Effects of Caffeine on Crayfish Muscle Fibers

I. Activation of contraction and induction of Ca spike electrogenesis

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ABSTRACT Contractions are evoked in single muscle fibers of crayfish by intracellular as well as extracellular applications of caffeine. Responses to external applications in concentrations above 2 mM could be induced indefinitely. With concentrations above 5 mM the caffeine-induced responses were highly repeatable. Tensions were transient even when the caffeine remained in the bath. There was no change in resting potential, but during the contraction the effective resistance decreased about 10%. A number of factors (change in pH, Ca, K, and Cl) modified the responses. The time course of the tension was greatly prolonged when the transverse tubular system (TTS) was swollen and was again shortened when the TTS was caused to shrink. An increased permeability to Ca induced by caffeine was evidenced by the transformation of the normally graded electrical responses to Ca spikes, which are insensitive to tetrodotoxin. The overshoot is a function of both external Ca and caffeine. A 10-fold change in Ca changed the overshoot by 19 mv in the presence of 10 mM caffeine and by 29 mv in 80 mM caffeine. The role of the increased permeability to Ca for caffeine-induced contractions will be analyzed in the accompanying paper.

INTRODUCTION

Caffeine induces contraction of muscle and has been extensively used for investigating contractile processes, mainly in muscles of frog (for references see Sandow, 1965). Caffeine penetrates the cell membrane rapidly and enhances the influx and efflux of Ca (Bianchi, 1961, 1962), and the increase of intracellular Ca is supposed to be the trigger for contractile activity (Sandow, 1952). The membrane potential is unaltered by caffeine and in fact, caffeine induces contractions in muscle fibers that are depolarized in media
with high concentrations of K (Axelsson and Thesleff, 1958; Lüttgau and Oetliker, 1968). Caffeine (1–10 mM) reduces sequestration of Ca in sarcotubular preparations (Weber and Herz, 1968; Weber, 1968). It has been suggested that this action of caffeine brings about a movement of Ca from the sarcoplasmic reticulum into the myoplasm, the rise of Ca in the latter thus accounting for the tension evoked by the presence of caffeine (Weber and Herz, 1968).

A considerable body of data is now available on the excitation-contraction coupling processes in crayfish muscle (Girardier et al., 1963; Reuben et al., 1967 a). The present study of the effects of caffeine on this preparation was undertaken with the expectation that it might provide additional information, particularly on the source of Ca for the chemomechanical processes of contraction. In some respects, the responses of single isolated crayfish muscle fibers to caffeine are similar to those observed in frog muscle, and in this paper the main emphasis is on the phenomenology of caffeine-induced contractions in the crayfish fibers. However, caffeine also produces effects on the electrogenic properties of the cell membrane. One that has not been reported for other preparations is the induction of spike electrogenesis in the crayfish muscle fiber. The spikes of these fibers are generated by an influx of Ca (Takeda, 1967; Ozeki et al., 1966). Thus this finding, which will be described and analyzed in the present paper, provides new evidence that caffeine can promote the entry of Ca into the cell. The accompanying paper deals with the observation that after a challenge with caffeine, crayfish fibers become transiently refractory to subsequent applications, but immediate recovery of responsiveness can be induced by increasing external K or by intracellularly applied currents. These data, therefore, bear on the relationship between contractile activation with a chemical agent, caffeine, and by electrical processes. Preliminary reports of some of this work have appeared (Chiarandini et al., 1967, 1970).

METHODS

All the experiments were done on single muscle fibers from *Orconectes virilis*. The study was carried out over a period of more than 3 yr and involved a total of more than 200 muscle fibers. Seasonal variation was not seen. Each of the figures included in this and the accompanying paper is illustrative of at least three similar experiments. In most cases, however, a given experiment and its variations were done on a series of 20 or more fibers. The work was done at room temperatures (19°–22°C).

Isolation of the muscle fiber from the flexor muscle of the meropodite was done following the technique described previously (Girardier et al., 1963), but with some modifications. The preparation was dissected in a small chamber containing 1–2 ml of saline. All the exoskeleton was removed except for a small segment of the ischiopodite distally to the tendon. In order to record tension the tendon was attached to the tip of a small stainless steel forceps fixed to an isometric force-displace-
A stretch of about 25% above the resting length was applied. The membrane potential was monitored with an intracellular microelectrode filled with 3 M KCl. Polarizing currents were passed through a second micropipette filled with 3 M KCl. The electrophysiological equipment was standard for the laboratory. Tension, membrane potential, and applied current were recorded on a four-channel Grass polygraph with curvilinear registration.

A standard crayfish saline modified from van Harreveld's (1936) was used (200 mM NaCl, 5 mM KCl, 13.5 mM CaCl₂, and 2 mM Tris Cl; pH 7.4). Cl-free solutions were made by replacing all the Cl by propionate which has been found to be impermeant for the crayfish muscle while still allowing a high concentration of ionized Ca in the solution (Reuben et al., 1964). Solutions with elevated concentrations of K (high K salines) were made hypertonic, by adding the necessary amount of the K salt or isotonic, by substituting an equivalent amount of K for Na. Salines containing an elevated concentration of Ca were prepared by substituting the necessary amount of the Ca salt for an osmotically equivalent amount of the Na salt. Ca-free media were obtained by omitting the Ca salt. Anions of the lyotropic series (Br⁻, NO₃⁻, I⁻, and SCN⁻) were tested by substituting them for Cl in different concentrations (20, 100, and 200 mM). Mn ions were added at a concentration of 20 mM, removing an osmotically equivalent concentration of NaCl.

