The Relationship between Caffeine Contracture of Intact Muscle and the Effect of Caffeine on Reticulum

A. WEBER and R. HERZ

From the Institute for Muscle Disease, Inc., New York 10021, and the Department of Biochemistry, St. Louis University, School of Medicine, St. Louis, Missouri 63104

ABSTRACT At concentrations between 1 to 10 mM, caffeine reduced the Ca-accumulating capacity of fragmented reticulum obtained from frog and rabbit muscle. With 8 mM caffeine enough Ca was released from frog reticulum to account for the force of the contracture. Caffeine did not affect all reticulum membranes equally. The fraction which was spun down at 2000 *g* was more sensitive than the lighter fractions. The percentage of the total accumulated Ca released by caffeine decreased with decreasing Ca content of the reticulum. In parallel with their known effects on the caffeine contracture, a drop in temperature increased the caffeine-induced Ca release while procaine inhibited it. Caffeine also inhibited the rate of Ca uptake, which may in part account for the prolongation of the active state caused by caffeine.

INTRODUCTION

It is generally accepted that the contractile activity of muscle is regulated by the level of ionized calcium in the sarcoplasm surrounding the myofibrils (Hasselbach, 1964; Weber, 1966; Ebashi, 1968). We have therefore tested whether caffeine, which is an agent that produces contracture, exerts its effect by releasing calcium from the sarcoplasmic reticulum. Relatively large amounts of Ca might be involved, because the tension developed during caffeine contracture approximates that of a tetanus (Lüttgau and Oetliker, 1968), indicating maximal activation of the actomyosin system (Weber et al., 1964).

It is already believed that the site of action of caffeine, which readily penetrates into the cytoplasm (Bianchi, 1962), is probably intracellular because caffeine contracture can be elicited by intracellular injection (Caldwell, 1964; Podolsky, personal communication). This belief is supported by the observation that the caffeine contracture is independent of depolarization (Axelsson and Thesleff, 1958), and of the integrity of the transverse tubular system (Gage

and Eisenberg, 1967), and, furthermore, by the observation that it does not depend on the inflow of extracellular Ca because it occurs in a Ca-free medium (Frank, 1960). The present experiments were designed to investigate this intracellular mechanism in some detail.

Some of the results of this study—but without experimental details—have been published previously (Herz and Weber, 1965; Weber, 1966).

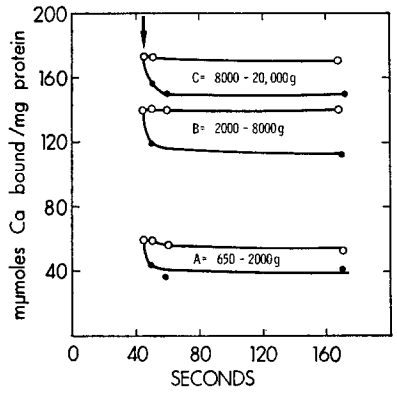

FIGURE 1. Caffeine-induced Ca release in different subcellular fractions of frog muscle. At arrow addition of 0.1 M KCl (open circles) or caffeine (final concentration 8 mM) in 0.1 M KCl (filled circles). Ca^{2+} at the time of addition, *A*, 0.065 μM, *B*, 0.075 μM, *C*, 0.098 μM.

MATERIALS AND METHODS

Preparation of Sarcoplasmic Reticulum The leg muscles of *Rana pipiens* (kept in a tank with running water in a cold room) and leg and back muscles of rabbits were used. Reticulum was isolated as previously described (Weber et al., 1966) with one modification. In addition to the fraction spun down between 8000–20,000 *g* we collected also the fractions spun down between 650–2000 *g* and 2000–8000 *g* from frog and the 2000–8000 *g* fraction from rabbit. The 650–2000 *g* fraction from rabbit had to be omitted because it was not possible to remove myofibrils at 650 *g*.

