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ACTION OF CAFFEINE IN EXCITATION–CONTRACTION COUPLING OF FROG SKELETAL MUSCLE FIBRES

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SUMMARY

1. Frog sartorius muscle bathed in 1 mM-caffeine generates brief asynchronous contraction of individual sarcomeres, 'sarcomeric oscillations', and propagated contractile waves.

2. Analysis of cinematographic records shows that during sarcomeric oscillations the sarcomere length decreases and the distribution of sarcomere lengths is wider than in controls.

3. Caffeine can produce sarcomeric oscillations in K depolarized muscle fibres and, to a limited extent, in glycerol-treated muscle fibres.

4. Treatment of muscle with dantrolene sodium blocks production of sarcomeric oscillations by caffeine.

5. In caffeine-treated muscle fibres, electrically produced depolarization could initiate or increase the frequency of sarcomeric oscillations, and electrical hyperpolarization diminishes the frequency or stops sarcomeric oscillations.

6. Caffeine solution bathing a muscle undergoing sarcomeric oscillations (the perfusate), when applied to a fresh muscle, initiates sarcomeric oscillations with a relatively short latency.

7. An U.V.-absorbance peak at 245 nm develops in the caffeine solution bathing a muscle undergoing sarcomeric oscillations.

8. It was found that a contraction-regulating substance (oscillogen) is released from a muscle undergoing sarcomeric oscillations. From results of selective dialysis and gel permeation chromatography, the molecular weight of the oscillogen is estimated to be between 700–1000 daltons.

9. It is proposed that the oscillogen is a normally occurring substance which functions in excitation–contraction coupling at the T-tubule terminal cistern junction in skeletal muscle.

INTRODUCTION

It is well known that application of 5 mM-caffeine to skeletal muscle produces a strong contraction (Axelsson & Thesleff, 1958). Although application of lower concentrations of caffeine does not cause an appreciable increase in muscle tension,

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the contractile system is observably affected. Marco & Nastuk (1968) showed that frog sartorius muscle bathed in 1 mM-caffeine generates brief asynchronous contractions of individual sarcomeres which they called 'sarcomeric oscillations'. These sarcomeric oscillations appeared at junctional and extrajunctional regions. With time the sarcomeric oscillations evolved into more orderly waves of contraction extending over localized groups of sarcomeres, and finally they became well developed propagated contractile waves. Coleman, Coleman, Griffin, Weltman & Chapman (1972) described the generation of trains of contractile waves produced by the application of low concentrations of methylxanthines to tissue cultures of skeletal muscle. These waves travelled at rates of 50–200 μm/sec. Mooseker, Pratt, Kiehart & Stephens (1977) observed wave-like contraction and relaxation of sarcomeres in isolated myofibrils treated with solutions containing ATP and low levels of free calcium.

Contractions propagating with a velocity of 3–4 μm/sec have been electrically induced in 'skinned' muscle fibres immersed in oil (Natori, 1965, 1975). These contractions were assumed to be controlled by membrane systems of the transverse tubules (T-tubules) and the sarcoplasmic reticulum (s.r.). Caffeine-containing solutions, when applied to these 'skinned' fibres, induced ripple-like contractions travelling at a much lower velocity. Endo, Tanaka & Ogawa (1970) recorded wave-like changes in contractile tension in caffeine-treated 'skinned' skeletal muscle fibres. These contractions were thought to be the result of drug-induced release of Ca^{2+} from the s.r.

Several possible mechanisms of action may be involved in the initiation of muscle contractions by caffeine. Contractions produced by injection of caffeine into single muscle fibres of crab (Caldwell & Walster, 1963) or crayfish (Chiarandini, Reuben, Brandt & Grundfest, 1970*a*; Chiarandini, Reuben, Girardier, Katz & Grundfest, 1970*b*) show that this compound has an intracellular site of action. Evidence bearing on these experiments was provided by Weber (1968) who found that caffeine inhibited uptake of Ca^{2+} by the s.r. In addition, participation of the surface sarcolemma was suggested by experiments of Bianchi (1962, 1968) who showed that caffeine increases the influx and efflux of Ca^{2+} in frog sartorius muscle. In interpreting results of their experiments on caffeine-treated muscles, Lüttgau & Oetliker (1968) suggested that this agent acts on T-tubules. Finally, because caffeine can inhibit phosphodiesterase, it could influence Ca regulation by altering the level of cyclic nucleotides (Rasmussen & Goodman, 1977).

In the present study we have provided more complete characterization of caffeine-induced sarcomeric oscillations and the later developing propagated mechanical waves. We have also provided further evidence to distinguish the various sites which may be involved in the initiation of sarcomeric oscillations and propagated waves. Finally, we have attempted to characterize a substance which is released from oscillating caffeine-treated muscle. We propose that this substance (which we call oscillogen) plays a role in excitation–contraction coupling (E–C coupling). Preliminary results have been reported (Kumbaraci & Nastuk, 1980).

METHODS

Most of the experiments were performed *in vitro* using frog sartorius muscle. A few were done on cruralis muscle (*Rana pipiens*). The muscles were kept at room temperature (18–20 °C) in Ringer solution of the following composition: 112·4 mM-Na^+; 2·5 mM-K^+; 1·8 mM-Ca^{2+}; 117·1 mM-Cl^- and 3 mM-HEPES, pH adjusted to 7·4 by addition of NaOH. All drugs tested were dissolved in Ringer solution.

The various solutions used in experiments to be described ordinarily were applied to the bath containing the isolated frog muscle preparation. However, in some experiments there was need to determine the oscillogenic activity of a small volume of solution. In these cases the solution to be tested was applied by microperfusion to a localized region of the sartorius muscle. An aliquot of the solution to be tested (approx. 0·5 ml.) was drawn up into a glass tube fitted at its lower end with a micropipette of tip diameter about 50 μm. This tube assembly was clamped in a micromanipulator. The fluid reservoir in the glass tube produced sufficient hydrostatic pressure to expel fluid from the pipette when its tip was lowered below the surface of the fluid bathing the isolated muscle. Immediately before the start of a microperfusion, the Ringer solution bathing the muscle was drained and the muscle was quickly washed two or three times with 1 mM-caffeine–Ringer solution. Thereafter it was bathed in approx. 3 ml. of 1 mM-caffeine–Ringer solution unless otherwise stated.

The latency and intensity of sarcomeric oscillations were estimated by observing the muscle preparation with a binocular microscope (magnification, 400 ×). If no sarcomeric oscillations were initiated, the microperfusion was discontinued after 20 min. In both the bath application and microperfusion experiments, the intensity of sarcomeric oscillations was rated according to the number of oscillating sarcomeres observed, the frequency of sarcomeric oscillations and the number of oscillating regions found scattered over the surface of the muscle.

