Evidence of Calcium Regulation by Mitochondria in Smooth Muscle

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(Received September 8, 1975)

ABSTRACT

The effects of caffeine on the calcium regulation by mitochondria were studied in connection with the contracture tension of the stomach smooth muscle.

Electron microscopic observation showed that the mitochondria in smooth muscle could take up calcium ion when the muscle was soaked in the excess calcium Ringer solution; in such solution intramitochondrial electron dense precipitates, probably calcium compounds due to either oxalate or pyroantimonate, were increased in the presence of caffeine. Their appearances exhibit electron dense granules, some of them showed annular aggregates. These results strongly suggest that the mitochondria in smooth muscle cell can function to regulate the cytoplasmic ionized calcium.

Physiological study showed that no contracture was induced by soaking the muscle in Ringer's solution containing caffeine (1 to 10 mM); prolonged exposure of the muscle to caffeine Ringer caused an appreciable reduction of the contracture tension which was induced by means of either electrical stimulation or potassium-depolarization.

It is hypothesized that the reduced tension caused by caffeine would primarily result from the greater accumulations of calcium by the mitochondria which in turn lead to the decrease of intracellular available calcium. Massive accumulation of calcium in the mitochondria might also cause the gradual impairment of the intramitochondrial energy production system.

INTRODUCTION

There are numerous evidences that contractile activity of smooth muscle, as that in other types of muscle[1], is regulated by the changes in the level of ionized calcium in the myoplasm[2,3,4]. Consequently, any stimulants of smooth muscle appear to act by increasing the intracellu-

lar calcium ion concentration. As to the subcellular calcium storage two cellular compartments[5,6] have been considered; the endoplasmic reticulum and the mitochondria.

It is generally acepted that membrane depolarization in all muscle types can lead to the removal or release of calcium from the reservoir and thereby lead to the muscle contraction[1-5]. Pharmacological agents, e.g. caffeine, can remove calcium from its storage sites in the skeletal muscle, thereby also lead to the contraction, and that this process appears unrelated to the membrane depolarization[7,8]. The author has also reported that isolated stomach muscle showed no response to additional caffeine (1 to 10 mM)[9,10]. In addition, when the muscle strips were exposed more than 30 min to caffeine Ringer subsequent stimulation by both electric current and potassium-depolarization caused the remarkable reduction of the contracture tension[9]. These results largely differ from those of the previous investigators[7,15]. The reasons, however, have remained unknown, and none of the similar findings have been reported till quite recently.

In recent years, there appeared a hypothesis that the mitochondria in the muscle might be one of the main regulator of the intracellular calcium activity[6,11,12]; these suggest that the calcium activity in the sarcoplasm is mainly determined by the intracellular events.

The present study was undertaken in order to investigate whether mitochondria are main regulator of the cytoplasmic calcium activity in smooth muscle and, if so, whether caffeine can exert its actions by altering calcium transport in and out of the mitochondria or not. To examine and demonstrate the electron-opaque precipitates of the intramitochondrial calcium ion, electron microscopy was undertaken. Localization of calcium ions in both the extracellular and intracellular components of the smooth muscle layer will be described separately and so will not be elaborated in this paper. Preliminary report of this work has been presented in abstract (1966, 1971).

MATERIALS AND METHODS

Most of the experiments were performed on the smooth muscle of the toad's stomach in winter. In summer, the stomach of bullfrog was also used. Small strips of the muscle were excised from the middle portion of the stomach and used for both physiological experiments and electron microscopic observations.

