraction collector. The ^{45}Ca activity of aliquots of the various fractions was determined in a Packard liquid scintillation counter, using Instagel as a solubilizer.

After effluxing, the preparations were ashed, dissolved in HCl, and the remaining activity was estimated by liquid scintillation counting. From these data the rate coefficient was calculated for each fraction, the rate coefficient being defined as the percentage of the average activity in the muscle that had emerged per minute during the collection interval (Isaacson & Sandow, 1967*a*).

The skeletal muscle preparations for these experiments consisted of small bundles of fibres (5–10 fibres) dissected from the coxal muscles of the cockroach (*Periplaneta americana*). The isolated fibre bundles were loaded and the efflux and rate coefficients determined in the same manner as described above for smooth muscle preparations.

For efflux experiments involving KCl depolarization, Krebs saline or insect saline containing 100 mM KCl, was substituted for normal saline in the constant flow apparatus reservoir. The experimental saline was prepared by substituting KCl for NaCl to maintain isotonicity with the muscle fibres. For salines containing caffeine, the caffeine was dissolved directly in the saline immediately prior to substitution in the flow apparatus.

Isolation of muscle fractions

Mitochondrial and sarcoplasmic reticular preparations were isolated from cockroach skeletal muscle by homogenization in KCl-imidazole buffer. Mitochondria were sedimented at 8000 *g* and sarcoplasmic reticulum at 28000 *g*. Both pellets were resuspended and purified by recentrifugation. The exact details of the methods used and the tests for purity have been published elsewhere (Huddart, Greenwood & Williams, 1974). The visceral muscle fractions used were the mitochondria and the membrane vesicular pellets. After homogenization of the isolated longitudinal ileal muscle, these fractions were sedimented at 8000 and 28000 *g* respectively.

Calcium uptake by muscle fractions

All isolated muscle fractions were resuspended in extraction buffer at pH 6·8, and adjusted to a concentration of 1 mg protein/ml, determined by standard biuret analysis.

All fractions were prepared in the absence of ATP, and they were allowed to take up ^{45}Ca in the incubation medium of Nakamaru & Schwartz (1972), consisting of 4 mM $MgCl_2$, 2 mM disodium ATP, and 40 mM tris-maleate buffer at pH 7·0, containing 0·1 μCi/ml ^{45}Ca. The calcium concentration of this loading medium was varied from 1 to 4 mM to provide a suitable range over which to determine Ca uptake. After incubation, the muscle fractions were collected by passage through a membrane filter with 0·45 μm pore size, and the material was washed with normal buffer. Dried filters were added to Instagel and counted in a Packard liquid scintillation counter, blank filters being used as controls. Further details of these methods have already been published (Huddart *et al.* 1974).

RESULTS

Depolarization-induced efflux

That calcium efflux is indeed associated with depolarization and activation of both skeletal and ileal smooth muscle is shown by the experiments summarized in Fig. 1. Here it can be seen that after effluxing for between 90–110 min (at which point the slow phase of efflux of intracellular origin is established), addition of 100 mM KCl to the medium results in a large rise in the rate of ^{45}Ca efflux from both types of preparation. In the case of skeletal muscle, the calcium efflux stimulation was from a rate coefficient of 0·2 %/min to 0·35 %/min, the corresponding figures for ileal smooth muscle being 0·2 %/min to 1·25 %/min. These figures refer to the ^{45}Ca activity in the fractions collected immediately after the addition of KCl. In ileal muscle, the stimulation