Preparation and analysis of photographic records

The chamber containing the muscle fibre was placed on the rotating stage of a Leitz microscope. A 40× water-immersion Zeiss objective lens (N.A. = 0·75) and 16× ocular provided a total magnification of 640×. During the initial adjustment, an incident light beam supplied by a tungsten source was passed through a polarizer plate. The longitudinal axis of the muscle fibre was placed parallel to the plane of polarization by rotating the specimen stage. Emergent plane-polarized light was extinguished by appropriate rotation of a polarized analyser plate. Small adjustments were made in the position of the muscle fibre and the analyser so that both the fibre and the background appeared dark. A mica quarter wave plate was then inserted in the light path below the analyser at 45° to the plane of polarized light from the polarizer. The A-bands and Z-lines of the muscle fibre appeared dark and the I-bands appeared bright. Minor adjustments of the polarizer were made until the best contrast was obtained. When single photomicrographs were taken, the tungsten light source was replaced by a flash unit (General Radio Strobulume Type 1532-D; flash duration 30 μsec).

Motion pictures were taken with a 16 mm high-speed motion picture camera, LOCAM Model 51-001 (Redlake Corp.). The camera was fixed in position on a bracket, without mechanical contact with the microscope. The light source was a Chadwick-Helmuth Point Source Strobex, Model 71, which provided a light flash approx. 55 μsec in duration and a beam having approx. 2° divergence. The camera, activated by a foot switch, triggered the flash in synchrony with the film advance. Frame rate of the camera could be adjusted from 16 to 500 frames/sec. 16 mm black-and-white films (Kodak Double-X-Negative and Plus-X-Negative) were used.

Sarcomere length was measured on a 2000× magnified image of the muscle fibre obtained by optical projection of selected frames from the film negative. The negative image was projected onto a Talos digitizing tablet connected to a Tektronix programmable calculator (model 31). Sarcomere length was measured as the distance between centres of adjacent I-bands. Z-lines were often not distinctly visible, probably because overlying connective tissue (which we were unable to remove) obscured fine details.

Special experimental conditions

1. Depolarization of sarcolemmal and T-tubule membranes was achieved by applying Cl^--free Ringer solution in which the extracellular K^+ concentration was elevated. In such solutions the

Na^+ concentration was reduced by an equivalent amount and Cl^- was replaced by propionate$^-$ (an impermeant anion). Isotonic K^+ Ringer solution had the following composition: K propionate, 115 mM; Ca propionate, 1·8 mM; HEPES, 3 mM; pH adjusted to 7·4 by addition of KOH.

2. Disengagement of T-tubules from the surface sarcolemma was carried out as described by Eisenberg, Howell & Vaughan (1971). An isolated sartorius muscle was placed for 1 hr in hypertonic Ringer solution containing 400 mM-glycerol. Then the preparation was allowed to recover for 1 h in Ringer solution containing 5 mM-Ca^{2+} and 5 mM-Mg^{2+}. Excess Ca^{2+} and Mg^{2+} was removed by bathing the muscle in normal Ringer solution for 2 hr. After such treatment the recorded resting potentials averaged 84 ± 6 mV. Action potentials could be elicited via nerve stimulation but no muscle twitch was observed.

3. *Characterization of the 'oscillogen'.* (*a*) *Molecular weight.* This was roughly estimated by testing the permeation of oscillogen through membranes having selected pore size. Caffeine–Ringer solutions that had been applied to muscles were withdrawn and dialysed using Spectropor 2 (mol. wt. cut-off 12–14,000) or Spectrapor 3 (mol. wt. cut-off 3500) dialysis tubing. Dialysis against 1 mM-caffeine–Ringer solution was continued for 48 hr at 4 °C with continuous stirring. Aliquots of the solution inside and outside the dialysis sac were each microperfused onto a fresh muscle to determine latency in production of sarcomeric oscillations. Alternatively, 3–4 ml. aliquots of each solution were applied to a fresh muscle. (*b*) *Separation of the oscillogen by gel permeation chromatography.* A Sephadex G-10 column (1·6 × 30 cm) was prepared and calibrated with haemoglobin and potassium dichromate. In this column, in which the void volume was 11 ml. and the elution flow rate was 0·1 ml./min, haemoglobin was eluted in the 12–13th ml. fractions and potassium dichromate was eluted mainly in the 33–35th ml. fractions. After calibration, the column was purged with five 10 ml. additions of Ringer solution.

Separation of caffeine was carried out as follows. The column was loaded with 1 ml. of 1 mM-caffeine–Ringer solution followed by elution with 40 ml. of Ringer solution at a flow rate of 0·1 ml./min. Caffeine concentration in the eluate fractions was determined by measuring U.V. absorbance at 272 nm using a Perkin-Elmer recording spectrophotometer (Coleman 572).

Separation of the oscillogen: A 1 ml. aliquot of 1 mM-caffeine–Ringer solution which had been drawn off from an oscillating muscle ('active perfusate') was loaded into the column followed by elution with 40 ml. of caffeine-free Ringer solution. The spectral absorbance of each eluate fraction was measured at 272 nm.

The capacity of an eluate fraction to initiate sarcomeric oscillations was tested by microperfusion of an aliquot of the eluate on to an isolated muscle as described above. Further details are given in the Results section.

RESULTS

Characterization of the response to caffeine application

Immersion of frog sartorius muscle in Ringer solution containing 1 mM-caffeine gives rise to the following series of responses. After approximately half an hour, localized twitch-like contractions of individual sarcomeres (called sarcomeric oscillations) occur at isolated regions along a single fibre. Plate 1 shows a region of a muscle fibre undergoing sarcomeric oscillations photographed in a series of successive cineframes 40 msec apart. An individual I-band indicated by an arrow in each successive frame has undergone a transient decrease in width. The decrease in I-band width occurs in a restricted region of the sarcomere near the edge of the fibre. This event resembles the localized I-band shortening produced by application of an electrical stimulus to the Z-line region of a muscle fibre (Huxley & Taylor, 1958). The distance between the centres of the A-bands adjacent to the arrow is 2·4 μm in *A*, 2·1 μm in *B*, 1·8 μm in *C*, 2·2 μm in *D* and 2·4 μm in *E* and *F*. Thus this oscillation is complete in about 100 msec.

After 1 hr of exposure to caffeine, sarcomeric oscillations appear in increasing numbers. Active regions are found scattered over the surface of the muscle and they

extend along a short length of several adjacent muscle fibres. The large number of oscillating sarcomeres in such an area give it a shimmering appearance. At the periphery of such an active area the number of oscillating sarcomeres diminishes.

Plate 2 shows two photomicrographs taken at an interval of 80 msec from a vigorously oscillating muscle fibre. In *B* the third I-band from the left end of the bar has been widened as a result of the shortening of the adjacent sarcomeres.