Physiological experiments

The muscle strips of which serosa was pield off were used; they

were usually 0.5 mm thick, 4.0 mm wide and 15 to 25 mm long in size when mounted vertically in the bath, where one end was tied to the glass rod and the other end was connected to the strain gauge (U-gage Type UL, Shinkoh) in order to record the isometric tension, which was recorded using the Hitachi-recorder (Model, QPD 33). In some experiments, isotonic contraction was recorded on the smoked drum. The electrical stimulating electrodes, which consisted of two platinum ring-wires, were placed near the each end of the muscle strip; a uniform and longitudinal electric field was produced through a variable transformers (60 cycles/sec, a-c field). The bath solution was bubbled through the air pump throughout the experiments. There were no significant differences on the results at various temperatures between 22°–28°C. The Ringer solution used consisted of (mM); NaCl 112, KCl 2.0, $CaCl_2$ 1.1, and buffered with Na-phosphate (pH 7.2); caffeine Ringer (2–10 mM) was prepared by the addition of anhydrous caffeine (Sigma Chemical Co.) to the basic Ringer solution; the elevated potassium Ringer is an isotonic solution in which sodium was replaced with potassium; isotonic KCl solution contains 114 mM KCl with 1.1 mM $CaCl_2$. Usually, first, the muscle strip was perfused with Ringer solution for, at least, 60 min before experiments. Potassium contracture, which will be referred to below as KCl contracture, was produced by replacing basic Ringer solution with high potassium Ringer; at the end of KCl contracture the test solution was replaced by the basic Ringer in which muscle strip was washed out over a 60 min period in four changes; and then, the muscle was exposed to caffeine Ringer. After 30 min in caffeine Ringer the muscle was tested again with high potassium Ringer, if needed.

Electron Microscopy

Precipitates of calcium ions were produced using the reagent of either potassium pyroantimonate[13] or ammonium oxalate[14]. The occurrence of these precipitates was assessed by soaking the muscle, prior to fixation, in the following solutions of varied ionic compositions; 1) basic (or normal) Ringer solution, 2) calcium free solution containing 0.2 mM EGTA, 3) isotonic solution consisted of 112 mM choline chloride and 3 mM $CaCl_2$, and 4) isotonic sucrose solution containing 7 mM $CaCl_2$. After perfusion of the muscle with these test solutions for 60 minutes, the muscle was then placed in the following fixatives; 1) 2.5% glutaraldehyde and 2% OsO_4 buffered with 100 mM potassium phosphate (pH 7.4). To each of the fixatives potassium pyroantimonate was added to yield a concentration of 2%; heating was usually needed for dissolution of this reagent, and 2) 2% OsO_4 containing 0.25 mM ammonium oxalate

buffered with veronal acetate (pH 7.0). Control muscle was treated with above fixatives prepared without pyroantimonate or oxalate. All the muscles were fixed in both glutaraldehyde and OsO_4 fixatives for 2 hrs respectively. After fixation they were quickly dehydrated in ethanol, embedded in Epon 812, sectioned with Porter-Blum-ultramicrotome, stained with uranyl acetate, and examined with JEM. T6S Electron microscope. Further details will be described elsewhere (in preparation).

RESULTS

Electron microscopy of mitochondria

General:

None of the precipitates were found in the control muscle fixed in glutaraldehyde alone or in that fixed in osmium tetroxide containing no reagent. After soaking the muscle in calcium free Ringer, however, occasional precipitates of pyroantimonate could be found, peppered throughout the myoplasm of smooth muscle. This problem may be concerned with the specificity of the reaction products, since potassium pyroantimonate probably produces an electron opaque precipitate when combined with other cations[13,16]. Prolonged perfusion of the muscle with isotonic solution devoid of both sodium and calcium ions, e.g. isotonic choline chloride solution or isotonic sucrose solution, usually

