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Caffeine Inhibition of Calcium Accumulation by the Sarcoplasmic Reticulum in Mammalian Skinned Fibers

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Summary. Oxalate-supported Ca accumulation by the sarcoplasmic reticulum (SR) of chemically skinned mammalian skeletal muscle fibers is activated by MgATP and Ca^{2+} and partially inhibited by caffeine. Inhibition by caffeine is greatest when Ca^{2+} exceeds 0.3 to 0.4 μM, when free ATP exceeds 0.8 to 1 mM, and when the inhibitor is present from the beginning of the loading period rather than when it is added after Ca oxalate has already begun to precipitate within the SR. Under the most favorable combination of these conditions, this effect of caffeine is maximal at 2.5 to 5 mM and is half-maximal at approximately 0.5 mM. For a given concentration of caffeine, inhibition decreases to one-half of its maximum value when free ATP is reduced to 0.2 to 0.3 mM. Varying free Mg^{2+} (0.1 to 2 mM) or MgATP (0.03 to 10 mM) has no effect on inhibition. Average residual uptake rates in the presence of 5 mM caffeine at pCa 6.4 range from 32 to 70% of the control rates in fibers from different animals. The extent of inhibition in whole-muscle homogenates is similar to that observed in skinned fibers, but further purification of SR membranes by differential centrifugation reduces their ability to respond to caffeine. In skinned fibers, caffeine does not alter the Ca^{2+} concentration dependence of Ca uptake ($K_{0.5}$, 0.5 to 0.8 μM; Hill n, 1.5 to 2.1). Reductions in rate due to caffeine are accompanied by proportional reductions in maximum capacity of the fibers, and this configuration can be mimicked by treating fibers with the ionophore A23187. Caffeine induces a sustained release of Ca from fibers loaded with Ca oxalate. However, caffeine-induced Ca release is transient when fibers are loaded without oxalate. The effects of caffeine on rate and capacity of Ca uptake as well as the sustained and transient effects on uptake and release observed under different conditions can be accounted for by a single mode of action of caffeine: it increases Ca permeability in a limited population of SR membranes, and these membranes coexist with a population of caffeine-insensitive membranes within the same fiber.

Key Words chemically skinned fibers · caffeine · Ca uptake · sarcoplasmic reticulum · Ca^{2+} · free ATP · MgATP

Introduction

Caffeine appears to have two distinct actions on the sarcoplasmic reticulum (SR) membranes of vertebrate skeletal muscle: it evokes Ca release, and it inhibits ATP-dependent Ca accumulation. The ability of caffeine to cause release of Ca from isolated SR vesicles, skinned fibers and intact fibers has been extensively documented. In spite of the diverse conditions used by different laboratories, caffeine-induced responses in all these preparations have a number of features in common: i) Release is half-maximal with 0.2 to 3 mM caffeine and maximal with 2 to 10 mM [4, 12, 21, 22, 34, 37, 54]; ii) a substantial fraction of Ca is retained by the SR even at the peak of a maximal response [21, 37, 47, 52]; iii) release is usually transient, and the preparation may be refractory to further stimulation [4, 10, 12, 17, 21, 22, 28, 32, 37–39, 47, 54; but *see* 24, 46]; and iv) release apparently depends on Ca load and on the concentrations of Ca, Mg and ATP in the medium [10–12, 15, 21, 22, 34–37, 39, 46, 47, 50–52, 54]. There is evidence from studies on isolated SR vesicles and on intact fibers that Ca release induced by caffeine and other agents is proportionately greater for the SR terminal cisternae than for the membranes of the longitudinal cisternae [15, 18, 23, 30, 37, 41, 52, 53]. This difference in responsiveness to caffeine has been demonstrated most clearly using isolated SR vesicle preparations, where membranes from these two morphologically distinct regions of the sarcomere can be separated by differential centrifugation into "heavy" and "light" fractions, respectively [29].

Caffeine-induced responses of isolated SR vesicles differ from those of the two types of fiber preparations in being difficult to reproduce, even within the same laboratory [52]. Recently, a careful study

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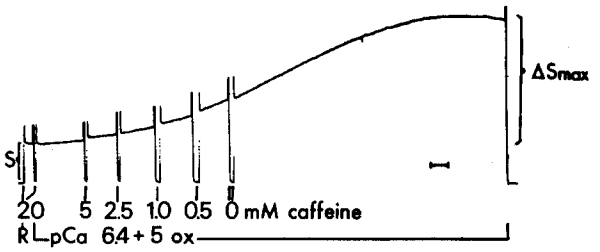

