The Mechanism of the Action of Caffeine on Sarcoplasmic Reticulum

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ABSTRACT   Evidence is presented that caffeine does not act on the mitochondrial Ca uptake system and that its effect cannot be attributed to the accumulation of adenosine 3',5'-phosphate. Two distinct caffeine effects are described. At high ATP concentrations caffeine decreases the coupling between ATP hydrolysis and Ca inflow. It either inhibits inflow without any inhibition of the rate of ATP hydrolysis, or it stimulates the ATPase activity without stimulating Ca inflow. These high ATP concentrations (much higher than needed for the saturation of the transport ATPase) greatly reduce the control of the turnover rate of the transport system by accumulated Ca. At low ATP concentrations when the transport system is under maximal control by accumulated Ca, caffeine inhibits the ATPase activity without affecting the rate of Ca inflow.

INTRODUCTION

Previous evidence indicated that caffeine-induced contracture of intact muscle results from the drug acting on the membranes of the reticulum where caffeine causes a decrease in the capacity for Ca storage and a reduction in the rate of Ca uptake (Weber and Herz, 1968).

In this study the effect of caffeine on the membranes was investigated in greater detail to gain a better understanding of its mechanism of action. The action of caffeine was studied under a variety of conditions and its effect on Ca release was compared with its effect on transport ATPase.

MATERIALS AND METHODS

The preparation of three fractions of reticulum, and the measurement of Ca uptake and release were carried out as described previously (Weber and Herz, 1968). Sr uptake was measured by the use of ^{89}Sr as described in an earlier paper (Weber et al., 1966).

ATPase Activity   The hydrolysis of ATP was measured under conditions similar to those used to measure the release or uptake of Ca: 0.4–0.6 mg reticulum protein per ml; 0.1 M ionic strength; 10–20 mM imidazole, pH 7.0; 1 µg oligomycin/mg protein; and a phosphate donor system consisting of either 2 mM phosphoenolpyruvate

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(PEP) and 75 μg phosphoenolpyruvate kinase (PEPkinase) or 1–2 mM creatine phosphate (CP) and 0.25 mg/ml creatine phosphokinase (CPK). The concentrations of MgATP and ionized Mg were varied as in the figure legends. When the concentrations of EGTA and Ca were as low as those used in the measurement of Ca release (between 10–100 μM EGTA and 100–250 mμmoles Ca/mg protein), the concentrations of ionized Ca in the medium were calculated from simultaneous Ca uptake measurements. In other assays the ionized Ca was kept at a fixed value by a high concentration of CaEGTA buffer. The experiments were started by the addition of a mixture containing the ATP and all of the Mg. After about 1 min incubation at 24°C further additions were made as indicated for each experiment. When PEP was the phosphate donor the experiments were terminated by the addition of trichloroacetic acid (TCA) to a final concentration of about 3 %. When CP was the phosphate donor, the experiments were terminated by the addition of parachloromercuribenzoate (to a final concentration of 10 mM), followed by protein precipitation according to Somogyi (1945). With PEP as the phosphate donor inorganic phosphate was determined according to the method of Taussky and Schorr (1953) after filtration of the TCA extract through charcoal in order to remove the caffeine. With CP as phosphate donor creatine was measured according to the method of Eggleton et al. (1943) without the removal of caffeine since the slightly altered color did not interfere with the absorption measurements at 520 mμ.

Use of Different Fractions   In all experiments involving the release of Ca one of the heavy reticulum fractions was used because they responded most strongly to caffeine. The light fraction (8000–20,000 g) was used for some studies of the ATPase activity. Caffeine significantly altered the ATPase activity of that fraction, which had the advantage of being available in larger amounts than the other fractions.