To study further the changes in sarcomere length in such oscillating fibres, we made single flash photomicrographs of selected areas of a muscle before and 1·5 hr after applying 1 mM-caffeine. The sarcomere length of 6–10 adjacent sarcomeres was

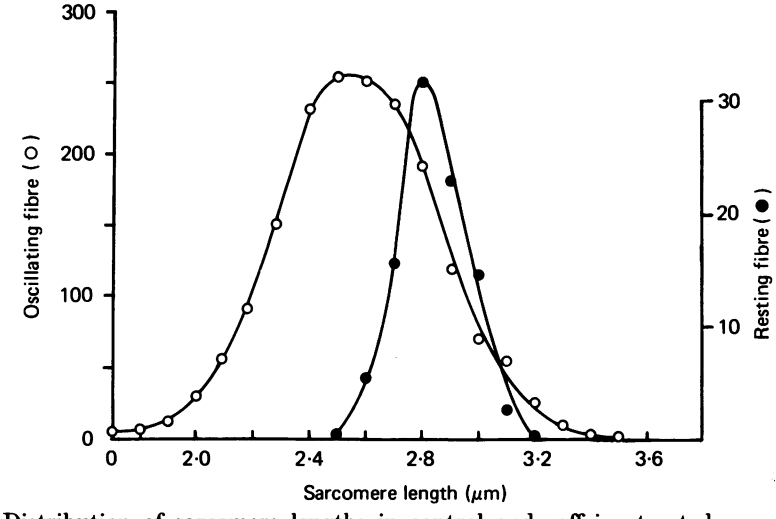

Fig. 1. Distribution of sarcomere lengths in control and caffeine treated muscle. ●, sarcomere lengths of non-oscillating control muscle fibre prior to application of caffeine; ○, sarcomere lengths of oscillating muscle fibre following application of 1 mM-caffeine. Ordinates show no. of sarcomeres.

measured using the projected negative images of these selected areas. Fig. 1 gives the distribution of sarcomere length from non-oscillating control fibres and from oscillating caffeine-treated fibres. For the controls the modal sarcomere length = $2{\cdot}83 \pm 0{\cdot}31$ μm and for the oscillating fibres the modal value is less, sarcomere length = $2{\cdot}56 \pm 0{\cdot}28$ μm. The sarcomere length of oscillating fibres has a relatively wide range (1·7–3·5 μm). The I-bands of the oscillating fibres apparently are not obliterated during each oscillation.

With time during the application of caffeine, sarcomeric oscillations become more organized. Contraction of one sarcomere appeared to initiate sequential contraction of adjacent sarcomeres, thereby producing a wavelet which travelled about 50–100 μm along the length of the fibre.

After 3 hr of exposure to caffeine, the wavelet activity became more fully developed. Contractile waves involving a group of contracted sarcomeres propagated along the length of the fibre at a velocity of 20–500 μm/sec. Plate 3 shows such a propagated contractile wave. At certain sites along the muscle fibre a group of sarcomeres contracted spontaneously more or less in synchrony, thereby initiating two

propagated waves which travelled away from the region of origin. Occasionally propagated contraction waves were observed travelling towards each other. As shown in Pl. 4, these waves annihilated each other upon collision.

Evidence for action of caffeine on T-tubule lateral cistern junctions

The fact that caffeine can cause portions of single sarcomeres to undergo oscillatory contractions could be taken to mean that this agent is acting at the T-tubule lateral cistern junction. Additional evidence consistent with this idea was obtained by Nastuk & Alexander from experiments conducted by them during 1968–70. Many of these results have not been published, and they will be described briefly here. Frog sartorius muscle *in vitro* was bathed in Tris (1 mM) or phosphate (3 mM) buffered Ringer solution containing caffeine (0·75 or 1·0 mM). Membrane potential was conventionally measured with 3 M-KCl-filled intracellular electrodes. A second 3 M-KCl-filled intracellular electrode placed 70–100 μm from the recording electrode was used to pass current across the membrane to produce variations in the membrane potential. Current was supplied by an optically coupled stimulus isolation unit driven by a Grass S44 stimulator whose output was varied manually. Results obtained from fourteen fibres (three muscles) were as follows. In non-oscillating caffeine-treated fibres, gradual reduction of the membrane potential from the resting value, approx. -90 mV, to values between -70 and -60 mV caused sarcomeric oscillations to be initiated within a few seconds, but sometimes the latency for appearance of sarcomeric oscillations was as much as 25 sec. Return of the membrane potential to the control value, or hyperpolarization to -115 mV, slowed the sarcomeric oscillations and sometimes stopped them completely. In one fibre showing vigorous sarcomeric oscillations, the resting potential was found to be -74 mV. Increasing the resting potential to -78 mV slowed or stopped the sarcomeric oscillations. In one trial, application of a 400 msec hyperpolarizing pulse stopped the oscillations promptly. More usually, the resting potentials of caffeine-treated muscle fibres exhibiting sarcomeric oscillations were found to be in the control range.

If caffeine causes transient localized changes in T-tubule membrane potential, the over-all effect of these events in many T-tubules might be manifested as noise in the membrane potential record. Our membrane potential recordings of fibres exhibiting sarcomeric oscillations were not obviously more noisy than those from non-oscillating fibres. However these records were made at relatively low amplification, and a more careful search at higher amplification might show significant differences. As yet this has not been done, and this interesting question remains open.

The following experiments were carried out to explore further the action of caffeine on excitation–contraction coupling.

1. Glycerol treatment was used to decouple T-tubules from the surface sarcolemma. After glycerol treatment, application of 1 mM-caffeine–Ringer solution produced sarcomeric oscillations in about 10 min (five muscles), which represents a shorter latency than that seen in controls (approx. 30 min). However, compared with controls, the individual sarcomeres of glycerol-treated muscle showed a lower frequency of sarcomeric oscillations, and fewer sarcomeres oscillated. Propagated contractile waves were observed in deep lying fibres.

In interpreting the meaning of these results, we note from the work of Franzini-Armstrong, Venosa & Horowicz (1973) that glycerol treatment seriously disrupts the morphology of T-tubules and increases the access resistance of T-tubule openings, but it produces little change in the s.r. These investigators estimated that 90% of the T-tubules become disconnected from the external solution. If one assumes that caffeine initiates sarcomeric oscillations in normal muscle by entering the T-tubules and diffusing down their length to act at the T-tubule terminal cistern junctions, then the changes produced by glycerol treatment would be expected to diminish the number of sites in the muscle fibre at which applied caffeine could initiate sarcomeric oscillations.

2. The action of caffeine in producing sarcomeric oscillations was also tested on muscle fibres in which E–C coupling was interrupted by intense depolarization of the sarcolemma.