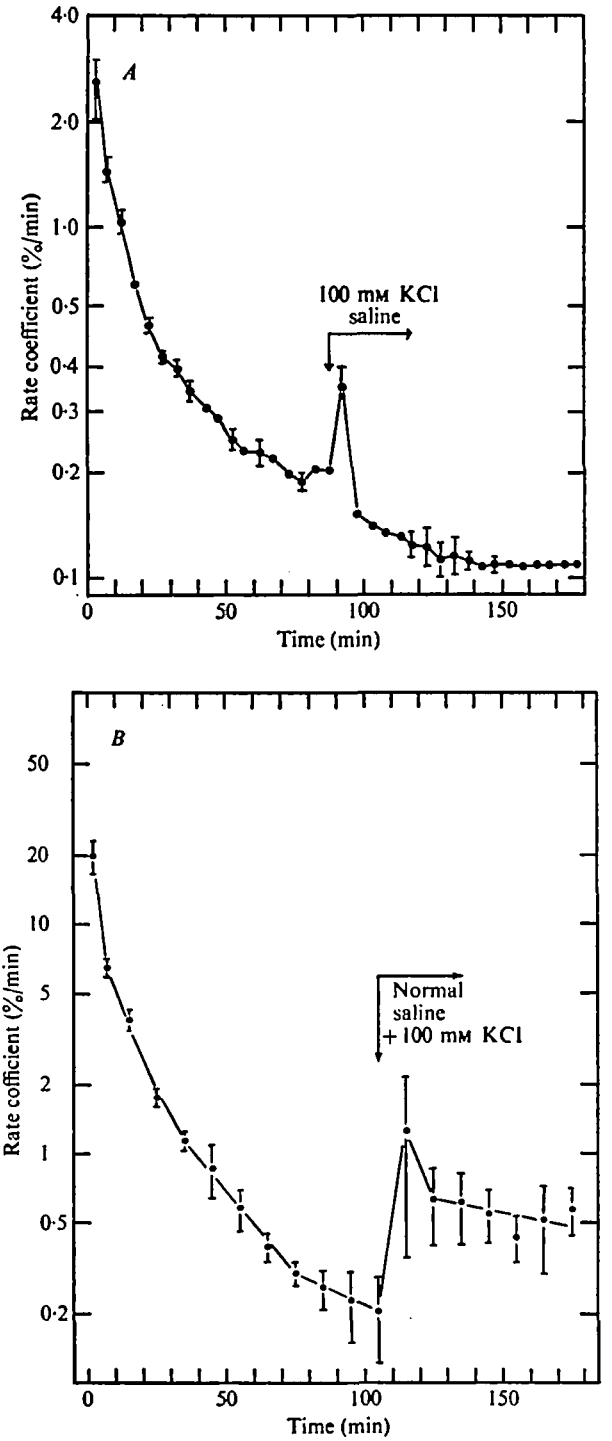

Fig. 1. *A*, the effect of KCl-induced depolarization on the ^{45}Ca efflux from cockroach skeletal muscle fibres. *B*, the effect of KCl-induced depolarization on the ^{45}Ca efflux from isolated rat longitudinal ileal smooth muscle. In both graphs, each point represents the mean ±S.E. ($n = 6$). Note in both cases the large stimulation of efflux by KCl.