Solutions containing up to 10 mM caffeine were made by adding to the saline the necessary volume of a stock solution of 0.5 M caffeine dissolved in warmed distilled water. When caffeine concentrations larger than 10 mM were required, the weighed drug was added to the saline. In order to apply the alkaloid 8–10 ml of saline containing the caffeine were flushed through the chamber in 4–8 sec. Usually the drug was removed 30–40 sec later by flushing large volumes of control saline. Caffeine concentrations below 5 mM were seldom used since they do not activate the fiber uniformly (see Fig. 2). Routinely for most measurements a concentration of 10 mM was employed. Procaine (procaine hydrochloride, Abbot Laboratories, North Chicago, Ill.) was added to the bathing solution in concentrations ranging from 10⁻⁴ to 10⁻² w/v. Tetrodotoxin (Sankyo Co., Tokyo, Japan) was used at a concentration of 10⁻⁶ g/ml.

Injection of Caffeine Fibers were also stimulated by local extracellular, or intracellular, applications of caffeine under microscopic control employing electroosmotic injection (Krnjević and Whittaker, 1965; Brandt and Grundfest, 1968; April et al., 1968). For this purpose a micropipette was filled with a low ionic strength (50 mM KCl) solution containing 1–10 mM caffeine. Due to the existence of a zeta potential arising from the negative fixed charges present on the surface of the glass wall of the micropipette, it is possible to induce an electroosmotic movement of fluid out of the micropipette by passing 10⁻⁷–10⁻⁶ amp of outward current. Low ionic strength solutions within the microelectrode are essential so as not to minimize the zeta potential. The amount of caffeine thus released was enough to cause localized contractions which could be photographed and their tension output recorded.
RESULTS

A. The Sites of Action of Caffeine

Axelsson and Thesleff (1958) found that intracellular injections of caffeine by diffusion from an internal pipette into frog muscle fibers were without effect. They therefore concluded that the primary site of action of caffeine is the cell membrane. Caldwell and Walster (1963) injected caffeine into crab fibers by hydrostatic pressure and obtained contractions. In the present work it was found that iontophoretic injections from an intracellular electrode filled with 0.5 mM caffeine citrate were ineffectual in the crayfish. Apparently the iontophoretic injections do not allow the caffeine concentration to attain an adequate level for inducing tension, since another method of applying caffeine intracellularly, as the uncharged molecule by electroosmosis, is quite effective in inducing tension. When an outward (cathodal, depolarizing) current was applied to the micropipette filled with 50 mM KCl and 1-10 mM caffeine the drug could be delivered into the fiber in sufficiently high

![Figure 1](image-url)
concentrations to evoke tension. The responses to these injections are in many respects similar to those that are elicited by iontophoretic applications of Ca (Brandt and Grundfest, 1968; April et al., 1968).

The photomicrographs of Fig. 1 show a fiber with such a microelectrode just outside the cell and then within the muscle fiber. The fiber was bathed in an isosmotic KCl solution and thus it was fully depolarized. Otherwise, the comparatively large currents \((10^{-7}-10^{-6} \text{ amp})\) required for ejecting the caffeine might themselves have caused contraction of the fiber. The approach of the caffeine-filled electrode did not itself induce a contraction, indicating that little or none of the caffeine diffused from the tip. However, when a current was applied (center) with the electrode still outside, a "contraction sphere" was elicited (Brandt and Grundfest, 1968; April et al., 1968). A localized contraction sphere was also obtained on intracellular application of the caffeine, after the electrode had entered the cell.

The records of Fig. 1 show the tensions evoked in another fiber with the application of caffeine through the exteriorized microelectrode (top) and after the electrode had entered the fiber (below). The latency and slow rise of the tension induced by the extracellular caffeine application suggest that the sites of caffeine action are intracellular. These results, although in accord with data implicating the sarcoplasmic reticulum (Weber, 1968; Weber and Herz, 1968), do not exclude other possible active sites such as surface or invagination membranes.

**B. Parameters of Caffeine-Induced Tension**

The foregoing experiments demonstrated that caffeine, whether applied to the muscle externally or internally, induced tension. The experiments reported in the rest of this paper employed only external applications, permitting a greater flexibility of experimental manipulation than is possible with intracellular applications. For example, there was now no need to restrict the experiments to K-depolarized fibers.

Fig. 2 shows the superimposed recordings of tension elicited in a single fiber bathed in control saline to which various concentrations of caffeine were added. In each sequence caffeine was introduced at the arrow and was removed after 50 sec. The changes in the bath required about 8 sec. In some of the registrations small "wash-in" and "washout" artifacts denote the periods during which the changes were made.

No appreciable tension developed when 1 mM caffeine was applied but 2 mM usually elicited a measurable response. Caffeine concentrations lower than 5 mM do not produce a uniform activation of the muscle fibers, as may be seen in the response induced by 2 mM caffeine. After the instability of the tension recording due to the wash-in artifact, several small elevations preceded the maximal tension evoked by this concentration of caffeine. Under
the microscope the fibers are then seen to undergo small discretely localized contractions before many such coalesce to give the larger response. Since the caffeine was introduced rapidly into the chamber, the nonuniform responsiveness suggests that the caffeine penetrated into some regions of the fiber preferentially, or that the contractile machinery in some regions is more sensitive to caffeine. The responses to low concentrations of caffeine have not yet been studied systematically. In order to ensure relatively uniform contractile activation of the fibers concentrations of caffeine greater than 5 mM were used normally in the present work.

![Figure 2](image-url)

**Figure 2.** Tensions evoked in a single muscle fiber by applying caffeine. The concentrations in the bath ranged between 1 and 40 mM as indicated on the superimposed recordings. The change from the control saline to one containing caffeine was begun at the time indicated by the arrow. It lasted about 8 sec and the end of the wash-in is shown by the artifact on the traces which is due to a small perturbation of the recording system. After 50 sec exposure to the caffeine the solution was replaced with control saline. The washout artifact is particularly evident on the lowest trace, in which the fiber was exposed to 1 mM caffeine. This concentration did not induce tension. Note that the decay of tension following 2 mM caffeine coincided with the corresponding portion of the decay curve after 5 mM caffeine. Inset shows a plot of the peak tensions evoked by the different concentrations of caffeine against the logarithm of the concentration.