Fig. 1. Caffeine inhibition of Ca oxalate accumulation in a chemically skinned psoas fiber. Fiber was stretched to 120% of slack length in relaxing solution (R) containing 170 mM K propionate, 10 mM imidazole (pH 7.0), 2.5 mM Mg acetate, 5 mM K_2Na_2ATP and 5 mM K_2EGTA. Thirty sec after the addition of 20 mM caffeine, the solution was replaced with the standard pCa 6.4 loading solution, identical to R except for the substitution of 2.15 mM CaEGTA for part of the K_2EGTA, and with 5 mM K oxalate and 20 mM caffeine added. The rates of increase in light scattering ($\Delta S/S \cdot$ min) were recorded at each concentration as caffeine was reduced to 0.5 mM. Two washes with a 0-caffeine medium preceded the completion of loading under control conditions, in which light scattering increased linearly for several minutes before levelling off at a plateau of $\Delta S_{max}/S = 3.3$. Calibration, 1 min

of several of the variables involved has established that optimal conditions for obtaining Ca release from heavy SR vesicles include maximal loading with Ca and the presence of a limited range of external Ca^{2+} concentrations (0.1 to 3 μM) as well as a minimum of 0.1 mM free ATP in the release medium [47]. Caffeine-induced Ca release was inhibited by higher or lower Ca^{2+} concentrations, whereas Mg^{2+} and MgATP did not affect the response. Others have reported that Mg^{2+} attenuates the action of caffeine, not only in isolated vesicles [15, 22, 36, 51] but also in skinned fibers [10, 46].

In spite of the large number of studies concerning the action of caffeine, it is not clear whether caffeine's inhibition of Ca uptake by the SR is related to its action in causing Ca release. The inhibition of Ca uptake differs by being sustained rather than transient [14, 18, 20, 24, 45, 52], but it appears to resemble release in being enhanced by free ATP [51], as well as in being greater for heavy SR than for light SR vesicles [14, 18, 24]. Detailed studies of caffeine concentration dependence and of the effect of caffeine at different Mg, ATP and Ca concentrations have not been done. The data presented here support the view that a single action of caffeine can account for both release of Ca and the effects on Ca uptake.

A major objective of this study was to examine more closely the parameters that govern inhibition of Ca uptake by caffeine. For this purpose we have used chemically skinned fibers isolated from fast-twitch muscles of the rabbit. Characteristics of oxalate-supported Ca uptake by the SR in these fibers in the absence of drugs have been described elsewhere, and it has been shown that under control conditions they resemble isolated SR vesicles with respect to Ca, ATP and oxalate requirements [45]. However, the skinned-fiber preparation has the important advantage of providing SR membranes *in situ* that are structurally and functionally intact and are strongly inhibited by caffeine. Furthermore, use of the skinned fiber allows us to test easily for reversibility of the drug effects. Finally, since the volume of a skinned fiber is small compared with the volume of the bath, Ca taken up or released by the SR has a negligible effect on bath Ca concentrations in the steady state. Thus Ca accumulation can be measured at constant external Ca^{2+} concentrations. Preliminary reports of some of the data have appeared [42–44].

Materials and Methods

Fiber segments were dissected from psoas and extensor digitorum longus (EDL) muscles of the rabbit, chemically skinned and stored at −15°C. Rabbits were either New Zealand white (from a New York supplier) or of mixed origin (from a Brazilian supplier), and weighed at least 2 kg. As previously described [9, 56], overnight exposure at 0°C to a "skinning" solution containing (in mM): 5 K_2EGTA, 2.5 K_2Na_2ATP, 2.5 Mg acetate, 172 K propionate and 10 imidazole propionate (pH 7.0) effectively eliminates the sarcolemma as a diffusion barrier to large solutes and makes it possible to vary the composition of the solution bathing the myofibrils and SR membranes. Within each sarcomere, the SR retains its characteristic morphological heterogeneity as well as its ability to accumulate and release Ca. Caffeine-induced Ca release was monitored as described by Wood [54], using a force transducer designed and built by P.W. Brandt [1].

Rate and extent of Ca accumulation were monitored by the increase in light scattering that accompanies formation of Ca oxalate crystals within the SR. By the use of ^{45}Ca, this increase (ΔS; *see* Fig. 1) has been shown to be proportional to the increase in Ca content [45], and in electron micrographs it can be seen that virtually every SR membrane profile is filled with Ca oxalate crystals once the level of light scattering reaches its maximum value (ΔS_{max}, Fig. 1). Light scattered by the fiber in the absence of Ca and oxalate (S, Fig. 1) is proportional to the volume of the fiber, and since ΔS and ΔS_{max} are also larger for larger fibers, they are routinely normalized by dividing by S [45].

Fibers equilibrated in relaxing solution (skinning solution containing 5 mM K_2Na_2ATP) were stretched to 120 or 180% of slack length in a one-ml chamber provided with stirring and temperature control (25°C). In the standard Ca loading solution, a mixture of 2.85 mM K_2EGTA and 2.15 mM CaK_2EGTA replaced the K_2EGTA of the relaxing solution to provide an ionized Ca^{2+} concentration of 0.4 μM (pCa 6.4). Other experiments were done with 1.73 mM K_2EGTA and 3.26 mM CaK_2EGTA (pCa 6.0). Higher and lower Ca^{2+} concentrations were obtained by varying the ratio of K_2EGTA to CaK_2EGTA, keeping the total EGTA constant at 5 mM. Association constants for CaEGTA and MgATP were those used earlier [45]; for all other ligands, the constants given by Fabiato and Fabiato [13] were used. The

Table 1. Caffeine inhibition of oxalate-supported Ca accumulation in muscle homogenates and isolated SR vesicles[a]