RESULTS

The Effect of Caffeine on Mitochondria   It has been shown previously (Weber and Herz, 1968) that subcellular fractions that are spun down at 8000 g and below release considerably more Ca on the addition of caffeine than do the lighter fractions. These heavy fractions contain, in addition to reticulum (Weber et al., 1966), the bulk of the mitochondria. Nevertheless Ca stored in the mitochondria is not the source of the Ca released by caffeine as was shown by an experiment using Dicumarol as an uncoupler of oxidative phosphorylation. Dicumarol, which inhibits mitochondrial Ca uptake (De Luca and Engstrom, 1961; Vasington and Murphy, 1962), was found (in concentrations of 50 μM) completely to prevent Ca uptake by rabbit heart and pigeon breast mitochondria when Ca uptake was supported by the hydrolysis of ATP. Dicumarol reduced Ca uptake to 20% in rabbit skeletal muscle mitochondria when uptake was supported by the oxidation of α-glycerophosphate. Even when Dicumarol had eliminated Ca storage in mitochondria, the amount of Ca released by caffeine was not diminished indicating that the released Ca did not originate from the mitochondria (Fig. 1). Even

without Dicumarol the mitochondria did not contribute significantly to the Ca uptake by the heavy fractions as indicated by the finding that Ca uptake was very little reduced in the presence of Dicumarol (Fig. 1). This inhibition of mitochondrial Ca uptake may be attributed to the method of preparation of the reticulum fractions, which tends to inactivate mitochondria, and to the low Ca concentration of the media (cf. Weber, 1966).
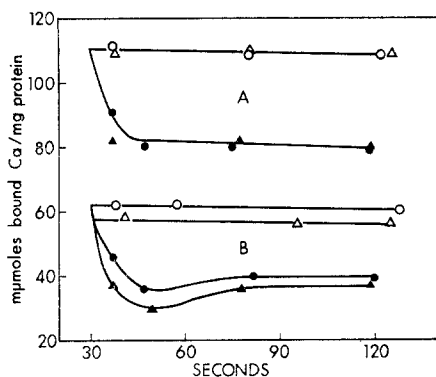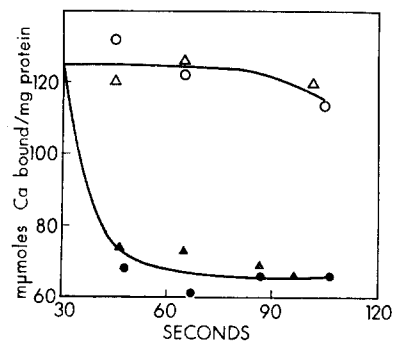

FIGURE 1   FIGURE 2

FIGURE 1.   The effect of Dicumarol on Ca uptake and on caffeine-induced Ca release of frog reticulum. A, 650–2000 g fraction; B, 2000–8000 g fraction. At 30 sec 0.1 M KCl was added without (open circles and open triangles) and with (filled circles and filled triangles) caffeine (8 mM). (Open and filled triangles) 50 μM Dicumarol present. 0.4 mM MgATP and 1.0 mM Mg^{2+}.

FIGURE 2.   The effect of adenosine 3′,5′-phosphate on Ca storage by frog reticulum (650–8000 g) in the presence and absence of caffeine. After 30 sec incubation 0.1 M KCl was added (open circles) alone, (open and filled triangles) with 0.01 mM adenosine 3′,5′-phosphate; (filled circles and filled triangles) with caffeine (8 mM). 0.4 mM MgATP; 1.0 mM Mg^{2+}.

Similar experiments showed that oligomycin, which inhibits ATP-supported Ca uptake by mitochondria (Brierley et al., 1963; Lehninger et al., 1963), did not influence the amount of Ca released in the presence of caffeine. Further, the inhibition of the rate of Ca uptake and the stimulation of the ATPase activity caused by caffeine (see below) persisted in the presence of oligomycin.

The conclusion that the observed effects of caffeine cannot be attributed to its action on the mitochondria is supported by the finding that caffeine did not inhibit the α-glycerophosphate–supported Ca uptake of rabbit skeletal mitochondria (Weber, unpublished data).