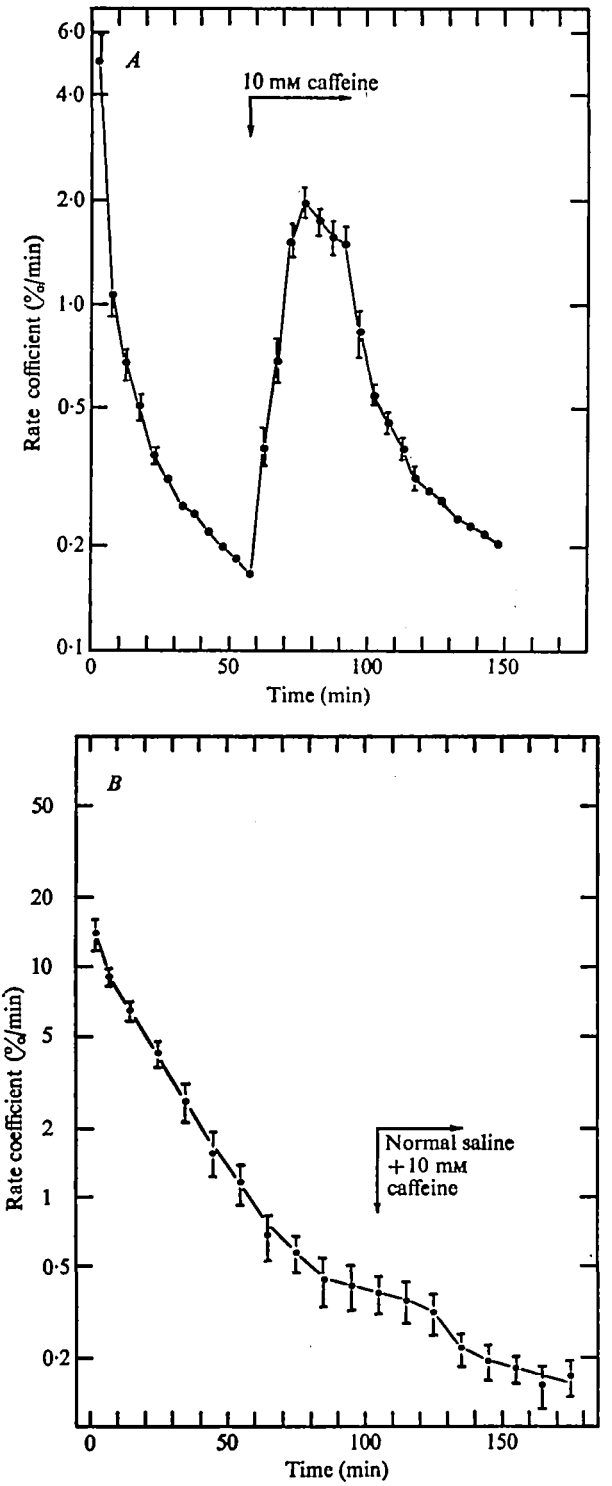

Fig. 2. The effect of 10 mM caffeine on the ^{45}Ca efflux from (*A*) cockroach skeletal muscle and (*B*) rat ileal smooth muscle. Each point represents the mean ±S.E. (n = 6). Note the massive caffeine-induced stimulation of efflux in skeletal muscle and the slight inhibition in ileal muscle.