Membrane fraction	Ca accumulation capacity		Inhibition
	Control	+10 mM caffeine	
Homogenate (4)[b]	80.3 ± 6.4	28.8 ± 2.7	64% (57–74%)
1500 × *g* supernatant (3)[b]	37.6 ± 7.0	19.0 ± 2.1	47% (35–60%)
Washed SR vesicles (4)[c]	3.05 ± 0.31	2.06 ± 0.22	36% (29–41%)

[a] Capacities are given as $\bar{x}$ ± SE of number of preparations indicated in parentheses in the first column; inhibition given as $\bar{x}$ and range. For preparations and assay conditions, *see* Materials and Methods.
[b] μmol/g wet weight of muscle.
[c] μmol/mg SR vesicle protein.

sodium added with ATP was treated like potassium for the purpose of these calculations. The equilibria involving potassium and oxalate, which were not included in our earlier publications, had negligible effects on free Ca^{2+}, but significantly affected the calculations for ATP and Mg species. The symbols ATP, Mg and Ca (without superscripts) are used to refer to the total amounts of these salts added to the solutions, whereas Ca^{2+}, Mg^{2+}, MgATP, CaATP and free ATP are used to refer to ionized Ca^{2+}, ionized Mg^{2+}, the sum of $MgATP^{2-}$ + $MgHATP^{-}$, the sum of $CaATP^{2-}$ + $CaHATP^{-}$, and the sum of ATP^{4-} + $HATP^{3-}$ + H_2ATP^{2-}, respectively. The standard *p*Ca 6.0 loading medium contained 138 μM Mg^{2+}, 2.12 mM MgATP, 7.3 μM CaATP, 1.52 mM free ATP, 1.35 mM $KATP^{3-}$, 210.6 mM K^+, 233 μM Mg oxalate, 4.6 μM Ca oxalate, and 4.76 mM free oxalate.

The accumulation of Ca oxalate was initiated by the addition of 5 mM K oxalate from a 1.0 M stock solution (pH 7.0), which was stored frozen until one or two days before use. Caffeine (Sigma Chemical Company, St. Louis, Mo.) was added from a 0.1-M stock solution, prepared fresh daily. EGTA, CaEGTA and propionate solutions were prepared as previously described [45]. The ionophore A23187 was the kind gift of Dr. R.L. Hamill of Ely Lilly Company (Indianapolis, Indiana) and was diluted to a working concentration of 0.01 mM in water just before being added to the bath. Control experiments established that the amount of ethanol added with the ionophore was without effect. A stock solution (5 mM in ethanol) was stored at −15°C.

Sarcoplasmic reticulum vesicles were prepared in the cold from 150 g rabbit dorsal or white hind leg muscles that were passed through a meat grinder and then blended at maximum speed for 75 sec in 3 volumes of a solution containing 100 mM KCl, 2.5 mM phosphate buffer, pH 6.8, and 2 mM EDTA [7]. Part of this solution was added in the form of ice cubes during the blending. The first supernatant resulting from sedimentation of this crude homogenate at 1500 × g_{max} for 15 min was subsequently centrifuged at 10,500 × g_{max} for 15 min. After filtration through two layers of gauze, this second supernatant was centrifuged at 70,000 × g_{av} for 45 min. Resuspended in 11.5 ml of a solution containing sucrose (1.3 M) and KCl (30 mM), the pellet was combined with 5 ml KCl (0.1 M) and centrifuged at 3650 × g_{max} for 10 min. The suspension of membranes in the upper part of the tube was collected, mixed with ATP, Mg and KCl (final concentrations 2 mM, 2 mM and 0.6 M after dilution to 80 ml), and centrifuged at 70,000 × g_{av} for 90 min. The pellet was resuspended in 40 ml 0.1 M KCl, centrifuged again for 40 min, resuspended in KCl and stored at 0°C at a concentration of 25 to 50 mg protein per ml [27].

On the day of preparation, or one day later, aliquots of the crude homogenate, the first supernatant and the final suspension of SR vesicles were tested for ability to accumulate Ca in a medium containing 10 mM $MgCl_2$, 50 mM Tris maleate, pH 7.0, 100 mM KCl, 4 mM K_2Na_2ATP, 0.05 mM $^{45}CaCl_2$, 10 mM Na azide and 5 mM K oxalate and 0 or 10 mM caffeine, at 30°C. In preliminary experiments, it was established that the effect of 5 to 10 mM caffeine was maximal. The crude homogenate was triturated using a Polytron PT 10 probe at half speed for 45 sec before use. Reactions were started by the addition of vesicles (0.006 to 0.01 mg/ml) or by the addition of an amount of crude homogenate or of the first supernatant that corresponded to 0.5 mg wet weight of muscle per ml. Ca accumulation was terminated by Millipore filtration after intervals of 50 to 450 sec, and the filtrates were counted in a liquid scintillation counter.

Results

Caffeine Inhibition of Ca Accumulation by Muscle Homogenates and Isolated SR Vesicles

Briggs et al. [2, 40; *see also* 19] have described optimal conditions for the study of oxalate-supported Ca uptake by the SR in unfractionated, whole-muscle homogenates. Under these conditions, which exclude the participation of mitochondria, the maximal steady-state capacity for Ca oxalate accumulation provides an index of functional SR volume.