Adenosine 3′,5′-Phosphate and Caffeine Action   Sutherland and Rall (1958) showed that caffeine inhibits the hydrolysis of adenosine 3′,5′-phosphate by 3′,5′-cyclic-nucleotide diesterase. Adenosine 3′,5′-phosphate may be formed in reticulum preparations during incubation with ATP, since adenylcyclase

activity has been found in such preparations (Rabinowitz et al., 1965; Seraydarian and Mommaerts, 1965). Therefore it was possible that the Ca release was caused by adenosine $3',5'$-phosphate which accumulates to higher concentrations in the presence of caffeine than when the diesterase is not inhibited. This possibility was ruled out by the experiment illustrated in Fig. 2 which showed that adenosine $3',5'$-phosphate did not cause any release of Ca from a reticulum fraction which did respond to the addition of caffeine.

The Influence of the Concentrations of ATP and Mg on the Action of Caffeine on Ca Movements Previous studies indicate that ATP has two effects on the transport system (Weber et al., 1966). First, it serves as a substrate for the
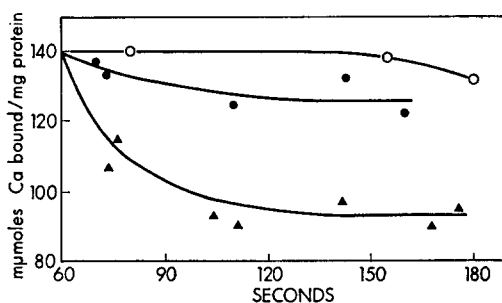

FIGURE 3. Caffeine-induced Ca release from rabbit reticulum (8000–36,000 g) in the presence of very low ATP (15 μM) and with elevated ATP (0.8 mM). Following 1 min incubation with 20 μM ATP and 1 mM Mg^{2+} 0.1 M KCl was added (open circles) alone; (filled circles and filled triangles) with caffeine (8 mM); (filled circles) without ATP; (filled triangles) with ATP to give a final concentration of 0.8 mM. After additions 0.75 mM Mg^{2+} in all assays; (open and filled circles) 15 μM MgATP.

transport enzyme that hydrolyzes ATP, thus providing energy for Ca accumulation (Hasselbach and Makinose, 1961). This enzyme has a high affinity for ATP as a substrate, since at 10 μM ATP the rate of hydrolysis and the rate of ATP-supported Ca net uptake are more than 80% of maximal (Weber et al., 1966). Second, in concentrations much higher than those required for substrate saturation, ATP plays a role as a modifier in the regulation of the turnover rate of the transport system (Weber et al., 1966). The turnover rate of the transport system is characterized by the rates of Ca inflow and ATP hydrolysis. It depends not only on the supply of Ca and ATP in the medium, but it is also regulated by the Ca accumulated in the interior of the reticulum. The accumulated Ca inhibits turnover: i.e., the transport system is subject to product inhibition. This product inhibition is alleviated by ATP in concentrations above 0.1 mM. Consequently (Weber et al., 1966) during steady-state conditions following Ca accumulation the turnover rate is very low when the ATP concentration is very low (10 μM) and increases up to 4-fold when the
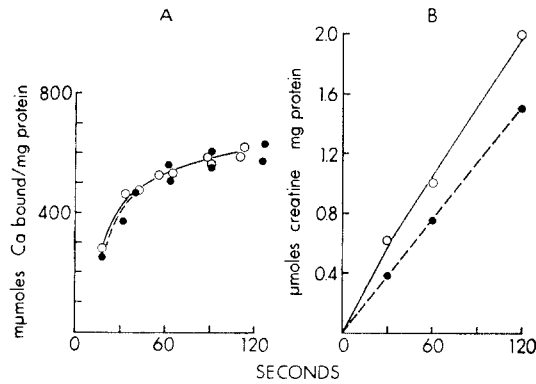
FIGURE 4.   The effect of caffeine on Ca net uptake and ATPase activity of rabbit reticulum (8000–20,000 g) in the presence of very low ATP concentrations. A, Ca uptake; B, ATPase activity measured as the release of creatine. Open circles control, filled circles 8 mM caffeine present. In addition to the usual mixture (described under Methods; protein, 0.2 mg/ml) the assays contained 6 mM oxalate, 4 mM $MgCl_2$, 10 μM MgATP and in A, 0.15 mM CaEGTA + 0.02 mM EGTA; B, 3.0 mM Ca EGTA + 0.4 mM EGTA. The initial Ca^{2+} of 1.4 μM in both A and B was immediately reduced to much lower values in A as the result of Ca uptake. Ca^{2+} remained high in B as a result of the strong buffering. Therefore the data in A and B do not correspond to the same Ca concentration and cannot be used to calculate a ratio of Ca uptake/ATP hydrolyzed.
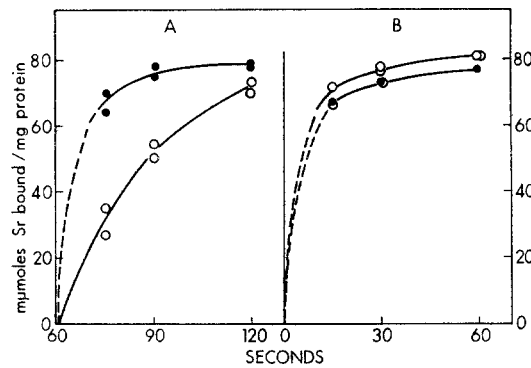