Ca Uptake or Release The assay mixtures contained reticulum in a concentration of 0.2–0.5 mg protein/ml, 10–20 mM imidazole, pH 7.0, 0.1 M ionic strength adjusted with KCl, 1–2 mM creatine phosphate, 0.25 mg/ml creatinephosphokinase, 1 mM Mg^{2+}, 0.4–2 mM MgATP, EGTA in the range 10–100 μM, and ^{45}Ca in amounts of about 100–250 mμmoles/mg reticulum protein. The reaction was started by the addition of a mixture containing MgATP and all the excess Mg. For measurements of Ca release, a 30–60 sec incubation with ATP was followed by the addition of caffeine in 0.1 M KCl. The rest of the procedures, including the determination of total Ca, has been described previously (Weber et al., 1966). Caffeine was found to be free of contaminating Ca. Protein determinations and other manipulations were previously described (Weber et al., 1966).

RESULTS

The addition of 8–10 mM caffeine to isolated frog reticulum that had accumulated Ca in the presence of ATP caused the immediate release of Ca in all but one of 12 preparations. The half-time for the release was usually about 10 sec.

The heaviest reticulum fraction was more sensitive to caffeine than the other two fractions. This can be seen from Fig. 1 in which the addition of caffeine

TABLE I

RELEASE OF CALCIUM

Fraction	Reticulum yield	Ca^{2+} before caffeine addition	Ca released, *mμmoles*	
× *g*	*mg/g wet muscle*	μM	*per mg reticulum protein*	*per g wet muscle*
650–2000	0.5*	0.19	28	14
2000–8000	0.5	0.07	30	15
8000–20,000	0.7	0.13	19	13
				—
				42
650–2000	0.4	0.13	23	9
2000–8000	0.6	0.11	45	27
8000–20,000	1.2	0.1	23	27
				—
				63
650–2000	0.6	0.14	25	15
650–8000	1.5	0.36	50	75

*Yield reduced because the muscle was homogenized for only 20 sec instead of 40 sec.

caused a release of 20% of the Ca accumulated by the heaviest fraction, while causing a release in the other two fractions of only 20% and 12%, respectively. In response to caffeine the heaviest fraction usually released about 30–50% of its accumulated Ca (most frequently about 40%), the next heaviest released from 25 to 30%, and the lightest from 0 to 12%. These values refer to reticulum that had accumulated a near maximal amount of Ca by equilibration with 0.1–0.4 μM ionized Ca in the medium. It has been shown previously—both for rabbit and for frog—that the amount of Ca accumulated by the reticulum increases as the concentration of Ca^{2+} in the medium is increased above 0.004 μM Ca^{2+} and reaches a maximum at about 0.2 μM (Weber et al., 1966).

In order to determine whether the amount of Ca released by caffeine is sufficiently large to account for the high tension of contracture we measured

the amount of Ca released by all three fractions. To make the findings applicable to living muscle undergoing a transition from rest to contracture we equilibrated the reticulum prior to the addition of caffeine with a Ca concentration in the medium that was low enough to permit myofibrils to be in the resting state (Weber and Herz, 1963; Portzehl et al., 1964). The amount of Ca released calculated per gram wet weight of muscle is given in Table I. The values are based on the yield of reticulum from muscle by our method of preparation, which for all three fractions together was about 2 mg reticulum protein per g muscle. Assuming tension to be proportional to the amount of

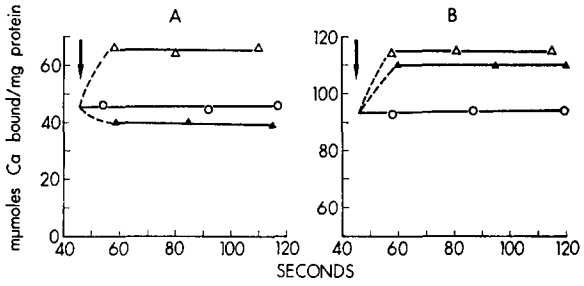

FIGURE 2. Reduction in the capacity for Ca uptake in two frog fractions. At arrow 0.1 M KCl was added without Ca (open circles) or with ^{45}Ca of the same specific activity as was present during preincubation (open and filled triangles). The specific activity during preincubation was calculated on the basis of the total Ca content of the assay, including the Ca contaminating the reticulum fraction which was measured by atomic absorption spectroscopy as previously described (Weber et al., 1966). The additional Ca was added without (open triangles) or with caffeine (8 mM) (filled triangles). A. 650–2000 *g*, Ca^{2+} of 0.05 μM before addition was raised to 2 μM. B. 2000–8000 *g*, Ca^{2+} of 0.03 μM was raised to 3 μM.

bound myofibrillar Ca (compare binding vs. pCa in Weber and Herz, 1963, with tension vs. pCa in Filo, Bohr, and Ruegg, 1965) the values for released Ca in Table I would allow for half-saturation of the myofibrils with Ca (Weber et al., 1964) and consequently account for half the maximal tension. But it is highly unlikely that our yield of reticulum was as high as 50%, so that the values in Table I should be at least doubled; it would then be possible to attribute all of the caffeine-induced tension to the caffeine-induced release of Ca from the various fractions of the frog reticulum.