In the presence of caffeine, both rate and capacity for Ca accumulation were attenuated. Table 1 shows the effect of 10 mM caffeine on capacity of the crude homogenate and of membranes obtained in intermediate and final steps of preparing SR vesicles. It can be seen that much of the original response to caffeine was lost. Inhibition of rate of uptake (*not shown*) was similar to inhibition of capacity, diminishing with each succeeding stage of purification.

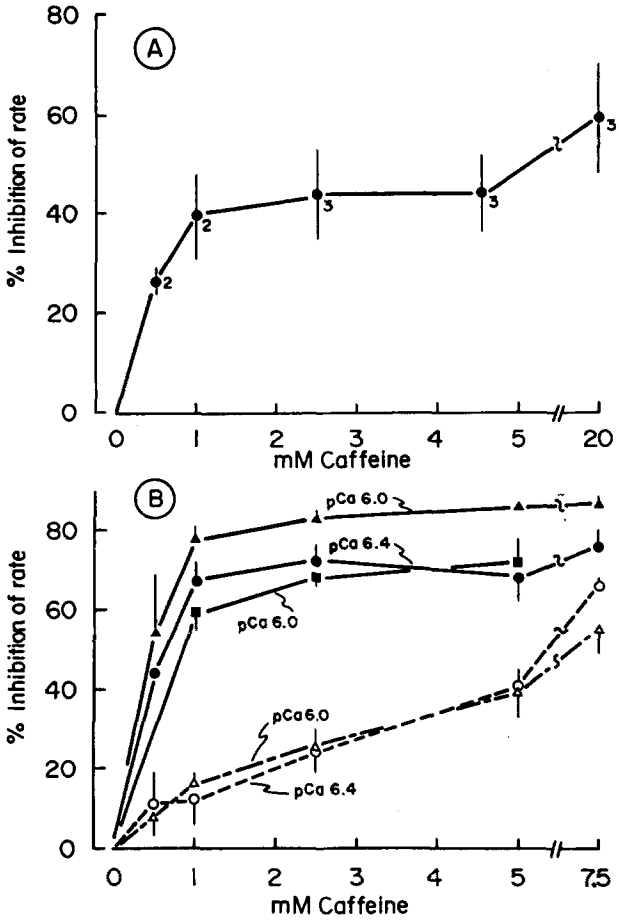

Fig. 2. Caffeine-dose-response curves. (*A*) Psoas fibers from New Zealand white (US) rabbits (4 to 13 fibers from each rabbit, measured at *p*Ca 6.4 as in Fig. 1). Values show $\bar{x}$ ± range or $\bar{x}$ ± SE of average values from two or three different rabbits at each point. (*B*) EDL fibers from Brazilian rabbits of mixed origin (circles, triangles) and from a guinea pig (squares). *Solid symbols*, Caffeine added together with oxalate to standard loading medium at *p*Ca 6.0 (▲, ■) or *p*Ca 6.4 (●), as in Fig. 1. *Open symbols*, Caffeine added after maximum control rate attained in the absence of caffeine at *p*Ca 6.0 (△) or *p*Ca 6.4 (○). Values show $\bar{x}$ ± SE of three to four fibers from one rabbit at *p*Ca 6.4 and one guinea pig at *p*Ca 6.0, and three to eight fibers from two rabbits at *p*Ca 6.0. Inhibition of the rabbit preparation shown at *p*Ca 6.4 was well above average; in fibers from four different animals measured at *p*Ca 6.4, average inhibition in the presence of 5 mM caffeine ranged from 48 to 68% when caffeine was added together with oxalate and from 25 to 45% when caffeine was added later

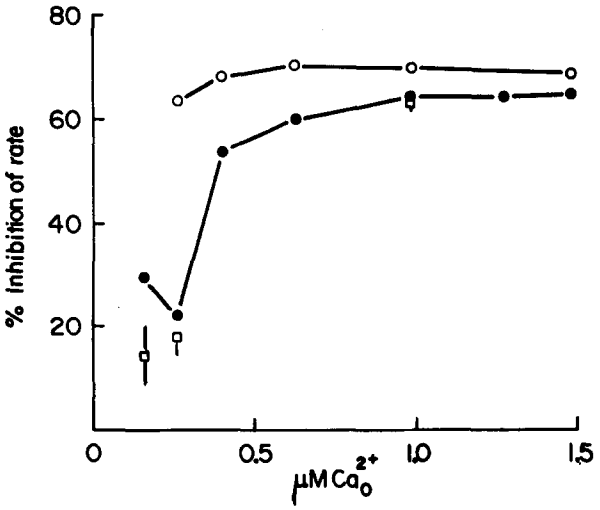

Fig. 3. External Ca^{2+} concentration dependence of caffeine inhibition. (●), Five fibers (EDL I, Table 2) were exposed to different Ca^{2+} concentrations in the presence of 5 mM caffeine, and the average rates compared to those obtained from the same sequence of Ca^{2+} concentrations applied to 10 fibers from the same muscle in the absence of caffeine. (○), Nine fibers (EDL II, Table 2) were exposed to different Ca^{2+} concentrations in the presence of 2.5 mM caffeine and compared to 10 control fibers from the same muscle, as described above. (□), Five fibers were exposed to different Ca^{2+} concentrations in sequence; at each point, the rate was recorded first in the absence of caffeine and then with 7.5 mM caffeine added to the bath. The inhibition was calculated from the ratio of the two rates. Values shown are $\bar{x}$ ± SE

Sarcoplasmic reticulum membranes were also lost during the preparation of SR vesicles. Based on the decreased capacity for Ca accumulation in the absence of caffeine (Table 1), it can be calculated that about one-half of the original SR was discarded in the first spin. Our final yields ranged from 0.5 to 2 mg of washed SR vesicle protein per g muscle. Recovery of one mg SR protein per g muscle represents only 15 to 20% of the original SR protein content, as estimated from measurements of total protein and phosphoenzyme formation in muscle homogenates and purified SR vesicles [31].