A sartorius muscle was first equilibrated for 10 min in Ringer solution in which priopionate$^-$ was substituted for Cl^- (Na^+, K^+ and Ca^{2+} concentrations had their usual values). The membrane potential of single muscle fibres measured at the end of this 10 min period averaged −89 mV. Addition of 1 mM-caffeine did not result in initiation of sarcomeric oscillations over a 60 min period of observation. When this experiment was repeated in the same manner but with the caffeine concentration increased to 1·7 mM, sarcomeric oscillations were produced in about 10–12 min (five expts.). Following this, the muscle was washed three times with caffeine-free propionate Ringer solution for 5 min. Thereafter 115 mM-K propionate Ringer solution was applied for 20 min. At the end of this time, 115 mM-K propionate containing 1·7 mM-caffeine was applied. Sarcomeric oscillations appeared in 2 min and propagated waves were seen after 10 min (five expts.). It was concluded that sarcomeric oscillations can be produced in totally depolarized fibres, but, to do so, the caffeine concentration must be raised above 1 mM, the usual threshold concentration. The reason for this reduction in sensitivity to caffeine in K propionate depolarized fibres is unknown, but perhaps the substitution of propionate$^-$ for Cl^- ions in the Ringer solution is a factor. It is known that the species of anion in the extracellular fluid has an influence on the contraction threshold (Hodgkin & Horowicz, 1960*a*; Kao & Stanfield, 1968). Determination of the contraction threshold for muscle fibres bathed in propionate-substituted Ringer solution would provide needed evidence bearing on the above speculation. We have no data on this point, and, to our knowledge, no published data are available.

Additional experiments were conducted on fibres depolarized to various levels by application of propionate Ringer solutions in which the K^+ concentration ranged from 2·5 to 115 mM. At all levels of depolarization addition of 1 mM-caffeine did not produce sarcomeric oscillations in any case (seven muscles). Evidently partial or complete depolarization produced by increased extracellular K^+ concentration does not potentiate the action of 1 mM-caffeine in initiating sarcomeric oscillations.

3. Dantrolene: 1-{[5-(p-nitrophenyl)-furfurylidene]amino}hydantoin sodium is used clinically as a muscle relaxant. From results of a number of investigations (Ellis & Bryant, 1972; Putney & Bianchi, 1974; Brocklehurst, 1975; Takauji, Takahashi & Nagai, 1975), it appears that this drug does not act directly on excitable membranes of the contractile system of muscle. Its action as a muscle relaxant is believed to result

from its capacity to block E-C coupling at the T-tubule lateral cistern junction. From various lines of evidence, Desmedt & Hainaut (1979) argued that dantrolene inhibits electrochemical coupling for submaximal membrane depolarizations and reduces the number of Ca^{2+}-releasing sites at the s.r. membranes activated by a given depolarization. We therefore thought that it would be interesting to test the effect of dantrolene on caffeine- induced sarcomeric oscillations.

Sartorius muscle was equilibrated for 30 min in Ringer solution containing 10 μM-dantrolene. Thereafter the bath was changed to Ringer solution containing 10 μM-dantrolene and 1 mM-caffeine. No sarcomeric oscillations resulted during the following 60 min (five expts.). Additional experiments showed that 10 μM-dantrolene blocks production of sarcomeric oscillations by 2 mM-caffeine. Further, after the applied dantrolene was washed out, the action of caffeine in producing sarcomeric oscillations was restored.

Evidence for the release of a contraction mediating substance from caffeine-treated muscle

During 1968–70 Nastuk & Alexander carried out a number of experiments to study mechanisms involved in the production of sarcomeric oscillations by caffeine. Some of their experimental results have not been published, and they will be included here, preliminary to the more recent work of our laboratory.

Nastuk & Alexander found that, as the volume of the caffeine–Ringer solution bathing a sartorius muscle was decreased, the latency for production of sarcomeric oscillations was shortened and the number of oscillating sarcomeres was increased. Results of more recent experiments, shown in Fig. 2, provide confirmation of this earlier work.

Nastuk & Alexander also discovered that when the caffeine–Ringer solution bathing a muscle undergoing sarcomeric oscillations was drained and fresh caffeine–Ringer solution was applied, sarcomeric oscillations temporarily ceased but returned with time. They also found that sarcomeric oscillations continued unchanged if the bathing solution was merely withdrawn and re-applied. Their earlier results were confirmed. Following withdrawal of caffeine–Ringer solution from muscle exhibiting sarcomeric oscillations and replacement with fresh caffeine–Ringer solution it was found that during the next 3 min the sarcomeric oscillations decreased in frequency and the number of oscillating zones also decreased. During the following 10 min full scale sarcomeric oscillatory activity resumed (twelve expts.). If a muscle undergoing sarcomeric oscillations was washed 3–4 times with fresh solution, sarcomeric oscillations stopped and resumed in 10 min (five expts.). If a muscle exhibiting propagated waves was washed several times, the motion slowly stopped in 5 min and low frequency oscillations resumed after 20 min (five expts.). If fresh 1 mM-caffeine–Ringer solution was locally perfused on a region exhibiting sarcomeric oscillations, the frequency of oscillations around the zone of perfusion decreased. After the perfusion pipette was withdrawn, it required 2–3 min for the activity to build up (four expts.).

In interpreting these experiments it might be supposed that, upon prolonged exposure of a muscle to caffeine in an unchanged bath, some agent accumulates in the unstirred layer on the surface of the muscle and acts to sustain sarcomeric oscillations. However, the following observations place this speculation in doubt. If

the solution bathing a muscle undergoing sarcomeric oscillations was drained and then reapplied to the same muscle, it was found that the oscillations did not diminish thereafter (ten expts.). Thus, even though the bathing solution is agitated and the unstirred layer above the muscle is disturbed, sarcomeric oscillations continue.

When a caffeine–Ringer solution bathing a muscle undergoing sarcomeric oscillations is applied to a fresh untreated muscle, sarcomeric oscillations are produced with reduced latency. This result is consistent with the supposition that an oscillogenic agent is accumulating in the bathing medium. We wondered whether the capacity of a muscle to produce this agent might be limited. To test this idea the following experiments were done. Five millilitres of 1 mM-caffeine–Ringer solution was applied

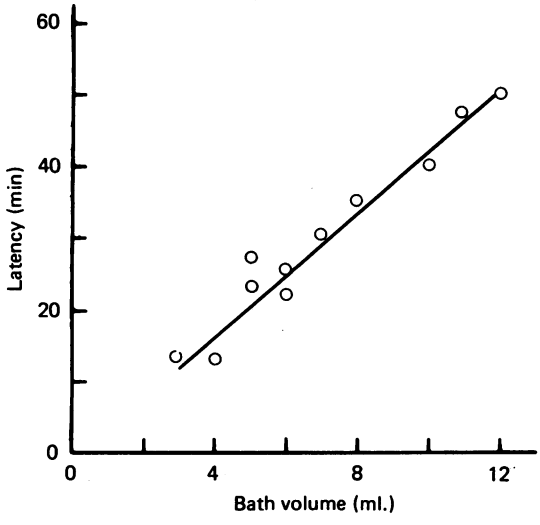

Fig. 2. Latency in production of sarcomeric oscillations in sartorius muscles bathed in various volumes of 1 mM-caffeine–Ringer solution. Each point represents average results obtained with 2–4 muscles.

to the bath containing a sartorius muscle for 2 hr. The bathing solution (perfusate) was then collected and fresh caffeine–Ringer solution was applied to the same muscle. This procedure was repeated three times. Each of these perfusates was then applied to fresh muscles. Fig. 3 shows that for the first perfusate the latency of sarcomeric oscillations is 5 ± 2 min. It can be seen that for the second and third perfusates the latency increases. For 1 mM-caffeine–Ringer solution which had not been in previous contact with muscles, the latency for sarcomeric oscillations is 21 ± 3 min. These results suggest that an agent is released from a caffeine-treated muscle and that its rate of release is decreased during successive bath applications of caffeine.