Release of Ca under identical conditions was also demonstrated in reticulum obtained from rabbit muscle. In this muscle the heavier fraction was much more sensitive to caffeine than the lighter one, which frequently did not respond to caffeine at all. Furthermore, whereas in 11 out of 12 frog preparations caffeine caused Ca release from one or more reticulum fractions several of our rabbit preparations showed no response to the addition of caffeine. In

two caffeine-sensitive rabbit preparations the amount of Ca released per gram of muscle was calculated to be 35–48 mμmoles Ca per g muscle.

The release of Ca may result from a reduction of the capacity for Ca ac-

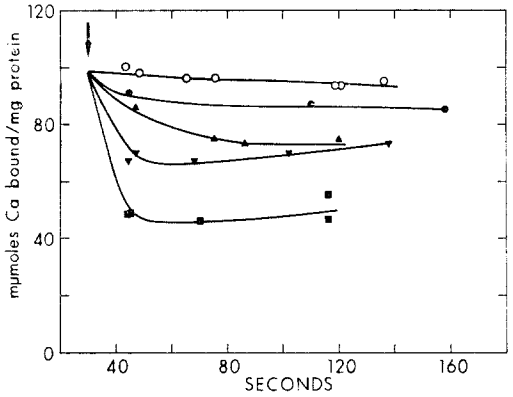

FIGURE 3. The effect of different caffeine concentrations on Ca release from the 650–8000 *g* fraction. At arrow 0.1 M KCl was added (open circles) alone, or with caffeine to give a final caffeine concentration of 0.83 mM (filled circles), 2.5 mM (filled triangles), 4.2 mM (reversed filled triangles), 8 mM (filled squares).

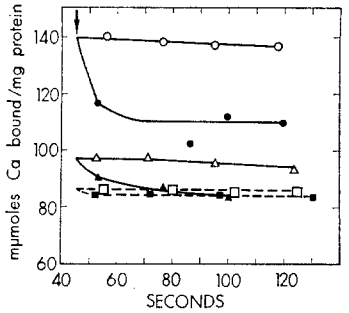

FIGURE 4. The effect of caffeine on rabbit reticulum (2000–8000 *g*) containing different levels of internal Ca. Additions at arrow as in Fig. 1. Open symbols designate controls, filled symbols indicate caffeine treatment. Ca^{2+} before addition: 0.075 μM (open and filled circles), 0.014 μM (open and filled triangles), 0.0095 μM (open and filled squares).

cumulation. Such a reduction in the capacity of the reticulum for Ca uptake is shown in Fig. 2. Caffeine and additional Ca were added to reticulum that had been equilibrated with a low Ca concentration in the medium so that the internal Ca level was less than maximal. Caffeine prevented the uptake of any of this additional Ca into the 650–2000 *g* fraction (Fig. 2 A). The lighter fraction, shown in Fig. 2 B, was, as mentioned above, less sensitive to caffeine and took up a large fraction of this Ca but less than did the control without caffeine.

The above experiments were carried out with a caffeine concentration of 8 mM. With lower caffeine concentrations less Ca was released (Fig. 3). The data in Fig. 3 were obtained from a rather sensitive frog preparation: 1 mM caffeine did not always cause a measurable release of Ca. Similar results were obtained with rabbit reticulum.