The tension rose and fell back to the resting level while the caffeine was still present. The rate of rise and the peak tension increased markedly as the caffeine concentration was increased. The peak increased approximately linearly with log (caffeine) between 2 and 20 mM of the agent (Fig. 2, inset). Tensions comparable to the maximal responses to high concentrations of K were induced by 40–80 mM caffeine, but even 10 mM of the drug evoked a tension close to the maximum. In the experiment of Fig. 2 the tension evoked by 10 mM caffeine was about 85% of maximal. Frog muscle fibers appear to be more sensitive to caffeine; maximal tension is induced by 5–10 mM of the agent (Lüttgau and Oetliker, 1968).

The responses to caffeine could be reproduced with numerous repeated applications of the drug, provided that an appropriate interval (15–20 min) was allowed between the applications. This interval can be changed by various experimental procedures (see the accompanying paper). The re-
producibility of the responses to successive challenges with caffeine in concentrations up to 40 mM under the stated conditions appears to be in marked contrast to the effects in frog muscle fibers (Conway and Sakai, 1960; Lüttgau and Oetliker, 1968).

The decay of the tension was approximately exponential but its rate was slightly faster for larger peak tensions. Thus, the half-time for the decay was 15 sec on exposure to 40 mM caffeine and 20 sec in 5 mM caffeine. The half-time for decay for K-induced tension also decreased with increasing amplitude of the contractions both in frog (Hodgkin and Horowicz, 1960) and in crayfish (Reuben et al., 1967).

C. Factors Modifying Caffeine-Induced Tension

**EFFECTS OF pH** The tension induced by 10 mM caffeine is markedly affected by the pH of the medium (Fig. 3). In comparison with the response at pH 7.4 the tension decreased when the pH was lowered and augmented when the pH was raised. The decay of the tension was not altered markedly by changes in pH. In the experiment of Fig. 3 a change from pH 7.4 to 8.3 increased the peak tension induced by caffeine by 30%. For comparison it may be noted (Fig. 2) that when caffeine (at pH 7.4) is increased from 10-40 mM the tension increases by only 15%.

The effects of changing pH are fairly rapid, reaching their full value within 15 sec. It is unlikely that the intracellular pH is changed significantly by the changes in the pH of the bathing medium (Caldwell, 1958). Thus, it is probable that changes in pH of the bath modify sites at the boundary of the muscle fiber and that these modifications influence intracellular processes that regulate contraction.

**EFFECTS OF CHANGING THE ANIONS IN THE MEDIUM** Muscle fibers were studied after having been equilibrated in media in which the Cl of the standard saline had been replaced totally or partially with other anions. When the substituting anions were those of the lyotropic series the tension induced by caffeine was not potentiated and might be reduced, particularly in the presence of I or SCN. On the other hand, substitution of propionate, an
impermeant anion (Reuben et al., 1964) for Cl, transiently enhances the tension evoked by caffeine (Fig. 4). In the propionate medium the peak tension initially increased and the duration of the contraction was prolonged. This contrasts with the effects obtained on tension evoked by elevating K. In the latter case the peak and the duration of the tension decreased when propionate was substituted for Cl (Reuben et al., 1967a). Subsequent challenges with caffeine, after the fiber had been equilibrated in the Cl-free saline, elicited tensions comparable to the control in the Cl medium, but the tension developed more slowly (Fig. 4).

**Effects of altering K.** When a muscle fiber is bathed in Cl saline an increase of K induces redistribution of KCl, no matter whether the change is made isosmotically or hyperosmotically (Boyle and Conway, 1941; Reuben et al., 1964). Water movements accompany the redistribution of KCl and the cell volume as well as the intracellular ionic strength changes to varying degrees. These factors, and particularly the second, modify the contractions that are evoked by intracellular applications of calcium or caffeine (April et al., 1968). In order to minimize these effects the fiber of Fig. 5 was initially equilibrated in the Cl-free control saline (i.e., one containing 5 mM K but with propionate substituted for all the Cl). The first record shows the tension evoked on applying 10 mM caffeine. The fiber was then exposed to a medium containing 50 mM K propionate (maintained isosmotic by removal of Na propionate) that evoked a transient contraction that was about 70% of the maximal response (Reuben et al., 1967a; cf. also Fig. 10 of the accompanying paper). After equilibration for 10 min in this medium the fiber was again challenged with 10 mM caffeine. The tension was slightly larger and more prolonged than in the control. This effect is in general agreement with the effects of high K on caffeine-induced tension in frog muscle (Axelsson and Thesleff, 1958; Lüttgau and Oetliker, 1968). However, subsequent challenges with caffeine, after intervals of 30-40 min and while
the fiber was still depolarized in the high K medium, elicited responses similar to that of the control.

**EFFECTS OF SWELLING THE TRANSVERSE TUBULAR SYSTEM (TTS)**

The first recording of Fig. 6 shows the caffeine tension elicited in the control saline. The fiber was then exposed for 30 min to an isosmotic Cl saline containing 100 mM K, and then returned to the control saline. As has already been described (Girardier et al., 1963; Brandt et al., 1965, 1968) this change induces a swelling of the TTS which is denoted by darkening of the fiber and the apparent disappearance of the prominent sarcomeric striations. Reapplication of caffeine now induced a tension of comparable peak amplitude, but which exhibited a prolonged plateau phase. The tension during this phase was rather asynchronous. In one fiber treated so that the TTS swelled, the plateau phase was observed for 30 min before it was terminated by the removal of the caffeine. Another challenge of the fiber in Fig. 6 with caffeine after 6 min evoked a similar long-lasting tension. Reversal of the swelling

![Figure 5](image1)

**Figure 5.** Changes in caffeine-induced tensions caused by a high K isosmotic saline. Fiber bathed in a Cl-free saline. Increase of K, to 50 mM resulted in a longer lasting and slightly higher tension initially. Subsequently, the time course of the response returned to the control in spite of the elevated K, (bottom records).