Caffeine Inhibition of Ca Accumulation by Skinned Fibers

In most of the skinned fibers tested, inhibition of oxalate-supported Ca accumulation was similar in magnitude to that observed in crude homogenates. With 20 mM caffeine present, a very slow accumulation of Ca oxalate was observed (Fig. 1). As the concentration of caffeine was reduced below 5 mM, Ca oxalate accumulated more rapidly. In psoas fibers from three different animals, inhibition of the rate reached a plateau at caffeine concentrations of 2.5 to 5 mM, with little change on increasing the concentration to 20 mM (Fig. 2*A*). The caffeine concentration dependence and extent of inhibition when Ca accumulation was supported by 5 mM pyrophosphate were similar to those shown in Fig. 2*A* for oxalate-supported uptake.

The extent of inhibition by caffeine was not af-

Table 2. Effects of caffeine on kinetic parameters of oxalate-supported Ca accumulation in skinned fibers[a]

No. of fibers	V_{max} ($\Delta S/S \cdot$ min)	$K_{0.5}$ (μM)	Hill (n)	Caffeine (mM)	Inhibition (% of V_{max})	V_{max} ($\Delta S/S \cdot$ min)	$K_{0.5}$ (μM)	Hill (n)
EDL I								
(10)(3)	0.87	0.68	2.10	2.5	31%	0.69	0.75	2.06
(5)				5.0	64%	0.31	0.68	1.54
EDL II								
(10)(9)	0.55	0.53	1.80	2.5	68%	0.17	0.59	1.45

[a] Values are obtained by applying nonlinear regression analysis (Eq. 1, *see* text) to average Ca^{2+} concentration curves measured either in the absence (10 fibers, columns 2 to 4) or in the presence of caffeine (3, 5 or 9 fibers, columns 5 to 9).

fected by the imidazole buffer, which is an analog of caffeine [3]. In five fibers, 5 mM caffeine was added after loading had reached its maximum rate at pCa 6.0 in the absence of imidazole. After caffeine had been washed out, the rate was recorded in the presence of imidazole and then caffeine was added again. The rates with and without buffer were not significantly different, and inhibition by caffeine ($\bar{x}$ ± SE) was 57 ± 3% in the first case and 50 ± 2% in the second.

In the concentration range of 0.5 to 7.5 mM, caffeine was equally inhibitory in psoas and EDL fibers of the rabbit, and in EDL fibers from a guinea pig. Inhibition was also similar for fibers from New Zealand rabbits and from rabbits obtained in Brazil (*cf.* Figs. 2*A* and 2*B*), although the latter accumulated Ca oxalate under control conditions at about one-half the rate of the former, had slightly lower steady-state capacities, and were prone to exhibit only partial reversal after prolonged exposure to caffeine concentrations above 7.5 mM. In each group of rabbits, there was one whose fibers under standard loading conditions were relatively insensitive to caffeine (less than 30% inhibition). These preparations were not studied further.

In a separate set of experiments, caffeine dose-response curves were obtained using two different protocols (Fig. 2*B*). One procedure was identical to that shown in Figs. 1 and 2A. In the second procedure, loading was allowed to reach its maximum rate in the absence of caffeine, and then 7.5 mM caffeine was added. After recording the new rate, the loading medium was replaced with one containing 5 mM caffeine, and thus rates were measured at each concentration as caffeine was progressively reduced to zero. Inhibition was calculated in terms of the control rate obtained at the end of the sequence on each fiber. Low concentrations of caffeine added after the maximum control rate had been attained (lower curves in Fig. 2*B*) were much less inhibitory than when loading was begun in the presence of caffeine (upper curves, Fig. 2*B*).

Calcium Concentration Dependence

In the experiments of Figs. 1 and 2A, the Ca^{2+} concentration was 0.4 μM, close to the $K_{0.5}$ for Ca transport [45]. At pCa 6.0, inhibition appeared to be slightly greater (Fig. 2*B*). In order to determine whether caffeine alters the Ca^{2+} concentration dependence for Ca transport, rates of Ca accumulation were measured at different Ca^{2+} concentrations in the presence or absence of 2.5 to 5 mM caffeine. The values for the parameters n, $K_{0.5}$ and V_{max} that best fit Eq. (1) were determined by nonlinear regression analysis [26] (Table 2).

$$v = \frac{V_{max}}{1 + (K_{0.5}/Ca^{2+})^n} . \quad (1)$$

The primary effect of caffeine was to decrease V_{max}, without altering Ca^{2+} affinity. Greater degrees of inhibition appeared to be associated with lower Hill n values (Table 2), but these changes in n were not statistically significant. Figure 3 (circles) shows that the rate of Ca uptake was equally inhibited at all Ca^{2+} concentrations between 1.5 μM and 0.3 to 0.4 μM, inhibition being less at lower Ca^{2+} concentrations. An effect of Ca^{2+} was confirmed in fibers from another preparation by measuring Ca uptake in the absence and then in the presence of caffeine in the same fiber at each of three different Ca^{2+} concentrations (squares, Fig. 3).