The following test was conducted to determine whether or not the amount of active agent released depended upon the duration of exposure of the muscle to caffeine. Single 5 ml. baths were continuously applied to individual sartorius muscles for 2, 4, 7 and 25 hr. Each perfusate was then tested by applying it to an individual fresh sartorius muscle and the latency of sarcomeric oscillations was determined. Results showed that the latency for sarcomeric oscillation production by all perfusates was approximately the same ($4{\cdot}2 \pm 1{\cdot}4$ min, eighteen expts.). From this evidence it

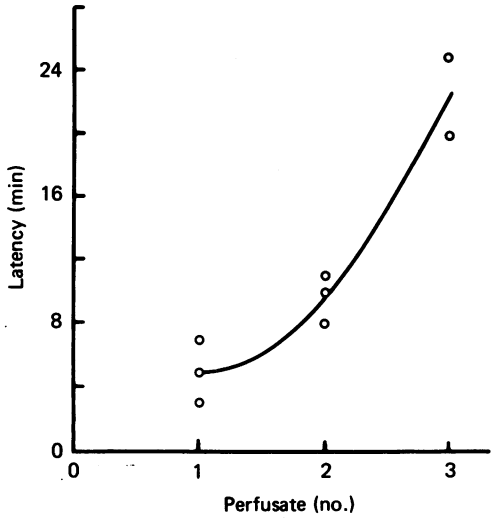

Fig. 3. Oscillogenic capacity of a series of perfusates collected from a single sartorius muscle bathed in 1 mM-caffeine–Ringer solution. Perfusate 1 represents 5 ml. of 1 mM-caffeine–Ringer solution which had been applied to the sartorius muscle for 2 hr before being removed and tested by bath application to three fresh sartorius muscles. Same remarks apply to the serially collected perfusates 2 and 3. Further details in the text.

appears that the concentration of the oscillogenic agent does not appreciably increase in the bathing solution beyond 2 hr of caffeine application.

Characterization of the oscillogen

Temperature stability of the perfusate containing the oscillogen was determined at 4 °C and at 80 °C. An active perfusate was stored at 4 °C for 5 days. Each day the perfusate was brought to room temperature and it was applied to a freshly dissected sartorius muscle. Latency in production of sarcomeric oscillations did not change over the 5 day test period from which it was concluded that the oscillogen is stable at 4 °C (twelve expts.). In other experiments the active perfusate was heated in a water bath at 80 °C for 15 min. A white precipitate formed which was presumed to be heat denatured proteins extracted from the muscle. The cooled, precipitate-free perfusate continued to initiate sarcomeric oscillations with short latency, 2–5 min (six expts.). Thus the oscillogen is not destroyed at a temperature of 80 °C.

The maximum molecular weight of the oscillogen was estimated by testing its permeation through dialysis tubing membrane with a mol. wt. cut-off above 3500 daltons. In a typical experiment a dialysis sac was filled with 5 ml. of active perfusate, following which the sac was tied off and immersed in 45 ml. of 1 mM-caffeine–Ringer solution. Dialysis was continued for 48 hr at 4 °C with continuous stirring, after which the solution was brought to room temperature. An aliquot of the solution inside the dialysis sac was microperfused onto a fresh muscle, and the same was done with a similar aliquot of the solution outside the dialysis sac.

In four such experiments it was found that the 'inside' and 'outside' solutions were equally effective in promoting the initiation of sarcomeric oscillations. With each solution the latency was between 10–15 min. In control experiments in which

1 mM-caffeine–Ringer solution alone was microperfused onto a muscle, the latency for production of sarcomeric oscillations was 30 min or longer. Thus from these results we concluded that the oscillogen was uniformly distributed between the solutions inside and outside the dialysis membrane and that the mol. wt. of the oscillogen was less than 3500 daltons.

More definitive experiments utilizing Sephadex G-10 column chromatography were performed to separate and further characterize the oscillogen. Sephadex columns were prepared and calibrated as described in Methods.

The goal of the first experiment was to determine whether the oscillogen could be separated from caffeine. The column was first equilibrated with Ringer solution. Then 1 ml. of a 1 mM-caffeine–Ringer solution was added to the column and followed by elution with 40 ml. of Ringer solution at a flow rate of 0·1 ml./min. The absorbance of each serially collected 1 ml. eluate fraction was determined spectrophotometrically at 272 nm (U.V. absorbance peak for caffeine). It was found that caffeine was eluted almost entirely in fraction numbers 26–31.

Separation of the oscillogen was carried out as follows. To increase the concentration of the oscillogen, 10 ml. of perfusate (1 mM caffeine–Ringer solution) was obtained from two vigorously oscillating muscles. The solution was lyophilized at −78 °C, and the residue was dissolved in 2 ml. distilled water. This concentrated perfusate was added to a Sephadex G-10 column and eluted with caffeine-free Ringer solution. Forty 1 ml. fractions were collected. Aliquots of these fractions were tested for oscillogenic activity by micro-perfusion on to freshly dissected sartorius muscles bathed in Ringer solution alone (see Methods). It was found that fractions 19–21 initiated sarcomeric oscillations with a latency of 2 min. These sarcomeric oscillations quickly evolved into contractile waves. This was a particularly interesting result because essentially caffeine is eluted in fractions 26–31 and fractions 19–21 do not contain caffeine. We concluded therefore that the oscillogen is distinguishable from caffeine. Additional evidence supporting this conclusion is given below.

From gel permeation chromatography we estimated the mol. wt. of the oscillogen to be between 700–1000 daltons. In order to provide further chemical characterization of the oscillogen and also an analytical technique which could be used for its estimation, we determined absorbance spectra on active oscillogen containing solutions in the U.V. region from 220–320 nm. A solution of 1 mM-caffeine–Ringer was used as a blank, and the active 1 mM-caffeine–Ringer perfusates represented the test samples. By this means the appreciable absorbance of 1 mM-caffeine solution in this spectral region was nullified. Fig. 4 is a plot which shows the difference in absorbance between the blank and various perfusates. It can be seen that the perfusates contain a substance with an absorption peak at 245 nm. Furthermore, with time the concentration of this substance increases in solution bathing oscillating muscle. Apparently a smaller quantity of this substance is also released from non-oscillating caffeine-treated muscle. The perfusates whose differential spectral absorbance is given in Fig. 4 were tested by microperfusing each on to freshly dissected sartorius muscles. In three such assays the first perfusate produced sarcomeric oscillations with an average latency of 3 min. Latencies for perfusates 2 and 3 averaged 7 and 14 min respectively. Thus the differential absorbance at 245 nm gives a measure of the capacity of a perfusate to produce sarcomeric oscillations when applied to muscle.