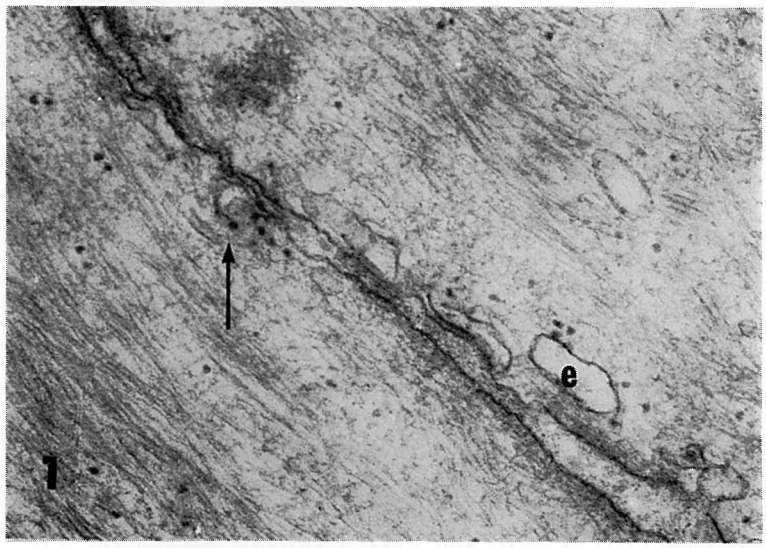


Fig. 1. Muscle was pre-soaked for 60 min in calcium-free Ringer solution containing 0.2 mM EGTA; pyroantimonate precipitates can be seen in myoplasm, most of them probably located in the cisternae of the endoplasmic reticulum and in the intermyofibrils. Antrum, ×60,000: e, dilated ER

caused the disappearances of the pyroantimonate precipitates. From these observations the precipitates produced in calcium free Ringer containing EGTA (0.2 mM) were thought to be those of sodium pyroantimonate; they seemingly consist of small, roundish aggregates of very fine grains, most of them appear to be located in or near the endoplasmic reticulum (Fig. 1); they never displayed annular profiles in the matrix of the mitochondria. In contrast, after load with calcium the precipitates seen in the mitochondria showed large annular profiles or ring-form which consisted of the cluster or aggregates of small granules (e.g. Figures 2 and 3); they were assumed to be the precipitates consisted of calcium compounds, because similar precipitates could be produced in the presence of the reagents after presoaking the muscle in the sucrose solution which contained excess calcium ions; they also disappeared after presoaking the muscle in calcium free Ringer containing EGTA.

Calcium uptake by mitochondria

Figures 2 and 3 show the pyroantimonate precipitates seen in the mitochondria in the muscles; both muscles were previously soaked in

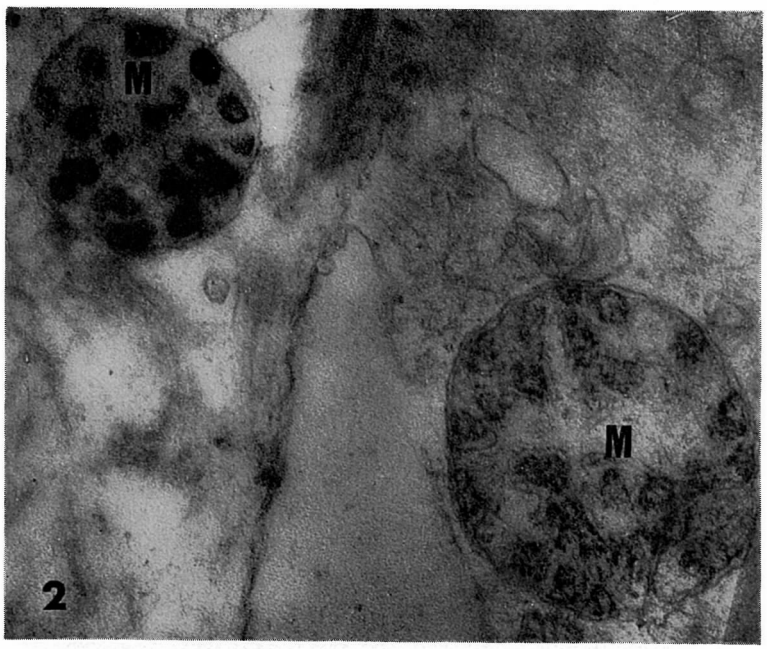


Fig. 2. Muscle was soaked for 60 min in caffeine Ringer containing 6 mM $CaCl_2$. Annular profiles of the pyroantimonate precipitates suggesting the increase of calcium uptake by mitochondria in the presence of caffeine. Antrum, ×60,000