Caffeine released only a very small fraction of the total accumulated Ca when the intravesicular Ca concentration became small. Thus in the experi-

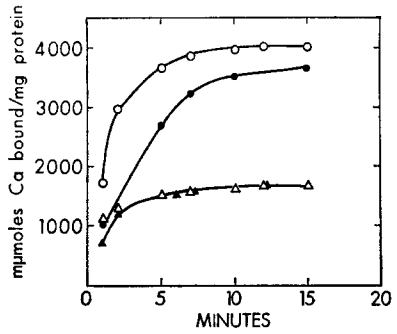

FIGURE 5. Inhibition of the rate of Ca uptake of rabbit reticulum (2000–8000 *g*) in the presence of 5 mM oxalate. The assay system differed from that used for the previous figures which was described under Materials and Methods in the concentrations of $MgCl_2$, 4 mM; ATP, 2 mM; EGTA, 0.17 mM, and protein 0.05 mg/ml. 10 mM caffeine was present from the beginning (filled circles and triangles). Initial Ca^{2+} (open and filled circles), 11 μM; (open and filled triangles), 2 μM.

ment described by Fig. 4 only about 3% of the accumulated Ca was released when the reticulum contained only a very low level of Ca corresponding to the equilibration with 0.0095 μM Ca in the medium, whereas the same vesicles released 21% of the accumulated Ca when they had been equilibrated with a considerably higher concentration of Ca in the medium (0.075 μM).

The finding that caffeine decreased the capacity for Ca does not necessarily mean that caffeine also inhibits the rate of Ca uptake. However, such an inhibition would account for the potentiation of the twitch by caffeine (Ritchie, 1954; Sandow and Brust, 1966; Sandow, 1965). With our method it is not possible to measure accurately the initial rate of Ca uptake at room temperature unless the total amount of accumulated Ca is greatly increased by the precipitation of Ca oxalate in the interior of the vesicles, a method introduced by Hasselbach and Makinose (1961). Fig. 5 shows that under these conditions the rate of Ca uptake was lowered by the presence of caffeine. However, the total amount of Ca accumulated was not reduced by caffeine. This can be seen clearly for the two lower curves and by extrapolation for the two upper curves, which probably would have met if more time had been allowed for the experiment. This latter finding seems at first to contradict our

previous finding (Fig. 1) that caffeine always reduced the capacity of frog reticulum heavily loaded with Ca. However, in spite of the large amounts of Ca accumulated this Ca does not represent a heavy load because it exists as the *inert* Ca oxalate precipitate. The level of ionized Ca in the interior is

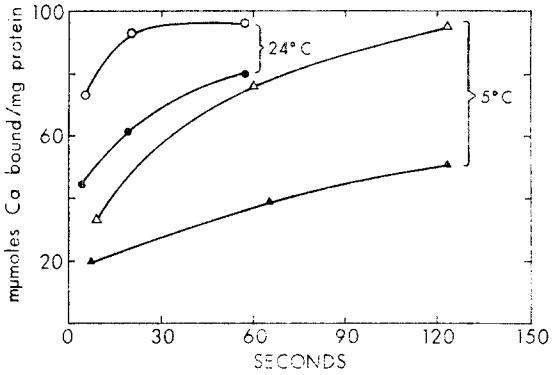

FIGURE 6. The effect of temperature on the inhibition of Ca uptake of frog reticulum (650–36,000 *g*). Open symbols controls, filled symbols 10 mM caffeine from the beginning. The pH was adjusted to 7 at the appropriate temperature. Initial Ca^{2+} 45 μM, final Ca^{2+} (open circles and triangles), 0.6 μM; (filled circles), 6 μM; (filled triangles), 20 μM.

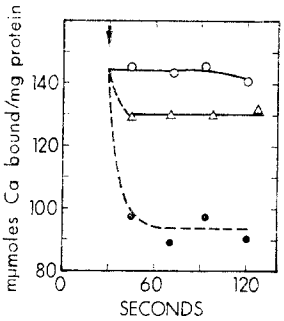

FIGURE 7. Inhibition of the caffeine effect on frog reticulum (650–8000 *g*) by procaine. (Open circles and open triangles), 3.3 mM procaine present from the beginning. At arrow addition of 0.1 M KCl (open circles); caffeine to final concentration of 8 mM (open triangles, filled circles).

actually quite low because the solubility product for Ca oxalate is so low. Therefore in the presence of 5 mM oxalate the vesicles are in equilibrium with Ca^{2+} levels in the medium of less than 0.01 μM. Under such conditions caffeine does not reduce the capacity for Ca as was illustrated by Fig. 4.