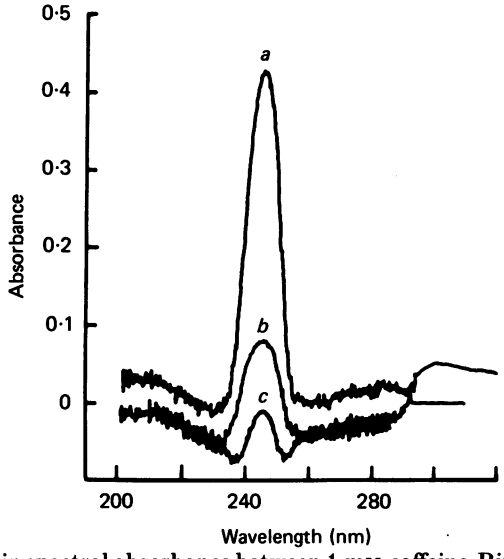

Fig. 4. Difference in spectral absorbance between 1 mM-caffeine-Ringer perfusate solutions and 1 mM-caffeine-Ringer solution as a blank. (*a*), 1 mM-caffeine-Ringer perfusate solution after 3 hr in contact with an oscillating muscle; (*b*) Same as *a*, but after 1 hr in contact with an oscillating muscle; (*c*) Same as *a*, but after 2·5 hr in contact with a non-oscillating muscle. (Absorbance curves traced from original records.)

The liberation, from oscillating caffeine-treated sartorius muscle, of a substance with spectral absorption at 245 nm was verified in four such additional experiments, and results similar to those shown in Fig. 4 were obtained.

DISCUSSION

Morphological aspects of sarcomeric oscillations and propagated waves

Our results show that the sarcomeric oscillations which are initiated by application of 1 mM-caffeine to frog muscle involve a transient diminution of sarcomere length. During this transient contraction the I-band width decreases but the change is insufficient to obliterate the I-band. Analysis of photomicrographs of oscillating regions of caffeine-treated muscle fibres (Fig. 2) shows that the distribution of sarcomere length is wider than in controls. There is also a suggestion that a few sarcomeres in caffeine-treated muscle are longer than the maximum length found in controls. We interpret this as evidence that contracting sarcomeres stretch adjacent relaxed sarcomeres.

Photographic evidence shows that, during generation of sarcomeric oscillations, only a portion of a sarcomere is involved. Thus, along the radial direction the sarcomeric oscillation is generated by contraction of a limited number of myofibrillar bundles. Such localized activity could be produced by a momentary release of Ca^{2+} from a restricted number of adjacent T-tubule terminal cistern junctions.

One of the goals of the present study was to measure the time required to complete an individual sarcomeric oscillation. A second goal was to determine whether contractile oscillations of a single sarcomere occur at random or exhibit some regular

periodicity. We also wished to estimate their frequency. Unfortunately our attempts to answer these questions were not successful. Plots of the lengths of a group of adjacent sarcomeres measured in successive frames of a high speed film record of oscillating muscle fibres showed irregularities which we did not regard as characteristic of sarcomeric oscillations. Similar irregularities of sarcomere length but of smaller amplitude appeared in analysis of non-oscillating muscle fibres. These changes we considered were artifacts produced by mechanical vibrations, some of which were generated by the high speed cine-camera. Additional problems in analysis arose from experimental errors in measuring small variations in sarcomere length.

During prolonged exposure to caffeine, sarcomeric oscillations evolve into slow propagated contractile waves which are readily observed visually and are also easily photographed (Plate 3). These contractile waves represent the contraction of an ensemble of adjacent sarcomeres. Each sarcomere appears to be active along its entire radial dimension. The contraction is vigorous and produces noticeable localized outward bulging of the muscle fibre. Similar contractile activity was observed by Coleman *et al.* (1972) in methylxanthine-treated muscle fibres in tissue culture.

The mechanism responsible for these contraction waves is unknown at present. Observation of the membrane potential of muscle fibres during the transit of contractile waves shows that this kind of activity is not accompanied by an action potential. In fact, electrical initiation of an action potential in caffeine-treated fibres produces a brief brisk twitch of much shorter duration than that shown by these contractile waves. However, the possibility that a change in membrane potential accompanies the contractile wave cannot be entirely ruled out at present, because during transit of the contractile wave a small reduction (1–2 mV) in membrane potential occurs. We believe that this depolarization is a mechanically produced artifact generated as a result of transiently increased electrical leakage at the impalement site. A further investigation of this question is required.

We considered the possibility that the mechanism responsible for propagated contractile waves in caffeine-treated fibres involves stretch activation of the contractile system. Stretch activation has been demonstrated in glycerol extracted insect muscle (Jewell & Rüegg, 1966). This property is also exhibited by glycerinated mammalian heart muscle (Steiger, 1971) and frog muscle (Rüegg, Steiger & Schädler, 1970) when the Ca^{2+} concentration is appropriately increased. Length dependence of the activation of skinned toad fibres by Ca^{2+} was shown by Endo (1972*a*, *b*). In untreated frog muscle fibres during tetanic contraction, stretch enhances mechanical performance (Edman, Elzinga & Noble, 1978) and gives rise to damped oscillatory tension changes (Armstrong, Huxley & Julian, 1966). Myofilament-generated tension oscillations during partial Ca^{2+} activation and activation dependence of the sarcomere length–tension relation has been shown in skinned cardiac muscle fibres (Fabiato & Fabiato, 1978). It appears that the property of stretch activation is exhibited when the intracellular Ca^{2+} concentration is raised either experimentally or by physiological means to the point where the contractile system is activated to some degree. Since frog muscle treated with 1 mM-caffeine exhibits sarcomeric oscillations and with time generates full contractile waves, it appears that the contractile system of these muscle fibres is effectively activated in some regions or is on the the threshold of activation in other zones. We therefore propose that caffeine-treated fibres can exhibit stretch

activation. An experimental study of stretch activation in caffeine-treated fibres is needed to provide evidence on this point.

Possible mechanisms involved in the generation of sarcomeric oscillations in caffeine-treated muscle fibres

Under physiological conditions, excitation–contraction coupling involves depolarization of T-tubule membranes which by an unknown coupling mechanism causes release of Ca^{2+} from terminal cisternae of the s.r. Thus if caffeine caused transient localized T-tubule depolarizations which initiated Ca^{2+} release from adjacent terminal cisternae, then localized transient activation of adjacent sarcomeres could result. There is no evidence that 1 mM-caffeine causes T-tubule membrane depolarization. As mentioned earlier, we have not detected any obvious increases in membrane noise in our membrane potential records, but this question should be investigated more carefully.

The fact that electrically produced membrane depolarization of caffeine-treated muscle fibres can initiate sarcomeric oscillations and that membrane hyperpolarization can slow or stop them does not necessarily provide critical support for the above mechanism. One might consider that electrically produced variation in T-tubule potential acts to create conditions more favourable or less favourable for the development of sarcomeric contractions by applied caffeine.