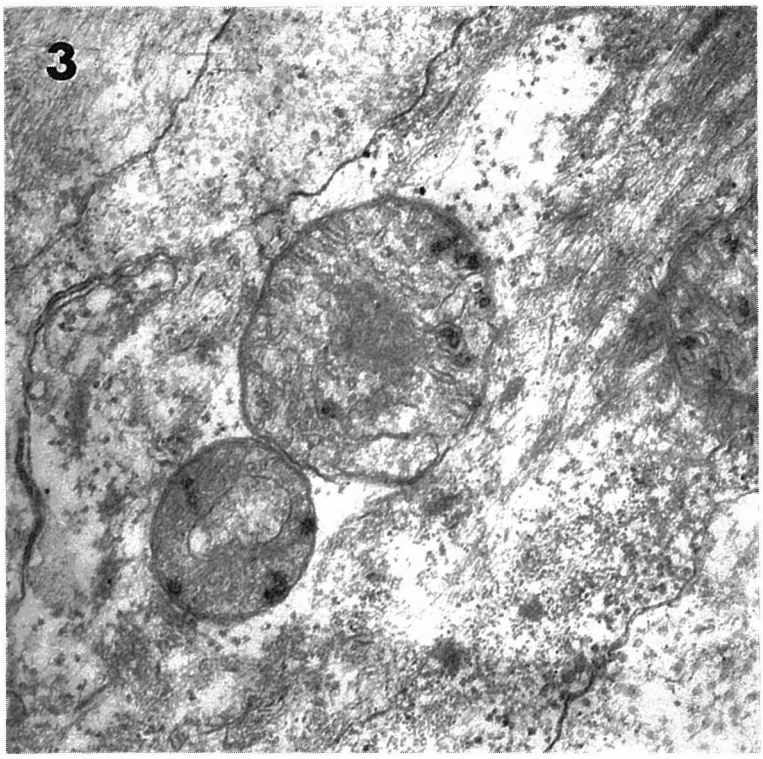


Fig. 3. Muscle was soaked for 60 min in Ringer solution containing 10 mM calcium (without caffeine). Note small number of pyroantimonate precipitates showing the annular profiles in the mitochondria. Antrum, ×45,000

Fig. 4. Muscle was soaked for 30 min in Ringer solution containing 6 mM calcium (without caffeine). Note small number of oxalate precipitates in the mitochondria. No precipitates in the myoplasm. bullfrog stomach, ×38,000

excess calcium Ringer: 8 mM calcium in Fig. 2 and 10 mM in Fig. 3. As can be seen in both photographs, presoaking the muscle in the excess calcium solutions caused a marked increase, in both the number and size, of the intramitochondrial precipitates. Similar results obtained with the use of the oxalate technique are also shown in both Fig. 4 and Fig. 5; as is clear in Fig. 5B, soaking the muscle in the solution containing ammonium oxalate often caused vacuolar degeneration in most of the mitochondria. The reason, however, remained unknown.

Localization of the precipitates in mitochondria

Localization of the intramitochondrial precipitates consisted of the oxalate salt appears to be essentially similar to that of the pyroantimo-