FIGURE 5 A.   The inhibitory effect of accumulated Ca on Sr uptake with high and low Mg concentrations. B, Sr uptake into reticulum not previously loaded with Ca in dependence on the Mg concentration of the medium. Open circles 4 mM Mg^{2+}, filled circles 0.1 mM Mg^{2+}. A, rabbit reticulum (8000–20,000 g) was incubated with Ca (200 mμmoles/mg protein) and 2 mM MgATP for 1 min. Then a mixture was added which contained EGTA (to remove Ca from the medium: EGTA/Ca = 3000/530 which allows a maximal Ca^{2+} of 0.02 μM), ^{89}Sr-EGTA (110 mμmoles/mg protein; Sr-EGTA/EGTA = 0.1), and either (open circles) Mg to increase the concentration of Mg^{2+} to 4 mM or (filled circles) free ATP to reduce the Mg to 0.1 mM. The ATP was diluted thereby to 1.6 mM. B, all reagents present from the beginning.

ATP concentration is raised to values above 0.1 mM (with the concentration of ionized Mg left at 1 mM).

Caffeine seemed to exert its effect preferentially on the transport system when the latter was modified by the presence of higher concentrations of ATP. With very low concentrations of ATP (15 μM) the release of Ca from the reticulum was minimal (Fig. 3) and there was no inhibition of net uptake (Fig. 4 A). Net uptake was measured in the presence of oxalate which greatly increased the Ca capacity of the reticulum thus prolonging the period of net uptake from less than 1 sec in its absence to several minutes in its presence (Hasselbach and Makinose, 1961).
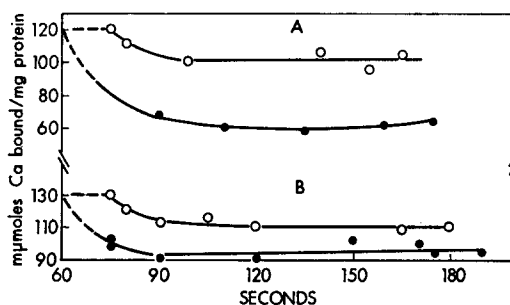

FIGURE 6. Caffeine-induced Ca release at low and at high Mg. Frog reticulum (2000–8000 g). *A*, 0.2 mM Mg^{2+} *B*, 4 mM Mg^{2+}. Open symbols control, filled symbols 9 mM caffeine added after 60 sec preincubation with 1.6 mM MgATP.

It was observed that elevated concentrations of ATP counteracted the inhibition of the transport system by accumulated Ca more effectively if the concentration of ionized Mg was low. This is shown by the experiment illustrated by Fig 5. Sr-Ca exchange was measured as an indication for the turnover rate of the transport system during steady-state conditions following Ca accumulation. It had been shown previously (Weber et al., 1966) that Ca-Ca and Sr-Ca exchange appear to be equivalent, as might be expected for ions which use the same transport system. Fig. 5 B shows the net uptake of Sr into reticulum that did not contain accumulated Ca; Sr entered rapidly independent of the Mg concentration. Fig. 5 A shows the entry of Sr in exchange for Ca previously accumulated. In spite of the elevated ATP concentration in the medium, the Sr exchange was relatively slow when the concentration of ionized Mg was high. Exchange proceeded rapidly, however, when Mg had been lowered to about 0.1 mM indicating that in the latter case the inhibition of the transport system by accumulated Ca was largely abolished. Again caffeine affected the transport system most strongly when the modification that liberated it from Ca control was most complete. As can be seen from Fig. 6, caffeine caused a greater release of Ca when the Mg con-

centration was lowest. Caffeine also inhibited considerably the rate of net uptake of Ca when the concentration of Mg was 0.2 mM (Fig. 7 A).