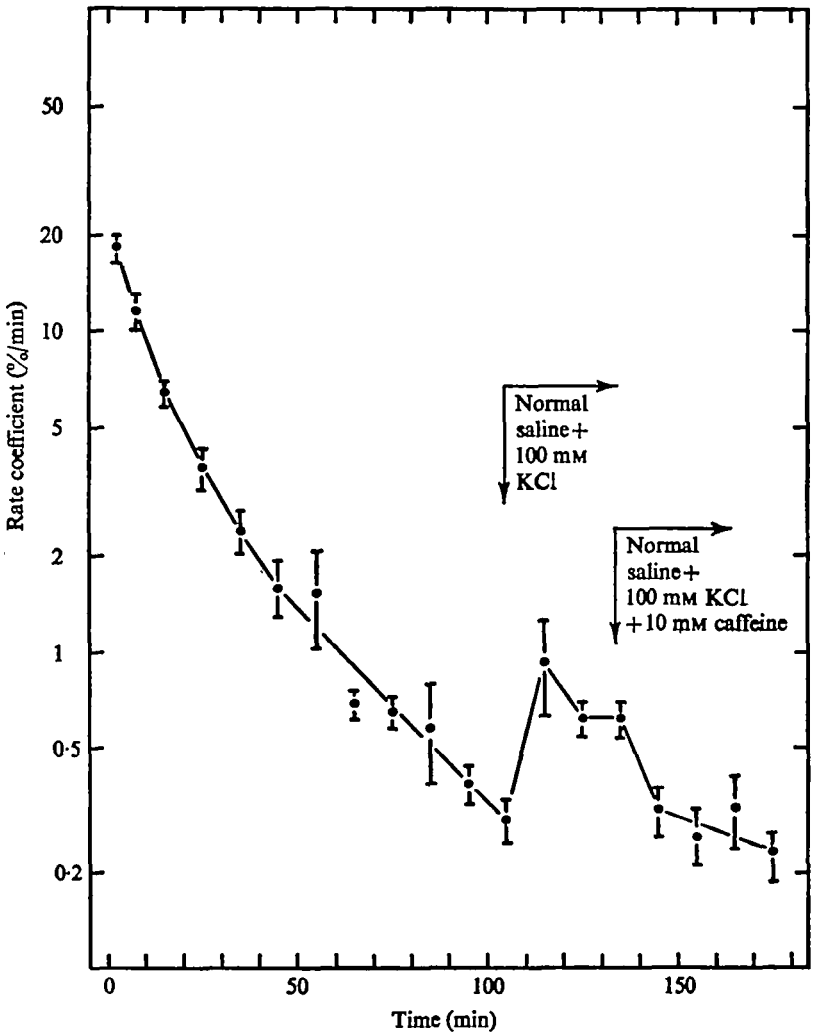

Fig. 3. The effect of 10 mM caffeine on the ^{45}Ca efflux from rat ileal smooth muscle, depolarized prior to caffeine application with KCl. Note that even in the presence of KCl efflux stimulation, caffeine causes a sharp inhibition of efflux. Each point represents the mean ±S.E. (n = 6).

of calcium efflux in the subsequent fractions remained fairly high (at a mean of 0·55%/min) for the rate coefficient, but in skeletal muscle fibres, the KCl-induced ^{45}Ca efflux stimulation was extremely transitory, the rate coefficient very rapidly returning to normal levels.

Effect of caffeine on calcium efflux

The effect of 10 mM caffeine (the concentration required to induce contractures in skeletal muscle) on the ^{45}Ca efflux from insect skeletal muscle can be seen in Fig. 2*A*. When this alkaloid was added during the slow intracellular compartment efflux phase, a massive stimulation of ^{45}Ca efflux was seen, from a mean rate coefficient of 0·17%/min to 1·9%/min. Unlike the response to KCl addition, the stimulation of efflux

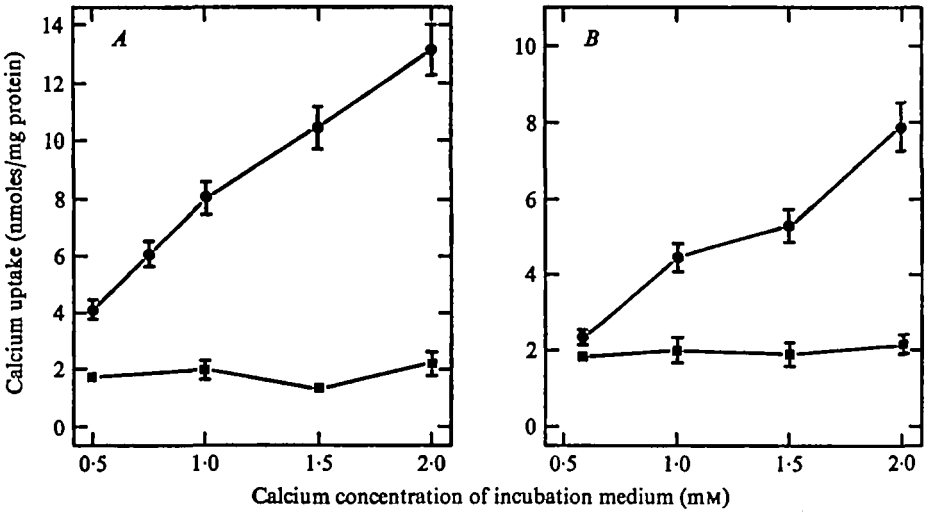

Fig. 4. Calcium uptake by (*A*) sarcoplasmic reticulum and (*B*) mitochondria isolated from cockroach skeletal muscle. Filled circles represent control conditions, filled squares with 10 mM caffeine. Notice that with both preparations, calcium uptake is greatly inhibited by caffeine. Each point is the mean ±S.E. (n = 10).

was not immediate, taking about 20 min to achieve peak conditions, but this high efflux stimulation effect was maintained during the time of caffeine application to the fibres, and recovery to normal efflux levels was relatively slow.