![Figure 6](image2)

**Figure 6.** Effect on the caffeine-induced tension of swelling the transverse tubular system (TTS). The first tension of the upper row was induced by 10 mM caffeine when the fiber was bathed in normal saline. In order to induce the swelling of the TTS, the fiber was then soaked in a 100 mM KCl isosmotic saline and after 30 min was replaced in normal saline for 20 min. When caffeine was applied, the peak tension was followed by a long-lasting plateau. A second application of caffeine, 6 min later, induced an almost similar response. The fiber was then soaked for 6 min in the 100 mM KCl isotonic saline to reverse the swelling of the TTS. When caffeine was applied the tension was smaller but the plateau phase was almost abolished.
of the TTS by reimmersing the fiber in the isosmotic 100 mM KCl saline (Brandt et al., 1968) eliminated the plateau phase of the response.

ROLE OF EXTERNAL Ca. An increase in the concentration of calcium from 13.5 to 50 mM rapidly caused a slight prolongation and increased the caffeine-evoked contractions. Removal of Ca from the bath causes a transient depolarization of the muscle fibers (Reuben et al., 1967b). Since depolarization affects the responses to caffeine (above, and the accompanying paper), studies on the effect of removing calcium were done with the fiber maintained in a high (220 mM) K propionate medium. The membrane potential thus was near zero and removal of the Ca did not produce a further depolarization.

The fiber for the experiment of Fig. 7 was equilibrated in the high K propionate Ca-free medium for about 1 hr before being challenged with 10 mM caffeine. The response (Fig. 7 A) was a nearly maximal tension for this fiber (ca. 3 kg/cm²). Similar responses were also obtained with fibers that were kept at rest in this medium for up to 3 hr. If the degree of tension elicited by caffeine reflects the available Ca store in the SR, then the depletion of this store in the Ca-free medium must be quite slow. However, repeated challenges with caffeine in all cases induced successively smaller and briefer tensions. In the experiment of Fig. 7 each application of caffeine lasted about 80 sec and there were 20 min intervals between the applications. The tension in response to the fourth challenge (D) was only about 10% of that in A. Responsiveness can be restored, but the introduction of 13.5 mM Ca propionate into the bathing medium is not in itself sufficient to do so. The foregoing experiments do indicate, however, that the source from which caffeine releases Ca to induce tension is dependent, at least secondarily, upon the concentration of the external Ca.

EFFECTS OF PROCAINE. Procaine (10⁻⁴–10⁻² g/ml) substantially reduces or abolishes the tension evoked in crayfish muscle fibers by intracellularly applied currents, or by elevating the K of the bathing medium (Reuben et al., 1967a). The caffeine-evoked tension is abolished within
15–30 sec after applying $10^{-4}$ g/ml procaine (see Fig. 5 of the accompanying paper). The prolonged responses induced by caffeine in muscle fibers with swollen TTS are also abolished by procaine.

**EFFECT OF Mn** The contractions induced by K are blocked within 5–15 sec after adding 20 mM Mn to the bath (Zachar and Zacharova, 1968; see also the accompanying paper). Caffeine-induced contractions are not affected by Mn, although Mn does exert other effects that will be described in the accompanying paper.

**D. Effects of Caffeine on Electrical Characteristics of the Muscle Fiber**

In crayfish muscle fibers the membrane potential is affected little if at all by addition of up to 40 mM caffeine, but there is a slow depolarization of about 10–15 mv during the exposure of the fibers to 80 mM caffeine for 1 hr. Low concentrations of caffeine which induce tension do, however, modify the electrical characteristics of the membrane. Effects of caffeine on the membrane resistance are small and transient, but there is a dramatic effect on the electrogenesis in response to electrical stimuli.

Tension is registered on the upper traces of Fig. 8. The membrane potential, recorded with an intracellular microelectrode, is seen on the middle traces and the lower traces monitor the inward (hyperpolarizing) currents that were applied through another intracellular microelectrode. As already shown (Fig. 2), 1 mM caffeine did not evoke tension (Fig. 8 A). This concentration of caffeine was also without effect on the membrane potential or resistance. The record shows only the initial 20 sec period of the application (note the wash-in artifact on the two upper traces), but the presence of 1 mM caffeine for 1 hr was without effect either on the membrane potential or on the voltage-current characteristics of the fiber. In Fig. 8 B 10 mM caffeine was added to the bathing solution, producing a tension which carried the registration off scale. During the tension response a movement of the recording microelectrode produced an artifact in the voltage trace. The initial and transient change in the base line represents a shift of about 5 mv, presumably due to this movement artifact. However, the hyperpolarizing pulses which tested the membrane resistance diminished about 10% during the contraction, indicating that permeability of the membrane to ions had increased somewhat.

An increase in both influx and efflux of Ca induced by caffeine has been shown by radioisotopic studies in frog muscle (Bianchi, 1961). Similar studies have not yet been done on crayfish muscle fibers. However, the small decrease in resistance illustrated in Fig. 8 B is consistent with the possibility that caffeine also causes an increase in permeability to Ca of these muscle fibers.
There is more decisive evidence, however, that caffeine induces increased permeability to Ca. After fibers had been soaked in caffeine for 5–10 min they invariably developed spikes in response to intracellularly applied depolarizing currents (Fig. 9). The ability to generate spikes disappeared within a few minutes after the caffeine was removed. Normally the electrogensis of these muscle fibers is a graded response (Reuben et al., 1967a), although some treatments, such as the application of procaine (Fatt and Katz, 1953; Takeda, 1967; Ozeki et al., 1966; Reuben et al., 1967a), can induce overshooting all-or-none electrogensis. The “procaine spikes” are dependent upon entry of Ca and are blocked by Mn (Takeda, 1967), but not by tetrodotoxin (Ozeki et al., 1966). The latter (10^{-8} g/ml) was also without effect on the “caffeine spike” (Fig. 9), whereas Mn eliminated the spikes.