The Dependence of the Action of Caffeine on Ca in the Medium The amount of Ca released from the reticulum on caffeine addition became very small when
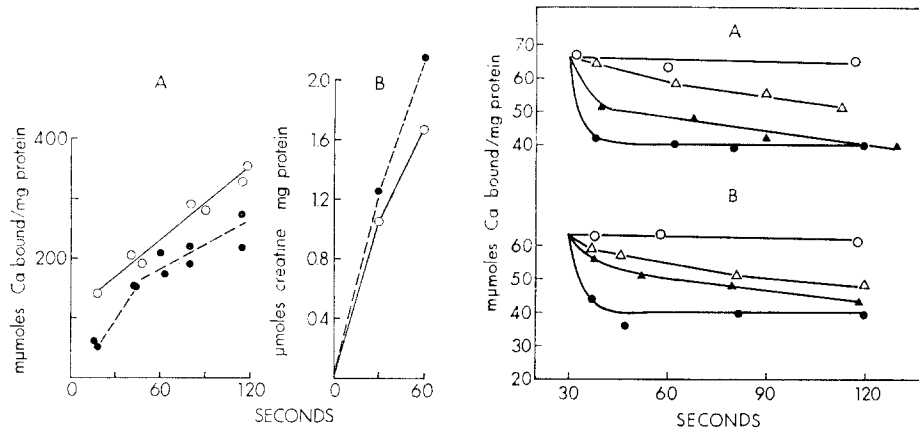

FIGURE 7   FIGURE 8

FIGURE 7. Inhibition of Ca net uptake and stimulation of ATPase activity by caffeine in the presence of low Mg. Rabbit reticulum (8000–20,000 g, 0.16 mg/ml). *A*, Ca uptake. *B*, ATPase activity measured as creatine liberation. Open circles, control; filled circles, 8 mM caffeine. 6 mM oxalate, 1 mM MgCl₂, 1.0 mM MgATP (resulting equilibrium concentrations, calculated as described previously (Weber et al., 1966): 1 mM Mg oxalate, 5 mM oxalate, 0.8 mM MgATP, 0.2 mM ATP, 0.2 mM Mg²⁺). The initial Ca²⁺ was 1.4 μM in *A* (0.15 mM CaEGTA/0.02 mM EGTA) and *B* (3.0 mM CaEGTA/0.4 mM EGTA). Ca²⁺ rapidly fell in *A* and remained high in *B* because of the difference in the CaEGTA buffer concentration. Therefore the rate of the ATP hydrolysis in *B* cannot directly be related to the rate of transport in *A*.

FIGURE 8. Inhibition of caffeine-induced Ca release by Ca deficiency of the medium. Frog reticulum (650-2000 g). After 30 sec incubation with 0.4 mM MgATP and 80 mμmoles Ca/mg the following additions were made (open circles), 0.1 M KCl; (open and filled triangles), EGTA to a final concentration of 0.2 mM (Ca²⁺ 0.0013 μM) in *A* and of 2.5 mM in *B* (Ca²⁺ 0.0005 μM); (filled circles and filled triangles), caffeine to a final concentration of 8 mM. MgATP 0.4–0.32 mM, Mg²⁺ 1.0–0.8 mM. All additions were adjusted to an ionic strength of 0.1.

the concentration of ionized Ca in the medium was very low even if the reticulum had been maximally loaded with Ca prior to the removal of Ca from the medium (Fig. 8). Although the amount of Ca released became very small the speed of caffeine action remained rapid with a Ca-deficient medium. It can be seen from Fig. 8 that the caffeine-induced Ca release was complete in about 10 sec. It was followed by a prolonged period of equilibration with the reduced Ca level of the medium, but this phase of Ca release was not accelerated by caffeine.