The addition of 10 mM caffeine during the slow efflux component of ileal longitudinal smooth muscle (Fig. 2*B*) caused little alteration to the efflux rate coefficient in complete contrast to the situation in skeletal muscle. Further insight into the actual action of caffeine on ileal smooth muscle was obtained when preparations were first exposed to the depolarizing action of 100 mM KCl prior to caffeine addition. The results of these experiments are shown in Fig. 3. Whereas KCl addition clearly results in a sharp increase in the rate coefficient of ^{45}Ca efflux, the addition of 10 mM caffeine in the *continued* presence of this depolarizing agent induces a fall in the efflux stimulation. In this preparation, KCl presence causes a sustained increase in ^{45}Ca efflux (Fig. 1*B*), hence it is obvious that caffeine is here causing an actual decrease in efflux, by some form of promotion of intracellular calcium binding. The effect of caffeine on ileal smooth muscle is thus the exact reverse of its action on skeletal muscle.

Calcium uptake and release by muscle fibre fractions

The experiments described above clearly indicate that caffeine has quite different actions on the ^{45}Ca efflux from skeletal and smooth muscle. The question therefore arose as to whether this alkaloid affected fibre calcium translocation in these muscles in different ways or whether the translocation mechanism itself was different in these muscles. In order to explain caffeine action on gross fibre efflux it became necessary to examine the action of caffeine on calcium movements within the fibre itself. To characterize the action of caffeine on calcium translocation at the intracellular level, the various calcium-sequestering subcellular fractions suspected of being involved in calcium movements during the contraction–relaxation cycle were isolated from the

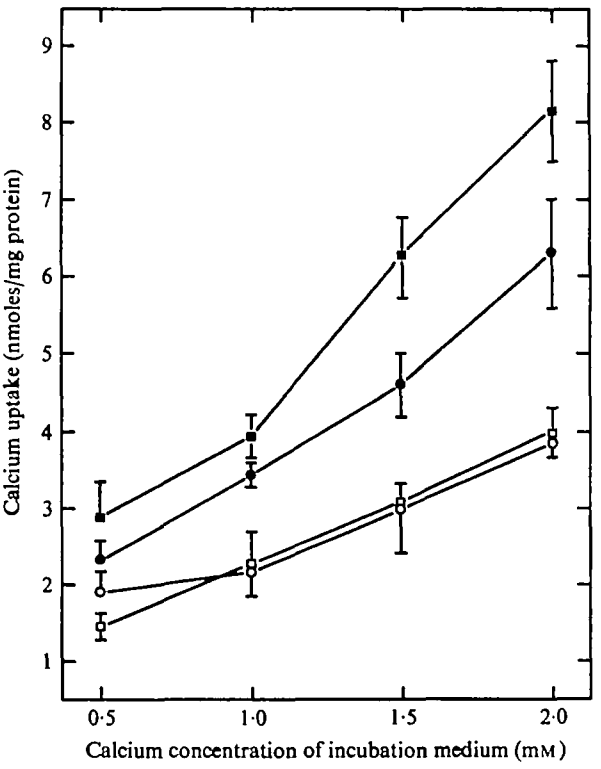

Fig. 5. The effect of caffeine on calcium uptake by mitochondria and plasma membrane vesicular fractions isolated from ileal longitudinal smooth muscle of rat. Filled circles, reticulum control; filled squares, reticulum plus caffeine. Open circles, vesicular fraction control; open squares, vesicular fraction plus caffeine. Each point represents the mean ±S.E. (n = 6). Note the lack of caffeine effect on mitochondria but the significant caffeine enhancement of calcium uptake in the membrane vesicular fraction.

muscle fibres. The calcium binding capacity of these fractions was determined over a wide range of calcium concentrations both in the absence and presence of caffeine. In the case of skeletal muscle, the mitochondria and sarcoplasmic reticulum were chosen for investigation, while in ileal muscle, in the absence of an organized sarcoplasmic reticulum, the mitochondria and membrane vesicular fractions were studied. This latter fraction has been shown to possess calcium-sequestering properties (see Syson, 1974).