In thinking along these lines we considered the possibility that in caffeine treated muscles there is an increased K^+ efflux which would raise the K^+ concentration in T-tubules. The resulting depolarization of T-tubule membranes might then initiate sarcomeric oscillations. One argument against such a mechanism is that sarcomeric oscillations are produced in caffeine-treated muscles whose membrane potentials (measured with an intracellular electrode) are in the normal range. A second argument is that in fibres depolarized to a variable extent, by stepwise increase of extracellular $[K^+]$, sarcomeric oscillations are not produced when 1 mM-caffeine is added to the bathing solution.

It is reasonable to suppose that sarcomeric oscillations are initiated by localized increases in intracellular Ca^{2+} concentration which brings about activation of the contractile elements. It may be noted that sarcomeric oscillations can be initiated at the neuromuscular junction by applying various quaternary ammonium compounds which depolarize post-junctional membranes (Marco & Nastuk, 1968). An increased Ca^{2+} influx occurs during such depolarization. (Takeuchi, 1963).

Caffeine initiates sarcomeric oscillations at all regions of the muscle fibre and it might do so by one or more possible mechanisms.

1. Caffeine might penetrate the sarcolemma and act directly on the s.r. to cause release of Ca^{2+}. Weber & Herz (1968) showed that Ca^{2+} accumulated by s.r. fragments is released when caffeine is applied.

2. Caffeine could cause a rise in intracellular Ca^{2+} by increasing Ca^{2+} influx across the sarcolemma (Bianchi, 1968).

3. Caffeine might act to increase intracellular Ca^{2+} via a second messenger such as cyclic AMP. It could cause an increase in cyclic AMP concentration by inhibiting phosphodiesterase. However, from experiments not detailed in this paper, we determined that extracellular application of cyclic AMP, cyclic GMP and dibutyryl

cyclic AMP in a wide range of concentrations had no influence on production of sarcomeric oscillations by caffeine.

4. Finally, caffeine might act directly on T-tubules, initiating a series of steps which result in release of Ca^{2+} from the adjacent lateral cisternae.

Perhaps caffeine can initiate sarcomeric oscillations by any of the above mechanisms, but there are reasons for believing that its principal site of action is the T-tubule terminal cistern junction (Lüttgau & Oetliker, 1968; McCallister & Hadek, 1973). This selectivity in the site of action of caffeine may be relatively prominent when caffeine is applied in low concentrations (1–2 mM).

Additional support for the above argument is provided by results obtained with dantrolene treated muscle. Several investigators (Ellis & Bryant, 1972; Putney & Bianchi, 1974; Brocklehurst, 1975; Desmedt & Hainaut, 1979) have concluded that dantrolene blocks E–C coupling at the T-tubule terminal cistern junctions. We have found that dantrolene blocks production of sarcomeric oscillations by 1–2 mM-caffeine, from which one might conclude that caffeine has the site of action stated above.

Possible action of the oscillogen in E–C coupling

Several mechanisms proposed to explain E–C coupling have been briefly reviewed by Mathias, Levis & Eisenberg (1980). In one of these, called the activator hypothesis, a change in T-tubule membrane potential mobilizes an intermediary substance which is involved in the release of Ca^{2+} from the terminal cisterns of the s.r. The suggestion that such an activator might exist was made by Hodgkin & Horowicz (1960*b*) and a detailed expansion of the activator hypothesis involving the postulated existence of a charged molecular complex at the T-tubule lateral cistern junction has been developed by Chandler and his colleagues (Chandler, Rakowski & Schneider, 1976).

We have tried to utilize the activator hypothesis in attempting to explain the action of caffeine in the generation of sarcomeric oscillations and the liberation of a chemical oscillogen. We estimate the molecular weight of the oscillogen as 700–1000 daltons. Its U.V. absorption at 245 nm suggests that the molecule contains a conjugated system. We have investigated the absorption spectra of the following compounds: carnosine, creatine, adenosine triphosphate, adenosine diphosphate, adenosine monophosphate, inosine triphosphate, cyclic AMP, dibutyryl cyclic AMP, cyclic GMP. None showed an absorption peak at 245 nm, as is the case for the oscillogen.

One possible mechanism we have considered is that caffeine may react with a charged molecular complex such as that postulated by other workers to be located at the T-tubule lateral cistern junction. Caffeine might cause dissociation of a low molecular weight subunit of the charged complex. This subunit might act on the lateral cistern membrane to cause release of sequestered Ca^{2+}. The subunit also could diffuse into the T-tubule lumen and pass out to the surrounding bathing fluid. Perhaps the oscillogen which we have detected is such a subunit molecule.

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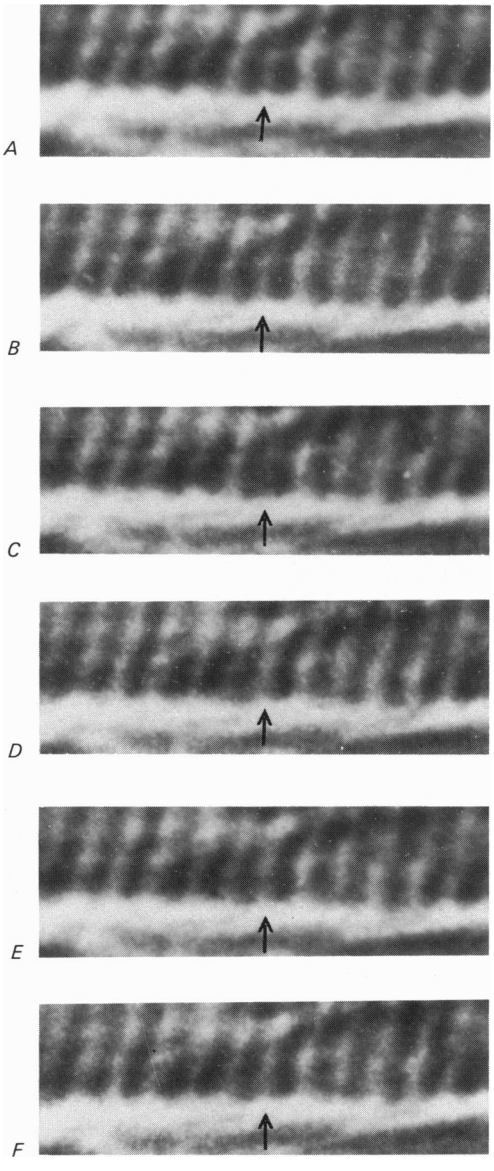
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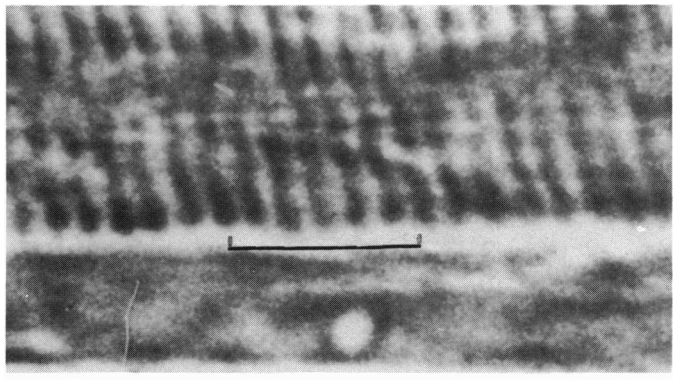