Sakai (1965) reported that cooling increased caffeine contracture, and his findings were confirmed by Lüttgau and Oetliker (1968) for single fibers. Fig. 6 shows the results of a single experiment to determine the effect of temperature on the action of caffeine on frog reticulum. Whereas in the

absence of caffeine a fall in temperature of 20°C does not alter the Ca capacity of frog reticulum (Weber et al., 1966), the data in Fig. 6 suggest that in the presence of caffeine the capacity was temperature-dependent and that caffeine caused a greater reduction of the Ca capacity at low than at high temperature.

Caffeine contracture can be prevented by local anesthetics (Schüller, 1925; Sakai, 1965; Feinstein, 1963; Lüttgau and Oetliker, 1968) which do not influence the entry of caffeine into the muscle (Bianchi, 1962). It was interesting, therefore, to find in the present experiment that procaine greatly reduced the release of Ca from the reticulum (Fig. 7). Procaine also reversed the effect of a short exposure to caffeine. In another experiment after 65 mμmoles of Ca had been released by 10 mM caffeine from 1 mg frog reticulum protein (2000–8000 *g*) 40 mμmoles were taken up again on the addition of 3.3 mM procaine. Procaine alone, at these concentrations, had no effect on Ca uptake by frog reticulum.

DISCUSSION

Our results (Table I) indicate that caffeine causes the release of Ca from frog reticulum in amounts large enough to account for the force of the contracture. Conditions that modify the caffeine-induced contracture affect the caffeine-induced Ca release correspondingly: both occur in a similar range of caffeine concentration; both are inhibited by procaine (the inhibition of Ca release by procaine in rabbit reticulum was shown indirectly by Carvalho and Leo [1967]); both seem to have a similar temperature dependence; and, finally, the speed of caffeine action on contracture and on Ca release is similar. Although the half-time for the contracture of single muscle fibers seems to be shorter than the half-time for Ca release (Lüttgau and Oetliker, 1968), it must be realized that the total amount of Ca released may be in excess of that required for maximal force. The evidence is strong, therefore, that the caffeine-induced release of Ca from the reticulum is the direct cause of the contracture, which accounts well for the finding that the caffeine contracture is absent in muscles depleted of Ca (Fujino and Fujino, 1964).

Furthermore, the inhibition of the rate of Ca uptake by caffeine explains, at least in part, why caffeine causes a prolongation of the active state (Ritchie, 1954) and also why caffeine causes an increase in twitch tension of rat muscle (Gutmann and Sandow, 1965).

However, some difficulties arise for mammalian muscle when the effect of caffeine on the Ca release from reticulum (rabbit, present experiments) is compared with the known effect of caffeine on contracture (rat muscle, Gutmann and Sandow, 1965). Although not all rabbit preparations responded to caffeine by Ca release, the amounts released from sensitive preparations were sufficiently large to be expected to cause the development of a high tension. Nevertheless, Gutmann and Sandow (1965) found that caffeine did

not cause a contracture in any of their rat muscles. It is not clear why they did not find a contracture in some of their muscles. One may speculate that the Ca content of rat reticulum in vivo is lower than that of rabbit in those of our experiments which produced a Ca release (cf. Fig. 4). It is also possible that the ionic conditions in intact mammalian muscle are such as to minimize the effect of caffeine on the reticulum (Weber, 1968).

Ca release was greater in the heavy fractions, which contain, in addition to reticulum (Weber et al., 1966), the bulk of the mitochondria. However, there is evidence that the Ca released in the presence of caffeine does not originate from the mitochondria (Weber, 1968).

The different response to caffeine demonstrated in the various fractions points to the heterogeneity of the Ca-transporting membranes. It should be checked whether the heavy fractions contain a significant number of triads, which were suggested by Lüttgau and Oetliker (1968) to be the site of caffeine action, or whether they contain a larger fraction of the terminal cisternae as compared to the longitudinal elements.

Our data also provide an explanation for why the caffeine effect on Ca uptake had been missed, or found to be very small, in some previous studies (Hasselbach and Makinose, 1964; Carsten and Mommaerts, 1964), in which the caffeine effect had been studied in the presence of oxalate to see whether the total amount of Ca uptake was diminished. As can be seen from Fig. 5 under these conditions caffeine did not affect the amount of Ca taken up but only decreased the initial rate of uptake. Furthermore, the measurements were taken with the light fraction obtained from rabbit muscle, which is not always sensitive to caffeine (Fig. 2).

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