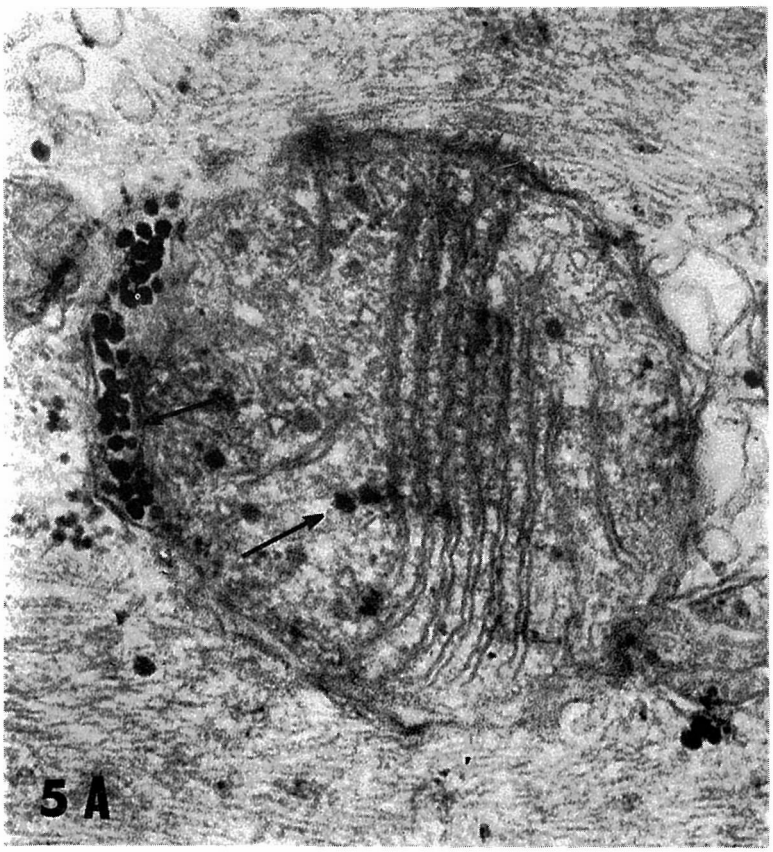


Fig. 5-A. Muscle was soaked for 60 min in caffeine Ringer containing 6 mM calcium. Note increase of electron dense granules consisted of oxalate precipitates; some of them locate in the mitochondria, others in the endoplasmic reticulum making contact with mitochondria. bullfrog antrum, ×96,000

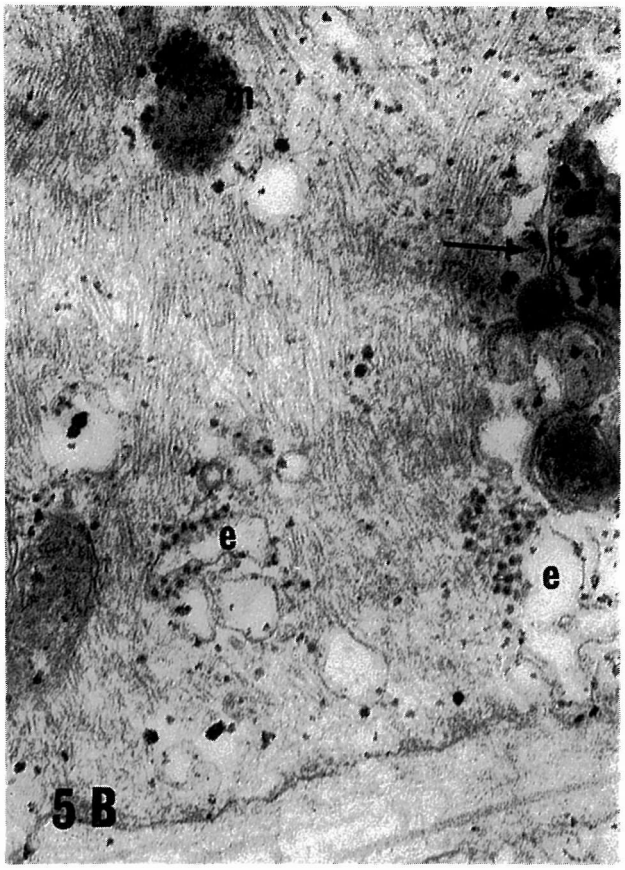


Fig. 5-B. Muscle was soaked for 60 min in caffeine Ringer containing 10 mM calcium; caffeine caused the increase of oxalate precipitates mainly in the mitochondria and the cisternae of endoplasmic reticulum. bullfrog antrum, ×60,000

nate precipitates: the pyroantimonate precipitates, probably calcium compounds, consist of multiple minute granules; these precipitates appear to be mainly located in the intercristal spaces, in contact with the outer leaflet of the cristae, and occasionally in the mitochondrial matrix. They generally showed annular profiles with the electron transparent cores (Fig. 2). In contrast, the intramitochondrial precipitates due to the oxalate salt showed no annular profiles (Figures 4 and 5); their appearances usually exhibited the electron dense precipitates composed of the aggregates of many fine grains located in the intercristal spaces and on the cristal surface of the mitochondria. These evidences suggest that after load with calcium the mitochondria in smooth muscle could probably take up the increased intracellular calcium ions; these calcium

might be bound to the different compositions in the mitochondria such as the proteins and lipids, as well as phospholipids, of the cristal membranes and the glycoproteins in the matrix of the mitochondria. This posibility also has been discussed in the previous literature[17].