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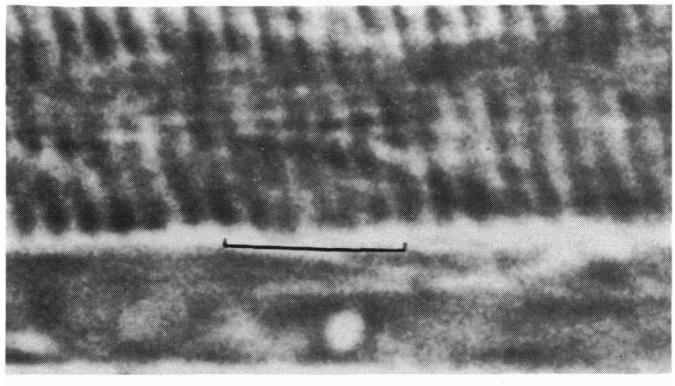

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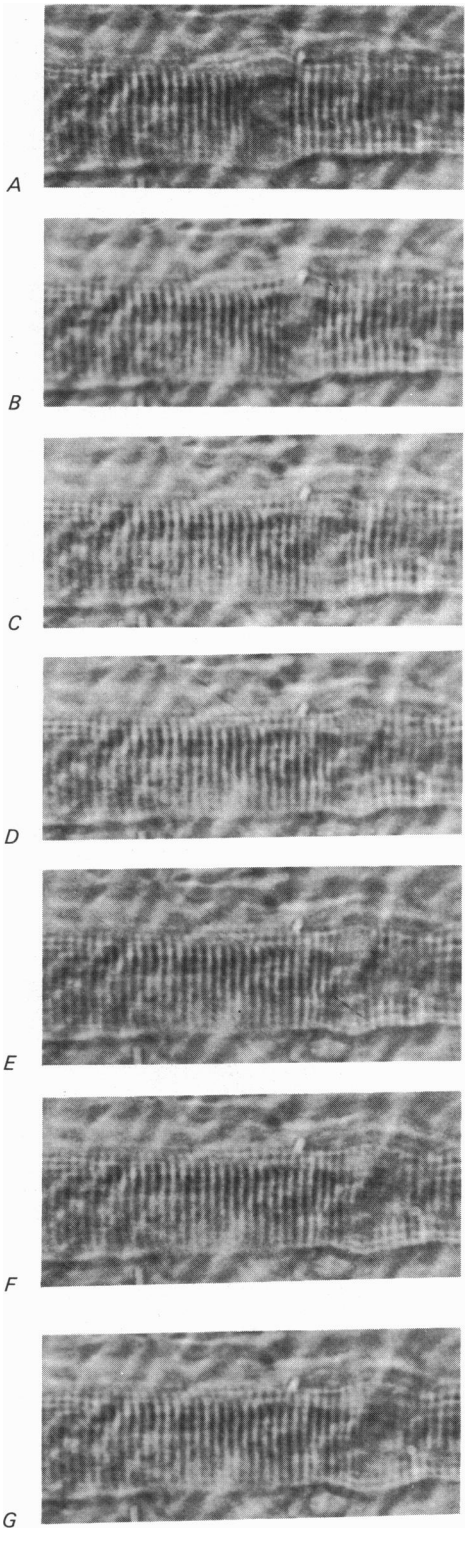
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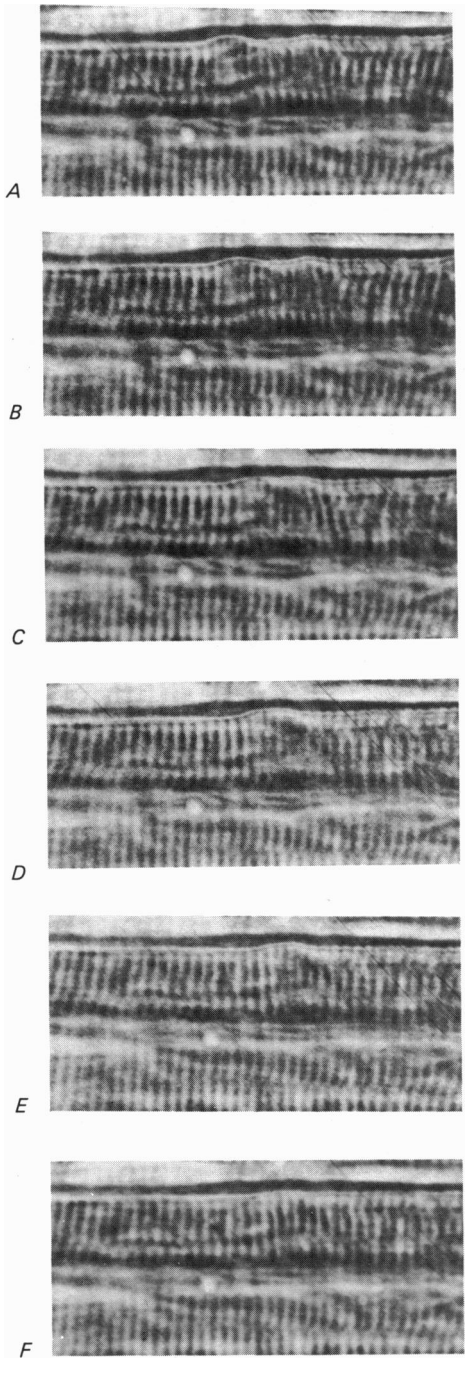
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EXPLANATION OF PLATES

PLATE 1

Photomicrographs taken at 40 msec intervals showing a region of a caffeine-treated muscle fibre early in development of sarcomeric oscillations (magnification = 2000 ×). The I-band indicated by the arrow in each frame undergoes a local decrease in width (frame C).

PLATE 2

Two photomicrographs taken 80 msec apart from a vigorously oscillating muscle fibre which had been exposed to caffeine for 1·5 hr (magnification = 1800 ×). The bar indicates a group of seven adjacent A-bands. As shown in *B*, the third I-band from the left end of the bar has been widened as a result of contraction of the adjacent sarcomeres.

PLATE 3

Sequence of photomicrographs taken at 40 msec intervals showing a propagated contraction wave involving several adjacent sarcomeres. Wave velocity is 400 μm/sec. Note the bulge in the fibre at the region of contraction (magnification = 600 ×).

PLATE 4

Two propagated waves travelling towards each other which become annihilated upon collision (magnification = 600 ×).