ATP Dependence of Ca Uptake and Caffeine Inhibition

As a preliminary to examining their effects on inhibition by caffeine, activation of Ca accumulation by

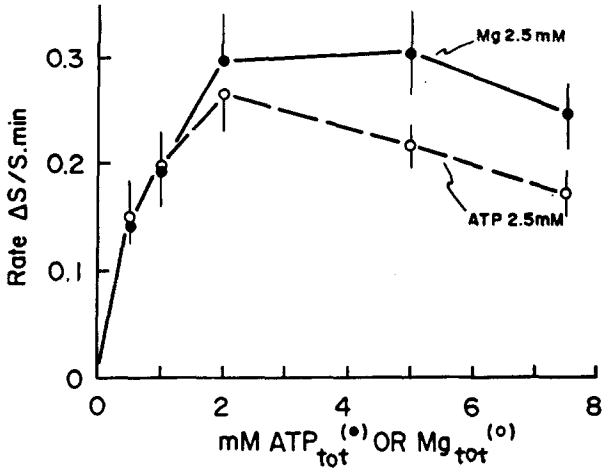

Fig. 4. Activation of Ca accumulation by MgATP. Rates of Ca accumulation were measured in the standard pCa 6.0 loading medium containing 2.5 mM Mg and various concentrations of ATP (●), or 2.5 mM ATP and various concentrations of Mg (○). Values show $\bar{x} \pm$ SE for eight fibers in each case

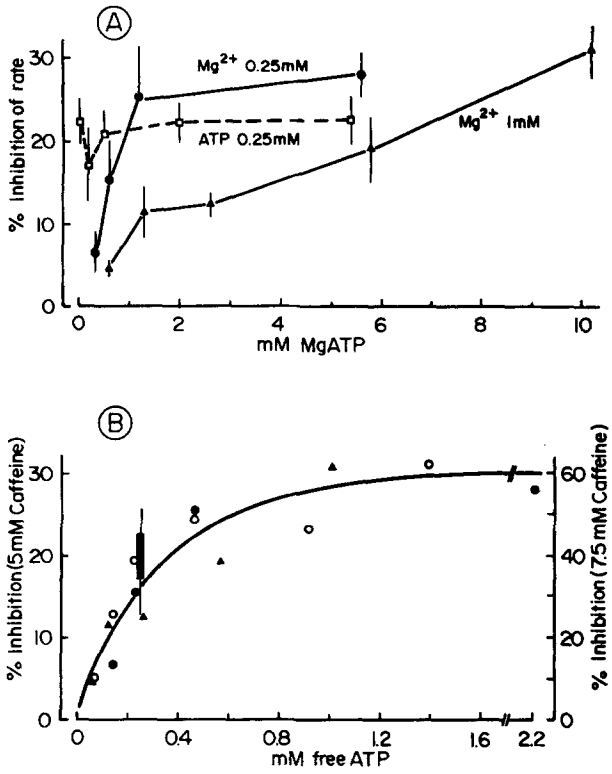

Fig. 5. Free ATP dependence of caffeine inhibition. Rates of Ca accumulation were measured in the standard pCa 6.0 loading medium, but with total ATP and Mg adjusted so as to maintain either 0.25 mM Mg^{2+} and 0.12 to 5.6 mM MgATP (●, ○); or 1.0 mM Mg^{2+} and 0.6 to 10.2 mM MgATP (▲); or 0.25 mM free ATP, variable Mg^{2+} (0.013 to 2.14 mM) and variable MgATP (0.033 to 5.42 mM) (□ in *A*; vertical bar in *B*). At each point, the rate was recorded first in the absence of caffeine and then with caffeine added to the bath to a final concentration of 5 mM (●, ▲, □) or 7.5 mM (○, right axis in *B*). Inhibition was calculated from the ratio of the two rates. Values show $\bar{x} \pm$ SE for three to eight fibers (five fibers in most cases)

Mg and ATP were studied at pCa 6.0 by increasing the ATP concentration with total Mg fixed, or by increasing the Mg concentration with total ATP fixed. The two procedures result in an identical sequence of increasing concentrations of MgATP, the substrate for the pump [49]. Ca^{2+} remains nearly constant, and each of the other species present (Mg^{2+}, free ATP and CaATP) varies in one direction with one procedure and in the opposite direction with the other. Both procedures for increasing MgATP led to increasing rates of Ca uptake (Fig. 4), and we infer that this increase reflects an activating effect of the MgATP complex at the low-affinity nucleotide binding site [8, 48]. We considered the possibility that rate of diffusion combined with loss of ATP due to hydrolysis within the fiber might have determined the low rates observed with low MgATP concentrations. However, this does not appear to have been the case, since the same low rates were found for both curves even though one was obtained with a large excess of ATP as a buffer for the MgATP complex. Since the two curves do not superimpose at the higher concentrations of MgATP (Fig. 4), one of the other ionic species may also modulate transport. Further experiments would be required in order to examine this possibility.