The graphs of Fig. 10 present other evidence to support the conclusion that caffeine alters the normally graded electrogensis to all-or-none spikes by promoting an increase in the movement of Ca when the fiber is depolarized by an applied current. The overshoots of the spikes increased linearly with log (Ca). In the presence of 10 mM caffeine the slope of the relation was 19 mv/decade, but the slope increased to 29 mv in the presence of 80 mM caffeine. The average slope value found in four fibers in this latter condition was 28.3 ± 0.5 mv (mean ± se). The amplitude of the overshoots at a given level of Ca was also increased when the caffeine concentration was raised, indicating that the conductance increase toward Ca is a function of the caffeine concentration. This was confirmed in experiments like that of
Fig. 11 in which the Ca concentration was kept constant while that of caffeine was changed. The graph of Fig. 11 is from an experiment in which Ca was kept at 13.5 mM and the caffeine concentration was varied from 5 to 80 mM. The latter value represents the practical upper limit since it is near the limit of solubility of caffeine at 20°C. In the experiments of Fig. 11 the slope was

26 mv/decade between the levels of 5 and 40 mM caffeine. The average value of the slope was 36.4 ± 5.4 mv for five fibers. The overshoot increased little more when the caffeine concentration was raised to 80 mM. It could be increased further, however, by raising the Ca, as has already been seen in the experiment of Fig. 10.

The records of Fig. 11 show the responses evoked in the presence of 10 and 80 mM caffeine, respectively (Ca kept at 13.5 mM). The duration of the spike clearly was prolonged by the presence of more caffeine. While this aspect has not been examined carefully as yet, the change in duration
Figure 11. Dependence of overshoot on caffeine concentration. The graph (solid circles) was obtained with 13.5 mM Ca in the bath. The open circle shows the increase in the overshoot when the Ca was increased to 53 mM in the presence of 80 mM caffeine. Insets, the spikes of this fiber when the caffeine concentration was 10 mM (upper record) and 80 mM (lower record).

Figure 12. Caffeine-induced spikes and tension. Three traces are shown in each recording: tension (upper), membrane potential (middle), and intracellularly applied stimulating current (lower). A, continuous sequence of records with the fiber in 10 mM caffeine. The stimuli were applied at 2 sec intervals in the upper section and at 4.5 sec intervals in the lower. The thin broken line indicates the level of initial tension. Although the small depolarizations evoked very small tensions, clear-cut “twitches” resulted when the fiber generated spikes. The tension of each contraction long outlasted the spike and there were marked summations with repeated stimulation. B, 30 sec after washing out the caffeine. Spikes were still evoked but were lower in amplitude. Both the amplitudes and the durations of the contractions were diminished dramatically. C, about 30 sec after applying 40 mM caffeine. Note the reduced sensitivities for recording the tension and membrane potential. The amplitude and duration of the spike were greatly increased. The peak tension was also increased, about fourfold, but the duration of the contraction was not significantly altered.
was always observed (Figs. 11 and 12). The increase in caffeine concentration may prolong the duration of the inward movement of Ca, or the repolarization, which is probably initiated by depolarizing K activation, may be delayed. The undershoot terminating the falling phase of the spike while the current was applied (Fig. 11) supports the view that depolarizing K activation underlies the repolarization. Thus, the prolongation of the spike as well as the increase in its amplitude indicates that higher concentrations of caffeine promote the entry of Ca into the fiber.

Depolarizations that are subthreshold for spike electrogenesis in the presence of 10 mM caffeine (Fig. 12 A) evoked little or no tension. When spikes were evoked, however, each in turn elicited tension which, though small, was very long lasting. The half-time for the decline from peak tension (about 8 mg) was 2–3 sec and the tension had not subsided completely after 7 sec following the first stimulus. Thus, when the fiber was stimulated at intervals of 5–10 sec there was marked summation.

However, when the caffeine was washed out the tension subsided rapidly. The records in Fig. 12 B were made less than 30 sec after removing the caffeine and the tension had returned to its original level. The fiber could still develop spikes, which had diminished from 87.5 mv in 10 mM caffeine to about 72.5 mv. Each spike now evoked a very small and very brief tension. Fig. 12 C shows the change in responsiveness about 30 sec after 40 mM caffeine was applied. The spike had now increased to 125 mv and lasted almost 0.5 sec. The tension had also increased, about fourfold, to 32 mg, but the duration of the tension was not significantly greater than in 10 mM caffeine. The interpretation of these findings will be discussed below.

DISCUSSION

The Sites of Action of Caffeine

Caffeine has been reported to modify the properties of sarcotubular preparations (Weber and Herz, 1968), surface membrane of muscle (Bianchi, 1961), mitochondria (Nayler and Hasker, 1966), and nerve terminals (Hofmann, 1969). It is believed that the common denominator for these different manifestations is an effect of caffeine that induces changes in the distribution of Ca and modifies the movement of Ca across cellular membrane components. The present experiments confirm the finding of Caldwell and Walster (1963) that caffeine produces tension when applied intracellularly (Fig. 1). Externally applied caffeine initiates tension that reaches its peak after some delay, and this is consistent with the view that caffeine acts upon sites in the depth of the fiber so that the tension appears only after a diffusional delay.