Effects of caffeine on calcium uptake by mitochondria

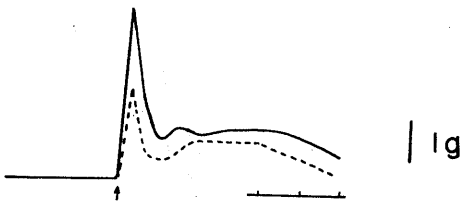
In the following experiments, the muscle was previously soaked in excess calcium (6 to 10 mM calcium) Ringer containing 2 to 10 mM caffeine; calcium precipitates were produced by changing the solution in the tissue bath with either solution or fixative containing the reagents.

Typical results are shown in Figures 2 and 5, where the production of the intramitochondrial precipitates was significantly increased when caffeine was present in the solution of the tissue bath. When the muscle was perfused with the solution containing 5 mM or more caffeine for up to 60 min, however, mitochondria in the muscle cell often showed swelling accompanied by the disorganization of their cristae and, finally, vacuolar degeneration. From these observations, it is conceivable that caffeine might cause the deterioration of the intramitochondrial metabolic system. This assumption will be discussed later.

Physiological study:

Effects of caffeine on the mechanical activity of the smooth muscle

When the muscle was exposed to caffeine Ringer, no spontaneous contraction was found. In the present study, therefore, the muscle contracture was induced by exposing the muscle to excess potassium Ringer (containing 30 to 114 mM KCl) or by electrical stimulation. Both typical patterns of the potassium-induced contracture and the effects of caffeine on its tension are shown in Fig. 6; control tension



----- pretreated with Caffeine (2×10^{-4}) in standard Ringer's solution
—— socked in standard Ringer's solution

Fig. 6. Typical patterns of the potassium-induced contracture and effects of caffeine on it: control tension, solid line. effects of caffeine, dotted line; both were recorded using the same muscle alternately.

is shown by the solid line (curve) and the effect of caffeine by the dotted line.

In these experiments the muscle was previously perfused with normal Ringer and then exposed to potassium-Ringer, thereby contracture shown by the solid line was obtained. After recording the contracture the muscle was washed out with normal Ringer and then exposed to caffein Ringer, which was renewed six time every five minutes. Afterwards, potassium contracture was again recorded and shown by the dotted line. The results suggest that exposing the muscle to caffeine Ringer caused the remarkable reduction of the contracture tension.

Similar result is also shown in Fig. 7, where the concentration of potassium chloride was varied from 30 to 90 mM; the threshold concentration of potassium was 30 mM in most of the present materials. After soaking the muscle in either normal- or caffein Ringer, the muscle was exposed to potassium Ringer; such an experiment was performed in a series of the elevated potassium solutions. The results indicate that the more concentration of potassium was used, the less tension developed when the muscle was tested after soak in caffeine Ringer. Similar result is also shown in Fig. 8, where the contracture was electrically induced; the alternative current was applied to the muscle strip in parallel with the circular muscles; the trains of impulses were usually

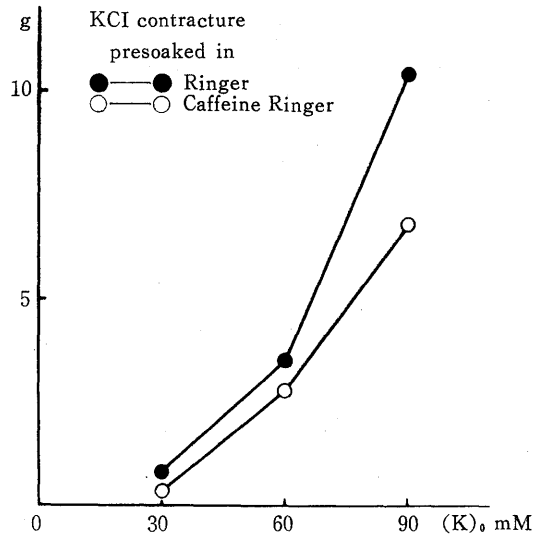


Fig. 7. Effects of caffeine on the potassium-contracture: the results were obtained using the same muscle. details in the text.