The Effect of Caffeine on the Transport ATPase   When the ATP concentration was above 0.1 mM, i.e. under conditions where caffeine caused the release of Ca or the inhibition of net uptake, caffeine never inhibited the ATPase activity. It either stimulated it or did not affect it at all (Tables I and II). The ATPase activity is considered to be stimulated when the rate of ATP hydrolysis exceeds that of a control of the same ionized Ca in the medium. The ATPase is not considered to be stimulated when the effect of caffeine can be accounted for by an increase in the concentration of the ionized Ca of the

TABLE I

THE INFLUENCE OF CHANGES IN THE
CONCENTRATIONS OF ATP, Mg, AND Ca ON THE EFFECT OF
CAFFEINE ON THE TRANSPORT ATPase

			ATPase activity	
Ca	MgATP	Mg^{2+}	Control	8–10 mM caffeine
μM	mM	mM	$\mu moles/min \times mg\ protein$	
		Frog, 2,000–8,000 g		
20	2	4	0.6	0.62
20	2	0.05	0.75	1.5
		Rabbit, 8,000–20,000 g		
18	0.008	0.9	0.09	0.08
18	1.3	3.3	0.25	0.29
18	1.3	0.15	0.67	1.2
0.04	1.3	0.15	0.19	0.19
0.001	1.3	3.3	0.14	0.14
		Rabbit, 8,000–20,000 g		
14	2.0	0.5	1.0	1.6

medium due to the release of Ca. The ATPase activity increases with increasing Ca concentrations until saturation has been reached at Ca concentrations above 1 μM (Weber et al., 1964, 1966). Activation of the ATPase activity—after a new steady state had been established with caffeine—can be seen in Fig. 9. Stimulation of ATPase never failed to occur when an elevated ATP concentration was combined with a low Mg; i.e., the condition where Ca release was maximal. For steady-state conditions this stimulation is demonstrated by the experiments presented in Table I, and for the period of net uptake by Fig. 7 B.

It has been reported in another study (Weber and Herz, 1968) that the effect of caffeine on the release of Ca is quite variable especially in rabbit reticulum preparations. The present experiments show that this also holds for the extent of stimulation of ATPase activity by caffeine, which varied from preparation to preparation. Furthermore, there was no correlation between

TABLE II

COMPARISON OF THE EFFECT OF CAFFEINE ON
Ca RELEASE AND ATPase ACTIVITY

	Control	Caffeine			
			Ca²⁺ increase		
Ca²⁺	ATP split	Ca release	From	To	ATP split
μM	$\mu moles/min \times mg$ protein	$m\mu moles/mg$ protein	μM	μM	$\mu moles/min \times mg$ protein
		Frog 650–2,000 g			
0.3	0.13	25	0.3	0.7	0.15
1.0	0.14				
12	0.18	14	12	14	0.26
18	0.10				
14	0.33	30	14	24	0.36
		Frog 2,000–8,000 g			
22	0.55	60	22	38	0.69
		Rabbit 2,000–8,000 g			
7	0.2	15	7	10	0.43
10	0.24				

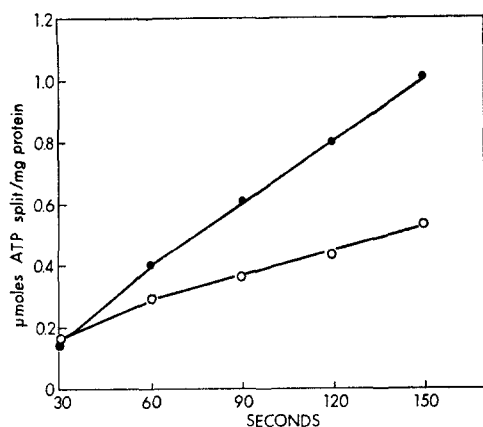The concentration of ionized Mg was 1 mM, that of MgATP varied between 0.2 and 0.4 mM.