The surface membrane of muscle, which includes the membrane of the TTS, has also been implicated as a site of action of caffeine (Axelsson and
Thesleff, 1958; Lüttgau and Oetliker, 1968). Intracellular injection of caffeine does not provide a means for clearly distinguishing between intracellular and surface membrane effects, since the intracellular applications decrease the distance for diffusion to the TTS membranes as well as to those of the SR. However, the induction of Ca-dependent spike electrogenesis in the presence of caffeine (Figs. 9–12) shows clearly that caffeine affects the Ca permeability of the surface membrane. Furthermore, the resistance of the fiber is decreased somewhat during the tension evoked by caffeine (Fig. 8). A change in resistance was also observed in frog muscles (Axelsson and Thesleff, 1958) and the Ca fluxes across frog muscle membrane are increased by caffeine (Bianchi, 1961). Thus, the present data are in agreement with others in showing that the surface membrane must be one of the sites of caffeine action. The action appears to involve a change in the permeability of Ca across the cell membrane. The present data do not allow conclusions to be drawn regarding a possible involvement of this change in the process of contractile activation. However, evidence on this score will be presented in the accompanying paper.

**Time Course of the Caffeine-Induced Tension**

The tensions elicited by 2–80 mM caffeine are always transient in crayfish muscle fibers. When the TTS is not swollen they last some 30–120 sec. They are prolonged somewhat by various treatments, such as increasing pH (Fig. 3), removal of Cl (Fig. 4), depolarization with increased K (Fig. 5), elevation of Cao and, most markedly, swelling of the TTS (Fig. 6).

Caffeine tensions in frog muscles have been compared to a state of rigor (Feinstein, 1963) and irreversible morphological changes have been reported (Conway and Sakai, 1960). Other investigators have found that low concentrations of caffeine elicit transient (or “phasic”) increase in tension (Caputo, 1966; Frank, 1962; Liittgau and Oetliker, 1968). However, Liittgau and Oetliker (1968) stress that the responses are variable and that lengthy exposures to even relatively low concentrations of caffeine cause irreversible effects, which they are inclined to ascribe to the morphological changes described by Conway and Sakai (1960). Electron micrographs of caffeine-treated muscle fibers of *Orconectes* (Brandt, unpublished data) confirm data on *Astacus* (Zacharova et al., 1968) that there are no obvious morphological alterations other than a swelling around and within the SR. Furthermore, the physiological evidence from *Orconectes* muscle fibers is not consistent with the view that caffeine induces irreversible changes in these preparations; an unlimited number of reproducible caffeine responses can be elicited in these muscles (see also the accompanying paper).

The fact that caffeine-evoked tensions are always transient in crayfish muscle, independent of the concentration of the drug, carries some implica-
tions as to the mechanisms of the action of caffeine within the muscle fiber. The major effect of caffeine is believed to be the reduction or block of sequestration of Ca by the sarcotubular membranes with the consequent increase of Ca in the myoplasm (Weber, 1968; Weber and Herz, 1968). A maintained elevation of the cytoplasmic Ca should be reflected in a maintained tension. The transience of the tension therefore indicates that if caffeine continues to block the sequestration of Ca by the SR, another mechanism must come into play. It is unlikely that relaxation results from the decrease of responsiveness of the contractile machinery to the maintained Ca level by some process that is analogous to desensitization of postsynaptic membrane by an activator drug. The fibers can still develop tension on depolarization with K or during spike electrogenesis (Figs. 9 and 12). It is more probable rather that the relaxation is caused by the uptake of Ca in a second system capable of sequestering Ca. With the uptake by the SR blocked by the caffeine, the Ca would eventually (and rather slowly) come to be sequestered by the second system. Since the decay rate of caffeine-induced tension is little affected by increasing the caffeine concentration from 2 to 40 mM (Fig. 2), the second sequestering system which would be responsible for the relaxation must be relatively insensitive to this range of concentrations. In sarcotubular preparations of frog muscle the rate of sequestration is markedly reduced by increasing the caffeine concentration in the range of 1 to 10 mM (Weber and Herz, 1968). Since this is also the range in which caffeine may induce sustained contractions in frog muscle the findings may indicate that the second sequestering system, if it is present in frog muscle, differs chemically from that of crayfish.

Some of the Ca that is released from the SR in the presence of caffeine may be lost by extrusion across the surface membrane. Under normal conditions the gradient for Ca is inward, but in the experiment of Fig. 7 it was diminished. Under this condition only a few challenges with caffeine were sufficient to render the fiber unresponsive to subsequent challenges. This finding cannot be ascribed to the direct effect on cell components of the absence of Ca. When fibers were equilibrated for many hours in the Ca-free medium the first challenge with caffeine always evoked a large response. Presumably, therefore, it is the high intracellular concentration of Ca, induced by caffeine, that leads to the rapid extrusion of this cation, perhaps by an “active” (or “pump”) mechanism.

It is not unlikely that the cell membrane plays a role in regulating Ca distribution. Many cells, including frog muscle (Bianchi and Shanes, 1959; Bianchi, 1961, 1968), are permeable to Ca to some degree, yet they usually maintain an inward gradient for Ca of some four to five orders of magnitude. Furthermore, various experimental treatments that modify contraction apparently change the membrane permeability toward Ca and/or the
Ca extrusion mechanism. In crayfish muscle, for example, lowering \((K)_o\) and raising \((Ca)_o\) induce a large maintained influx of Ca which is accompanied by tension (unpublished data). The accumulation of Ca by the SR under such conditions is readily seen in electron micrographs (cf. Grundfest, 1966, Fig. 25).

Factors Modifying Caffeine-Induced Tension

Removal of Cl (Fig. 4) or elevation of \(K_o\) (Fig. 5), variation of pH (Fig. 3) and Ca, and the swelling of the TTS (Fig. 6) all modified the caffeine-induced tensions.

Removal of Cl, with substitution of an impermeant anion, causes a transient depolarization of the muscle fiber and the reintroduction of Cl induces a transient hyperpolarization (Hodgkin and Horowicz, 1959; Reuben et al., 1964). Both these transient changes modify the caffeine tension in crayfish muscle fibers. It is not clear from the present data whether the enhancement of the caffeine tension brought about by Cl withdrawal is related to the transient depolarization or to the ionic redistribution initiated by this treatment.