In a second group of experiments, 5 mM caffeine was added to the bath after the rate had been recorded in the absence of the drug at each combination of Mg and ATP concentrations. In one set of fibers, free Mg^{2+} was fixed at 0.25 mM and MgATP varied 16-fold. In another set, free Mg^{2+} was 1 mM and MgATP concentrations were higher than in the first set. In both cases, free ATP increased in proportion to the total ATP added. In a third set of fibers, free ATP was fixed at 0.25 mM and free Mg^{2+} varied together with MgATP. Figure 5*A* shows that the inhibition is not determined by the MgATP concentration, whereas in Fig. 5*B*, the data from all three sets superimpose when they are plotted as a function of the free ATP concentration. The effect of free ATP saturated in the millimolar range, and was half-maximal between 0.2 and 0.3 mM free ATP (Fig. 5*B*). In fibers from other preparations tested (*not shown*), inhibition was half-maximal with similar or lower concentrations of free ATP. When a concentration of caffeine that caused twice as much inhibition was used (open circles and right axis, Fig. 5*B*), the dependence on free ATP concentration was not significantly altered.

Table 3. Effects of caffeine and $(Ca^{2+})_o$ on rate and capacity of oxalate-supported Ca accumulation in skinned fibers

*p*Ca	Caffeine (mM)	Rate ($\Delta S/S \cdot$ min)	Capacity ($\Delta S_{max}/S$)	Fractional uptake rate (min^{-1})	No. of fibers
6.4	0	0.25 ± .02	3.81 ± .45	0.069 ± .005	7
6.4	2.1	0.12 ± .01	1.78 ± .13	0.072 ± .009	8
6.8	0	0.16 ± .02	3.53 ± .46	0.048 ± .005	3

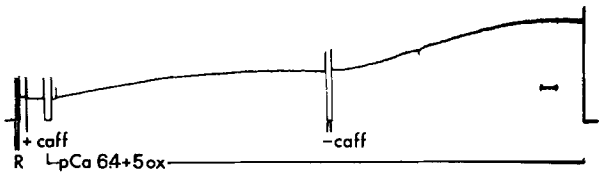

Fig. 6. Effect of caffeine on Ca oxalate accumulation capacity. A psoas fiber was exposed for one min to 2.1 mM caffeine in R and then to the standard *p*Ca 6.4 loading medium containing caffeine until loading reached a steady plateau of 1.3 times S, the signal in R. After two washes with 0-caffeine loading solution, the capacity increased to $\Delta S_{max}/S = 3.6$. In seven control fibers loaded without caffeine, the capacity was 3.8 (Table 3). Calibration, 1 min

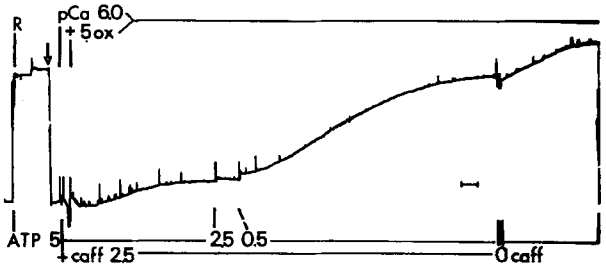

Fig. 7. Effect of free ATP on capacity in the presence of caffeine. An EDL fiber was stretched to 180% of slack length in R containing 5 mM ATP and 2.5 mM Mg. After downward re-adjustment of the baseline (arrow), the fiber was exposed to the standard *p*Ca 6.0 loading medium containing 5 mM ATP, 2.5 mM Mg and 2.5 mM caffeine, and 5 mM oxalate was added. With caffeine present, identical low capacities were recorded in 5 mM ATP and 2.5 mM ATP, but in 0.5 mM ATP the capacity increased 4.6-fold. A further 28% increase occurred after caffeine was washed out. Calibration, 1 min

After testing various combinations of Mg^{2+}, MgATP and free ATP, we were not convinced that free ATP was the unique determinant of inhibition for all combinations. However, it does appear that free Mg^{2+} can vary from 0.1 to 2 mM and MgATP from 0.03 to 10 mM without significantly affecting inhibition over the range of free ATP concentrations shown in Fig. 5.

Caffeine-Insensitive SR

In a previous study of oxalate-supported Ca accumulation in skinned fibers, it was shown that lowering the concentration of oxalate in the loading medium caused a decrease in rate of net uptake that was exactly proportional to the decrease in final steady-state capacity. Electron micrographs taken after loading had reached its final plateau in the presence of a low oxalate concentration showed SR membrane profiles either without crystals or filled with crystals, side by side in the same sarcomere [45]. Apparently the use of lower oxalate concentrations prevented some elements of SR from contributing to the formation of Ca oxalate precipitates. Elimination of these elements at low oxalate concentrations entirely accounted for the decrease in rate, while the remainder of the SR continued to accumulate Ca oxalate at the same rate as with higher oxalate concentrations.