Fig. 4 shows the effect of caffeine on calcium uptake by skeletal muscle sarcoplasmic reticulum and mitochondria. Both fractions, under control conditions, bound calcium in a manner similar to that demonstrated in mammalian sarcoplasmic reticulum (Carvalho, 1968*a*; Nakamaru & Schwartz, 1972), and it can be seen that in the presence of 10 mM caffeine, calcium uptake was considerably depressed over the whole calcium range employed. Comparable results from ileal longitudinal smooth muscle vesicular and mitochondrial fractions can be seen in Fig. 5. Here, as in the case of skeletal muscle, the mitochondria bind calcium far less avidly than the other fraction investigated. In the case of smooth muscle mitochondria, 10 mM caffeine is clearly without significant effect on calcium binding, and since binding is only slight even in control

conditions it seems unlikely that mitochondria are significantly involved in fibre calcium translocation during the contraction–relaxation cycle.

The behaviour of the membrane vesicular fraction from this preparation is of considerable interest since caffeine clearly significantly *increases* calcium binding, the reverse of the skeletal muscle condition. Since caffeine action on intact fibres is to inhibit ^{45}Ca efflux, associated with relaxation of the fibres (Syson, 1974), it is probable that the membrane vesicular fraction of the fibres is the compartment most centrally involved in fibre calcium modulation during contraction and relaxation.

DISCUSSION

In the interpretation of efflux data it is essential to be certain just what calcium efflux measurements mean in terms of myoplasmic calcium concentration. In theory, application of an external agent could stimulate efflux by augmenting calcium release from intracellular sites or by increasing influx from the bathing medium. In these experiments, the ^{45}Ca loaded preparations were continually bathed in isotope-free saline, so it is difficult to see how any potential influx of calcium from this medium could influence ^{45}Ca efflux during drug application. Furthermore, efflux measurements made in calcium-free external salines differ little from those in normal calcium media (Bittar *et al.* 1974; Huddart, unpublished observations). This evidence suggests that drug-induced stimulation of efflux results from an increased myoplasmic free calcium content, derived from intracellular sites.

It is clear that in both insect skeletal muscle and mammalian ileal smooth muscle, depolarization and subsequent contractile activation is intimately associated with calcium efflux from the fibres. These two types of muscle, however, differ considerably both in the sensitivity of their intracellular calcium binding sites to KCl depolarization and in the total kinetics of calcium mobilization during activation. KCl-induced depolarization results in a massive increase in calcium efflux in ileal muscle, but this increase is slower in onset and far more protracted in duration than that seen in insect skeletal muscle. This is most probably related to the great natural differences in the time course of their contractile responses, ileal muscle exhibiting slower and more sustained contractions than insect skeletal muscle. The differences seen in the time course of onset and duration of efflux stimulation caused by KCl and caffeine in these two types of muscle could simply be a measure of the relative permeability of the fibre membranes to potassium and caffeine. Additionally, there may be differences in the sensitivity to caffeine and KCl of the calcium binding sites in these muscles since there is reason to believe that the actual sites of calcium binding in visceral muscles may differ from those in skeletal muscle (see Somlyo, 1972; Huddart, 1975). What is clear, however, is that in both types of muscle, a rise in myoplasmic free calcium is the central agency responsible for the activation of contraction.

Caffeine application to these two types of muscle results in quite different ^{45}Ca efflux responses. In the skeletal muscle, where a massive caffeine-induced stimulation of calcium efflux is seen, this is obviously associated with the contracture-induction effect of this drug, a phenomenon previously reported in both vertebrate (Bianchi, 1961; Isaacson & Sandow, 1967*a*, *b*) and arthropod (Huddart, 1969*a*, *b*; Huddart & Abram, 1969; Chen *et al.* 1972) skeletal muscle. In the ileal smooth muscle preparation,

caffeine application slightly inhibits ^{45}Ca efflux, but if the preparations are previously depolarized by KCl in order to stimulate ^{45}Ca efflux, a dramatic inhibitory effect of caffeine on the efflux is seen. This is a situation which is in complete contrast to caffeine action on skeletal muscle.