Elevation of \(K_o\) transiently enhances the caffeine tensions. This enhancement can be observed a number of times if the fiber is alternately exposed to the high K saline and the control medium. The lack of a maintained enhancement in fibers left in the high K rules out a correlation between the effect of resting potential and caffeine-induced tension that has been reported in frog muscle fibers (Lüttgau and Oetliker, 1968).

The change of external pH is not likely to produce variations in the pH of the muscle interior (Caldwell, 1958). Nevertheless, the change of pH by steps as small as 0.4 unit within less than 10 sec affected the contractions induced by injection of Ca or caffeine (Brandt and Grundfest, 1968; Reuben et al., 1969; and unpublished data). This finding makes unlikely a possible effect of pH on the rate of penetration of caffeine across the cell membrane in experiments such as that of Fig. 3. Rather, it suggests that the surface membrane itself plays a modulating role in contractile activation by caffeine.

The surface membranes are evidently involved in regulating contractile activation other than that initiated by caffeine since Ca injections were also modified by pH changes (Brandt and Grundfest, 1968, and unpublished data). The enhancement of caffeine-induced tension by increasing pH has been observed also in rat muscles (Frank and Buss, 1967), but in frog muscle this effect is absent (Lorkovic, 1967). The rapid effect of an increase in \(Ca_o\) on caffeine-induced tensions must also be related to the surface membrane's contribution towards contractile activation. The effects of low Ca suggest that the membrane contribution is not a major one, however. Fibers soaked for long periods of time in Ca-free salines still responded with near maximal
tensions to the first challenge with caffeine, but the tension was reduced by repetitive applications of the drug (Fig. 7). This observation favors the concept that the large fraction of Ca that becomes available for contractile activation by caffeine is from intracellular sites that do not rapidly equilibrate with changes in Ca. A similar conclusion was reached by Frank (1962) based on data obtained from frog muscles.

In fibers in which the TTS has been swollen, the surface area of the membrane has been greatly increased and the properties of the bounding membrane must now make a large contribution to the caffeine-induced contractile responses (Fig. 6). Tension induced by elevating K+ or by intracellular injections of Ca in fibers with swollen TTS is not prolonged or enhanced (Reuben et al., 1967 a, and unpublished results). How the swelling of the TTS leads to the profound modification of the caffeine tension is unknown.

Substitution of the permeant anions of the lyotropic series for Cl does not seem to alter the caffeine tensions substantially. Enhancement of the tensions was not observed, but depression was seen in some cases when I or SCN was completely substituted for Cl. This observation in crayfish muscle fibers agrees with the results obtained by Frank (1961) in frog muscle, but disagrees with the findings of Matsushima et al. (1962) in the same preparation. In contrast to the present findings with caffeine is the marked enhancement of responses to Ca injections by the lyotropic anions (Reuben et al., 1969). Procaine blocks both K- and caffeine-induced tensions (Reuben et al., 1967 a). However, procaine has limited analytical usefulness in this context since it has several sites of action. For example, it modifies the Ca permeability of the surface membrane of crayfish muscle fibers (Takeda, 1967). It also interferes with the release of Ca from the sarcotubular fraction of muscle (Weber and Herz, 1968). Only one component of the tension evoked by injection of Ca is blocked by procaine, the other remaining essentially unaffected (Reuben et al., 1969). Mn, on the other hand, specifically blocks tension induced by current (Orkand, 1962) and K (Zachar and Zacharova, 1968; see accompanying paper), without modifying caffeine-induced tensions. The specificity of Mn as an investigative tool will be shown in the accompanying paper.

In summary, it seems necessary to assume that some of the factors which modify the responses to caffeine do so by changing the properties of the surface of the cell and thereby modulate the Ca concentration within the cytoplasm. The effect of pH would seem to be of this type, since it is unlikely that there is a substantial change in the intracellular pH when that of the bathing medium is altered. Other modifying factors appear to act by affecting the action of caffeine itself. The change in the form of the caffeine-induced tension effected by swelling of the TTS would seem to be an example of the latter type.
The Effects of Caffeine on the Surface Membrane

In agreement with the data on frog muscle (Axelsson and Thesleff, 1958; Lüttgau and Oetliker, 1968) it has been found that caffeine does not significantly alter the resting potential of crayfish fibers. It does increase the membrane conductance somewhat during a contraction (Fig. 8). Presumably, this is in part a reflection of an increased Ca flux (Bianchi, 1961), but in that case other ionic conductances must also have altered, since depolarization did not occur.

The presence of caffeine certainly augments the availability of cation-permselective sites which can be activated by depolarization so as to cause spike electrogenesis (Figs. 9–12). This electrogenesis is apparently due to influx of Ca, since the overshoot of the spike is Ca-dependent and the spike is not blocked by tetrodotoxin but is blocked by Mn. The further dependence of the overshoot on the caffeine concentration indicates that the number of Ca-permselective sites that can be activated by depolarization is increased with increasing caffeine concentration. Whether or not the depolarizing Ca activation is related to the increased resting permeability to Ca that is observed in vertebrate muscle (Bianchi, 1961) is not clear. The spike electrogenesis disappears soon after the caffeine is removed. Thus, the enhanced permeability to Ca evoked as a response to depolarization is only temporary.