FIGURE 9. Stimulation of the steady-state ATPase by caffeine. After 30 sec incubation with 1 mM Mg ATP and 174 mμmoles Ca/mg protein (filled circles) caffeine (8 mM) was added; and after incubation with 218 mμmoles Ca/mg (open circles) 0.1 M KCl was added. With caffeine the new steady state was established after 20 sec at a Ca²⁺ of 8 μM. The steady-state Ca²⁺ of the control was 4 μM. Both Ca²⁺ are saturating for the ATPase (Weber et al., 1964). MgATP 1.0–0.85 mM; Mg²⁺ 1.0–0.85 mM. Hydrolysis of ATP measured as the liberation of phosphate.

the extent of Ca release and the extent of the stimulation of the ATPase activity even though both responses to caffeine are enhanced by the same set of conditions. The lack of correlation is illustrated by the data in Table II and by the finding that the rabbit reticulum (Table I) which showed a considerable stimulation of the ATPase activity did not release any Ca at all on the addition of caffeine.

Stimulation of ATPase activity in this preparation was not accompanied by an increase of the Ca flux across the membrane as measured by Sr-Ca exchange; i.e., the ratio between the rate of Sr entry and the rate of ATP hydrolysis was decreased. Unexpectedly, inhibition of the rate of ATP hydrolysis was found with very low ATP concentrations, a condition in which

TABLE III

THE EFFECT OF PROCAINE ON THE STIMULATION
OF THE ATPase BY CAFFEINE

| | ATP hydrolyzed | | |
Ca^{2+}	Control	9 mm caffeine	6 mm procaine
μM		$\mu moles/min \times mg\ protein$	
20	0.75	1.5	0.76

The ATPase was determined following a preincubation with ATP and Ca for 50 sec at which time either 0.1 m KCl or caffeine in 0.1 m KCl was added. Procaine was present from the beginning. 2.0 mm MgATP, 0.15 mm Mg^{2+} frog reticulum 650–2000 g.

caffeine has a minimal effect on Ca movements. This can be seen in Fig. 4 B for the period of net uptake of Ca. During steady-state conditions in the presence of 10 μM ATP (Table I) the ATPase activity was too low to provide a significant difference in the absence and presence of caffeine.

A Ca deficiency in the medium (Table I) not only kept the release of Ca at a minimum but also prevented any effect of caffeine on the ATPase activity.

Procaine prevented the stimulation of the ATPase activity by caffeine (Table III) just as it prevented the release of Ca from reticulum (Weber and Herz, 1968).

DISCUSSION

Although caffeine does not always cause the release of Ca from reticulum (Weber and Herz, 1968), some effect of caffeine can be found when other parameters of reticulum activity, such as ATPase activity, are measured.

Under conditions where the regulation of the turnover rate of the transport system was modified by elevated ATP concentrations one may describe the caffeine effect as partial uncoupling. The inflow of Ca is coupled to the

hydrolysis of ATP; and in the absence of caffeine maximally two Ca ions are transported inwards for each molecule of ATP hydrolyzed. A ratio of Ca/ATP of 1 or 2 was found for net uptake by Hasselbach and Makinose (1961, 1963) and was found to be maintained during steady-state conditions by Weber et al. (1966). This ratio was reduced (and thus partial uncoupling had taken place), either when caffeine caused a decrease in the rate of Ca inflow without a corresponding inhibition of the rate of ATP hydrolysis (Fig. 7), or when caffeine caused a stimulation of the ATPase activity without a corresponding stimulation of Ca inflow (the rabbit experiments, Table I). If one assumes that the caffeine;induced Ca release is an indication of an inhibition of Ca inflow, uncoupling took place in all experiments with elevated ATP concentrations because in those experiments ATPase activity was never inhibited.

An inhibition only of inflow entails the release of Ca until the reduction of outflow (which is a function of the concentration of accumulated Ca) is sufficient for a new steady state to be established. There are several reasons to assume that Ca release is caused by an inhibition of Ca inflow.