The experiments on calcium uptake by isolated muscle cell fractions were designed to detect the loci of calcium binding in these two muscles, and the effect which caffeine had on these muscle compartments responsible for cyclical modulation of myoplasmic free calcium. The results from skeletal muscle material show that caffeine considerably inhibits normal calcium uptake in both sarcoplasmic reticulum and mitochondria. Our experiments show that isolated sarcoplasmic reticulum has a far greater calcium binding capacity per unit weight of protein than does the mitochondrial fraction. However, the greater overall mitochondrial weight in the muscle fibres (see Huddart, 1971*b*) suggests that mitochondria may play a significant role in the modulation of muscle cell calcium during the contraction–relaxation cycle. This latter view, which is still somewhat speculative, has received support from recent work on a variety of different skeletal muscles (Batra, 1974; Bittar *et al.* 1974; Huddart, 1975). The inhibitory effect of caffeine on reticular and mitochondrial calcium binding is clearly related to the caffeine contracture induction effect and the stimulation of intact whole fibre ^{45}Ca efflux.

In the case of ileal smooth muscle, caffeine is without effect on mitochondrial calcium uptake, but it significantly enhances the calcium binding by the membrane vesicular fraction. Mitochondria have recently been proposed as a possible locus of calcium binding and release for the purposes of excitation–contraction coupling in some smooth muscles (Somlyo, 1972). The results of the experiments reported here, on the other hand, suggest that mitochondria probably play little role in the cyclical modulation of fibre free calcium. Not only is their uptake small, but caffeine itself, which induces strong relaxation in this muscle, has no effect on binding. The membrane vesicular compartment, however, must certainly be considered as a source of calcium for excitation–contraction coupling purposes. The caffeine-induced increase in ^{45}Ca binding by this fraction, which correlates well with the caffeine-induced relaxation of the muscle and the inhibition of whole fibre ^{45}Ca efflux makes this suggestion plausible. Experiments must, however, be extended to other types of smooth muscle to check for a correlation between vesicular binding and contractile activation or inhibition.

The exact way in which caffeine affects the myoplasmic calcium pool of visceral muscle is little understood. Methylxanthines such as caffeine and theophilline are known to inhibit the phosphodiesterase system, resulting in raised levels of adenosine 3,5-monophosphate (cyclic AMP) (Sutherland & Robison, 1966; Pastan & Perlman, 1971). Raised levels of cyclic AMP are known to be involved in the relaxation of visceral muscle (Robinson, Butcher & Sutherland, 1967; Aberg & Andersson, 1972) as seen typically with beta adrenergic amines, a situation in complete contrast to that in skeletal muscle where c-AMP injection stimulates activity (Bittar *et al.* 1974). Much of the available evidence does seem to suggest that cyclic AMP may stimulate intracellular binding sites to accumulate calcium and prevent or reduce the release of membrane bound calcium (see Somlyo & Somlyo, 1968; Somlyo, 1972) resulting in relaxation, although visceral muscles do differ among themselves in their responses to

caffeine (Robison *et al.* 1971). In our experiments on rat ileal muscle, caffeine significantly stimulates membrane vesicular calcium binding (Fig. 5), but the situation is so little understood as yet to directly implicate an increased level of cyclic AMP in this action.

Why caffeine should have opposite effects on the Ca^{2+}/Mg^{2+} activated ATPase binding sites in ileal smooth muscle and in skeletal muscle is not clear. Preliminary studies of skeletal muscle sarcoplasmic reticular proteins separated from the reticulum by polyacrylamide gel electrophoresis (Huddart, 1975) have shown that caffeine causes a virtual elimination of the 96500 mol. wt. ATPase band and the 42000 mol. wt. calsequestrin band (see MacLennan, Ostwald & Stewart, 1974). This finding is consistent with the observed caffeine-induced inhibition of reticular calcium binding. No studies have yet been published on the protein constituents of the membrane vesicular calcium transport system of smooth muscle. This work is now under way in our laboratory since the effect of caffeine on the proteins of this compartment must be examined before an explanation can be offered to explain caffeine-induced promotion of calcium binding.

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