SPIKE-INDUCED TENSION IN THE PRESENCE OF CAFFEINE

The caffeine spikes are associated with tension, which, though small, is long-lasting. Spikes induced by procaine also evoke tension in crayfish muscle fibers, but these responses are brief as well as small (Reuben et al., 1967a, Figs. 16 and 24). The difference is of considerable interest since it may be related to the different effects of the two drugs on the sarcotubular preparations (Weber and Herz, 1968). Under the conditions of Figs. 9 and 12 the Ca released from the SR by caffeine had been taken up by the second, caffeine-insensitive, sequestering system. The spike-induced tension signals the entry of Ca into the fiber during the electrogenesis. Since the caffeine is still present the uptake of this Ca by the SR is inhibited and the long-maintained tension suggests that the second sequestering system operates much more slowly than does that of the SR. This is also in agreement with the finding (e.g., Figs. 2 and 7) that a caffeine-evoked tension subsides “spontaneously” with a slow time course. The sustained tensions elicited by even low frequency stimulation of the fiber (Fig. 12) are in accord with this conclusion. However, when the caffeine was washed out the accumulated tension subsided rapidly, indicating that the activity of the primary Ca uptake system of the SR had been restored. The spikes which could still be elicited now generated very small and brief tensions as would be expected if the Ca uptake system of the SR were now operative.
It seems likely that procaine slows or blocks the efflux of Ca from the SR but not the uptake mechanism that is sensitive to caffeine (Weber and Herz, 1968). In that case the tension evoked by procaine spikes should be shortened by the active Ca uptake system.

The data of Fig. 12 also provide grounds for some other conclusions as well, on the basis of the working hypothesis that caffeine blocks the primary Ca-sequestering system and that this effect is reflected in the time relations of the spike-evoked tension. In the presence of caffeine only the slower Ca uptake by the second sequestering mechanism is available. It has been suggested (Weber and Herz, 1968) that some of the SR is insensitive to caffeine. If that is the case also in crayfish muscle fibers, then this second component has very different Ca uptake kinetics than does the primary system which is believed to control contractile activation. In fact, the second component of the SR must be so insensitive that the kinetics of its Ca uptake are not affected by increasing caffeine from 10–40 mM (Fig. 12 C).

Although the decay of the tension was no slower in 40 mM caffeine than in 10 mM the amplitude of the tension was four times larger. This increase probably resulted not from a direct effect of caffeine upon the contractile system itself. Rather, the increase in amplitude and duration of the spike probably made for a greater influx of Ca during the depolarizing electrogensis. There is a similar increase in tension when procaine-induced spikes are prolonged (Reuben et al., 1967 a).

Noteworthy is the fact that there can be a dissociation between the amplitude of the spike electrogensis and the contractile response. Soon after the caffeine was washed out the tension became very small and disproportionately brief relative to the spike electrogensis (Fig. 12 B). The dissociation reflects the different systems that control the two responses of the fiber. Spike electrogensis is determined by the properties of the cell membrane. The tension is affected very strongly by the kinetics of the intracellular Ca-regulating systems. However, when the major Ca-sequestering mechanism is eliminated by caffeine the relation between spikes and tension (Fig. 12 A and C) comes into clearer focus.

**The Spike Electrogensis** The 29 mv/decade Ca slope shown in the upper graph of Fig. 10 indicates that the cell membrane should behave as a nearly ideal Ca electrode during spike electrogensis in the presence of 80 mM caffeine. However, the cytoplasmic ionized Ca is probably of the order of $10^{-7}$ M (cf. Weber, 1966). The external Ca is about $10^{-2}$ M and the overshoot, calculated from the Nernst relation, should be of the order of +150 mv. The observed value, when Ca is 13.5 mM, is about one-tenth of this (Figs. 9–12). Adequate voltage clamp data on crayfish fibers are still unavailable, but Hagiwara et al. (1964) and Hagiwara (1966) have reported such studies on barnacle muscle fibers, in which the intracellular ionized Ca is also of the order of $10^{-7}$ M. The overshoot of the spikes, although Ca-dependent
with a slope of nearly 29 mv/decade, was small, as in crayfish. They suggested that the overshoot is small because the peak of the spike is set by contributions of both the Ca and K batteries of the fiber. Helfferich and Ocker (1957) have given a theoretical treatment for a homogeneous fixed charge system in which a change of the divalent cation in a bionic system can yield a slope of 29 mv/decade, but the absolute potential is not related to the emf of the Ca battery alone (cf. also Tasaki, 1968, p. 56). It is doubtful, however, whether such a model, derived from a homogeneous system, can be applied to the case of the muscle fibers. It appears quite likely that the excitable membranes of living cells are heterogeneous electrochemical systems (Grundfest, 1966, 1969). In particular, the crayfish muscle fiber membrane has anion-as well as cation-permselective components, not only in the synaptic regions, but also in the nonsynaptic (Girardier et al., 1963). Thus, the classical models of the Nernst-Planck homogeneous regime, or its elaborations still as a homogeneous system, as for example by Teorell (1953), do not apply rigorously (Finkelstein and Mauro, 1963), although under certain limiting conditions these models provide good approximations to many varieties of electrophysiological data (Grundfest, 1969).

Induction of spike electrogenesis by caffeine has not been reported for other preparations. In crayfish fibers the observed effect is presumably related to the nature of the depolarizing electrogenesis which predominantly involves Ca influx (Takeda, 1967; Ozeki et al., 1966). Caffeine might also cause an enhancement of Ca influx in other membrane systems including those in which spikes result from Na influx exclusively or predominantly. However, if the Na current is much the larger the contribution of the Ca influx would be missed. A number of reports are now coming to light which indicate that spikes may be generated by a combination of Na and Ca currents (Geduldig and Junge, 1968; Hagiwara and Nakajima, 1966; Rougier et al., 1969). Even in squid axons there is an influx of Ca during the spike (Hodgkin and Keynes, 1957) and under a variety of special treatments squid axons may generate spikes in the absence of Na by an influx of Ca or some other cations (Tasaki, 1968; Tasaki et al., 1969; Yamagishi, 1970). The conductance increase and the inward currents may be very small, but the spikes have large amplitudes. Thus, the possibility that caffeine promotes spike electrogenesis due to Ca entry in other cells is testable, but such studies have not yet been carried out.

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