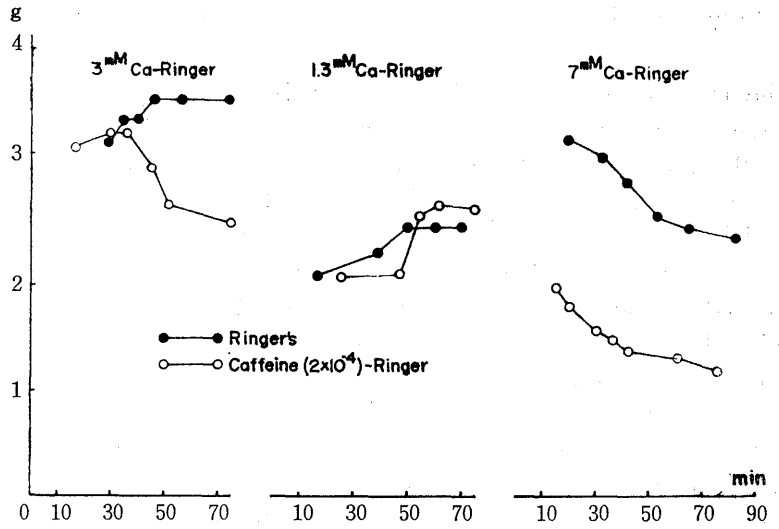


Fig. 8. Effects of caffeine on the electrically induced contracture: both were recorded using the same muscle. details in the text.

of 5 sec duration, at 5 v/cm and 50 c/s. In Fig. 8, electrically induced tension was first recorded in the control Ringer solution, in which the concentration of calcium ions was varied from 1.3 to 7 mM; afterwards again the tension was recorded. Results obtained from three different muscles are shown in Fig. 8. Soaking the muscle in Caffeine Ringer caused an appreciable decrease of the contracture tension induced by the electrical stimulation; the rate of reduction of the tension seems to depend on the concentration of external calcium ions; the more external calcium ion increased, the less tensions developed. In the experiments shown in both Figures 6 and 7; the results may be summarized as follows; (1) the tension reduction is closely related to the effects of caffeine, and (2) its effects may be related to both cytoplasmic calcium activity and the mitochondrial function in calcium transport, in calcium-loaded muscle.

DISCUSSION

Composition of the precipitates

To date many authors have described that not only calcium but also other cations in various tissues probably cause the formation of an electron opaque precipitate in the presence of potassium pyroantimonate[13,16]. Therefore, most important problem which arises out of the

present study may be concerned with the specificity of the reaction products.

In the present study, however, the oxalate precipitates as well as pyroantimonate precipitates were assumed to be the calcium compounds based on the following evidences: 1) no or very little precipitates were found in most of the muscles after removal of calcium from Ringer solution. In addition, two or more hours after calcium-chelation with EGTA no intramitochondrial precipitates were observed, and 2) after the load with calcium the intramitochondrial precipitates markedly increased. These results are in agreement with those of the earlier investigators, who suggested that the mitochondria in various tissues probably posses the ability to accumulate calcium ion under a variety of experimental condition[6,11,12]. Recently, X-ray microanalysis method has provided the evidences that both pyroantimonate and oxalate precipitates contain large amount of calcium[18,28]. Thus, it appeared that sodium ion in mitochondria was too small to be detected under the condition of the elevated concentrations of calcium.

The suppressive action of caffeine on the contracture tension

It has been assumed that caffeine penetrates across the membrane into the cytoplasm[5,8] and exerts its effect by releasing calcium from the intracellular calcium storage without induceing membrane depolarization[7,8]. Depolarization to the threshold level can also lead to the release of bound calcium in all muscle types, whereby also lead to muscle contraction[1,2,3,5]: Thus, contractile activity in all muscle types has been assumed to be controlled by the intracellular free calcium concentration[2,3,4].

As is evident from the present results, the responses of smooth muscle to caffeine quite differed from those of skeletal muscle: After longer perfusion with caffeine Ringer smooth muscle, but not skeletal muscle[7,19], showed the reduction of the contracture tension induced by the application of electric current or of high potassium Ringer. So far valid hypotheses accounting for the effect of caffeine on skeletal muscle have been proposed[7,8,19]. From the above mentioned discrepancy, however, these hypotheses appear not to be available for the explanation of the present results.