First, there is evidence that caffeine is capable of inhibiting inflow as shown by the inhibition of net uptake under conditions where outflow had been prevented by the precipitation of accumulated Ca by oxalate (Fig. 7 A; Weber and Herz, 1968). Second, although Ca release could also be caused by an increase in the rate of outflow it was found that caffeine did not significantly affect the rate of outflow from reticulum into a Ca-free medium (Fig. 8). Further, it has been shown previously (Carvalho and Leo, 1967) that caffeine does not diminish the number of binding sites for Ca. Therefore, it is plausible to tentatively attribute Ca release to the inhibition of inflow and to conclude that caffeine acts on the mechanism for energy transfer by reducing the coupling.

However, it is still not clear what kind of action on energy transfer might sometimes reduce the transport rate, sometimes stimulate ATPase activity, and sometimes do both.

The finding that the uncoupling effect of caffeine was greatly reduced by the presence of high Mg may be applied to intact muscle. It would mean that caffeine may not cause a contracture if the level of ionized Mg in the sarcoplasm is very high.

Although the *uncoupling* effect of caffeine was restricted to conditions where the transport system was modified by elevated concentrations of ATP, caffeine also affected the ATPase activity of the transport system under conditions when it was not modified by ATP and the turnover rate of the transport system was maximally controlled by accumulated Ca. Since caffeine improved the coupling under conditions (10 μM ATP) where caffeine inhibited the rate of ATP hydrolysis without decreasing the rate of Ca uptake, it would be of interest to determine the ratio of Ca/ATP with and without caffeine to see

whether caffeine increases the value above 2. Such measurements were not undertaken in this study; it is therefore possible that all these preparations were somewhat uncoupled and that caffeine only restored coupling to the original value instead of increasing it above normal.

The discussion on coupling is based on the assumption that caffeine modified the transport ATPase and not some other contaminating ATPase activity. The ATPase that was modified by caffeine shares with the transport ATPase the dependence on Ca for activation; in the absence of Ca in the medium caffeine had no effect on the ATPase activity (Table I). However, there exist Ca-activated ATPases; e.g., actomyosin and enzymes in mitochondrial fragments which may contaminate reticulum preparations. Any contribution by mitochondrial enzymes to the ATPase activity of the reticulum fractions was suppressed by oligomycin which was present in all ATPase assays. Actomyosin is not influenced by caffeine (Hasselbach and Weber, 1955; Weber, unpublished results). A further indication that caffeine affected the transport ATPase is the close correlation between conditions which modify the action of caffeine on Ca inflow and on ATPase activity. Procaine inhibited the effect of caffeine on Ca release and on the stimulation of ATPase activity; a low concentration of Mg enhanced both caffeine effects. The ATPase activity that was inhibited by caffeine must also be attributed to the transport system. In the presence of very low concentrations of ATP the rate of ATP hydrolysis by the transport ATPase is high only during net uptake of Ca; when the steady state is reached it falls to very low values even if the Ca concentration in the medium remains high. (Cf. Fig. 4 with Table I, line 3, or cf. Weber et al., 1966.) Contaminating ATPase activity would by definition be independent of the state of the reticulum. Since the caffeine-induced reduction of the ratio (0.4 μmole/min·mg) was much larger than the total persisting rate (0.1 μmole/min·mg), caffeine must have inhibited the transport ATPase.

The effect of caffeine on the reticulum is reversible. There was no residual effect of caffeine on either Ca uptake or the ATPase activity of reticulum after a 5 min preincubation. Further, it has been shown (Weber and Herz, 1968) that the caffeine-induced release of Ca is reversed when procaine is later added.

Two conclusions may be drawn. First, unlike ADP—another purine which causes the release of Ca (Weber et al., 1966)—caffeine does not seem to be a general inhibitor of the transport system but seems specifically to affect the energy transfer.

The second conclusion is based on the existence of two quite different caffeine effects. Caffeine inhibited ATPase activity under conditions where the transport system was subject to maximal control by accumulated Ca. When this control had been largely abolished by high ATP, caffeine caused uncoupling of ATPase activity from Ca transport. This selectivity of caffeine

action enforces the view that the transport system exists in two different conformational states comparable to the allosteric modification of enzyme systems (Weber et al., 1966).

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