It has been also reported that in the taenia coli of guinea pig caffeine produced the contracture and the membrane depolarization of the muscle cells[20], although this description has been quite revised lately. In contrast to this, recently, Osa[21] has found and discussed that caffeine might cause an inhibition of both mechanical and electrical

activities in smooth muscles, possibly through the metabolic activation of a mechanism which concerns calcium accumulation to some sequestering sites; the discussion of this author has based on the findings reported by Butcher et al.[22], who proposed that theophylline probably caused an increase of cyclic AMP by inhibiting the hydrolytic enzyme phosphodiesterase which in turn inhibited the muscle contraction. Takagi et al.[23] have also suggested that cyclic AMP inhibits smooth muscle by decreasing the supply of calcium to the contractile elements. However, conflicting views exist between the different tissues as to the role of cyclic AMP on the intracellular calcium movement; In intact kidney cells, administration of cyclic AMP increases calcium efflux from both the mitochondria and cytoplasm[12]. Of these, the latter appears to conflict with the former as well as the present results in which caffeine enhanced the accumulation of the intramitochondrial precipitates. It has been postulated that calcium can possibly reduce the phosphodiesterase activity, causing an increase of cyclic AMP[22]; additional cyclic AMP also appears to reduce the ATP and CrP contents in the normal but not in the calcium poor smooth muscle[24]. Thus, it is conceivable that actions of caffeine on the calcium transport in smooth muscle may not be unidirectional in determining which way the calcium ions will move.

According to Urakawa and Holland[25], potassium-induced contracture could be differentiated into two components in taenia coli; phasic and tonic contractions. They assumed that the phasic component would be related to releasing the intracellular bound calcium responsible for initiating the contraction. Based on this postulation it appears that in the present experiments caffeine could possibly inhibit the development of the phasic component by inhibiting the intracellular release of calcium from the reservoir. The tonic component may be metabolically dependent and maintained by an increased transmembrane calcium influx[25]. These postulations on the potassium contracture are in partial disagreement with those of the authors[26]; In the previous experiments, the phasic component of the potassium contracture could mainly be inhibited by addition of DNP (10^{-5}M) or by presoaking the muscle in the cold Ringer solution (4°C)[26]. Based on this findings it may be postulated that the reduction of the contracture tension brought about by caffeine might be metabolically dependent and also reflect the decreased intracellular level of ionized calcium.

The question why only in excess calcium but not in normal calcium Ringer caffeine could suppress the contracture tension induced by electrical stimulation yet remains unanswered. One hypothetical explana-

tion, however, may be proposed based on the electron microscopic observations.

As is evident from electron microscopy, when the muscle was soaked in the excess calcium solution additional caffeine had enhanced calcium uptake by mitochondria (Fig. 2 and 5); these calcium ions could be precipitated either as pyroantimonate or as oxalate precipitates. These precipitates invariably increased both in the mitochondria and the cisternae of the endoplasmic reticulum, but not in the myoplasm (see e.g. Fig. 4). However, in this conditions and even in a case devoid of the reagents needed for precipitating calcium caffeine often caused a variety of the degenerative changes in most of the mitochondria; less electron dense matrix and swelling of the mitochondria as well as disintegration of their cristae. Greenwalt et al[27] suggested that excessive accumulation of calcium in mitochondria might cause the uncoupling of oxidative phosphorylation.

There would be a considerable discrepancy and confusion on the possible interpretation of the effects of caffeine between the previous workers. Therefore, based on the present findings the author would like to propose a following assumption; Calcium uptake by mitochondria in smooth muscle is primarily dependent on the concentrations of extracellular calcium ions, which also depend on those in the external solution; hence, greater accumulation of calcium precipitates by the mitochondria could invariably be found only in the calcium-loaded muscle but not in normal muscle. This accumulation of calcium could be enhanced in the presence of caffeine, possibly through utilizing ATP. It is also plausible that degenerative changes in the mitochondria caused by the massive accumulation of calcium is suggestive of impaired metabolic activity in the mitochondria. It has also been suggested that uncoupling of the mitochondrial oxidative phosphorylation occurs when massive calcium is taken up by mitochondria and deposited within them as a dense granule[27].

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