Similarly, inhibition of Ca uptake by caffeine affected capacity to the same extent as rate of uptake. The fiber of Fig. 6 accumulated Ca oxalate slowly and reached a low steady-state plateau in the presence of 2.1 mM caffeine. When the caffeine was washed out, Ca oxalate accumulation immediately resumed. The final capacity was twice that attained in the presence of caffeine. Measurements on 15 fibers that were loaded either in the presence or in the absence of caffeine showed that both rate and capacity were reduced to the same extent, so that the fractional loading rate was the same under both conditions (7% of the final capacity per min) (Table 3).

Figure 7 shows that free ATP enhancement of inhibition by caffeine was related to caffeine's ability to alter the capacity. The fiber was allowed to accumulate Ca in the presence of 5 mM oxalate, 5 mM ATP, 2.5 mM Mg and 2.5 mM caffeine until it reached a plateau. When ATP was reduced to 0.5 mM, Ca uptake immediately resumed, even though caffeine was still present. Removal of caffeine with ATP held at 0.5 mM led to a small further increase in capacity. Control experiments showed that in the

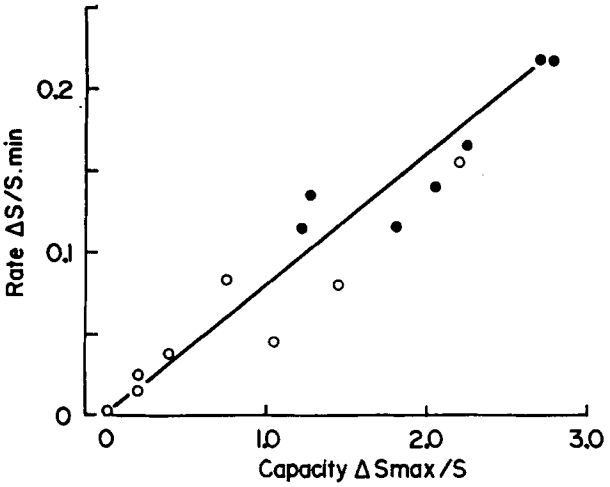

Fig. 8. Proportional reduction of rate and capacity in fibers exposed to A23187. (○) Psoas fibers were pre-incubated in 0.02 μM A23187 (4.5 to 8 min in R, followed by 4 to 8 min in standard *p*Ca 6.4 loading medium), or were pre-incubated in 0.1 to 0.2 μM A23187 (1 to 1.5 min in *p*Ca 6.4 loading medium). Rate and capacity were measured at *p*Ca 6.4 in the presence of the same concentration of ionophore, after the addition of 5 mM oxalate. (●) Controls, without ionophore

absence of caffeine, changes in ATP from 0.2 to 7.5 mM had no effect on capacity.

Effect of Caffeine on Efflux

The experiments described above do not indicate whether inhibition of net uptake by caffeine reflects reduced influx or enhanced efflux. This question was approached by comparing the results obtained using caffeine with the results obtained in two different experimental situations in which influx and efflux could be manipulated separately.

In the first experiment, the rate of uptake was decreased by lowering the Ca^{2+} concentration from *p*Ca 6.4 to *p*Ca 6.8 (Table 3). In contrast to the effect observed with caffeine, the capacity was unaltered. As a result, the fractional rate of uptake decreased from 7.2 to 4.8% per minute (Table 3). A similar reduction in fractional rate of uptake with no effect on capacity was observed when the MgATP concentration was decreased by lowering the ATP from 5 to 0.5 mM in the absence of caffeine (*not shown*).

In the second experiment, fibers were loaded in the presence of the divalent cation ionophore, A23187. In these seven fibers, which were pre-incubated with different concentrations of ionophore for various periods before adding oxalate, rates of net uptake were reduced to varying extents ranging from 0 to 100% of the average control value. Figure 8 shows that the reduction in rate caused by ionophore treatment was proportional to a reduction in capacity, as shown in Table 3 for the reduction in rate caused by caffeine. On the average, the seven ionophore-treated fibers in this experiment accumulated 8.0 ± 1.0% of their maximum capacity per minute, compared to 8.1 ± 0.4% for the fractional rates of uptake in the seven control fibers.

Another test for an effect of caffeine on efflux is shown in Fig. 9. A fiber was first exposed to the standard Ca-loading medium for 2 min in the absence of oxalate (Fig. 9*A*). Then the EGTA-buffered solution was replaced with W, a solution containing only contaminating Ca and no EGTA. The addition of caffeine induced a rapid but transient tension, indicating that Ca^{2+} around the myofibrils had increased at least to several micromolar and then returned to a subthreshold value. Ninety sec after the caffeine was washed out with W, a second addition of caffeine evoked a similar but smaller tension. This was repeated three more times while the fiber remained in W, and finally the original tension was reproduced after the fiber had been re-exposed to the standard loading medium for 2 min.

In Fig. 9*B*, the same fiber was allowed to accumulate Ca oxalate in the standard loading medium until a steady plateau of light scattering was reached. Then the solution containing CaEGTA and oxalate was replaced with W (lacking these two components), and in Fig. 9*C*, caffeine was added. The resulting tension was sustained until the caffeine was washed out, indicating a substantial Ca efflux. This caffeine-induced tension was repeated four more times, and the last tension was identical to that obtained at the end of the experiment after re-exposing the fiber to the original buffered Ca medium.

Discussion

In this investigation, caffeine-induced Ca release and caffeine inhibition of oxalate-supported Ca uptake were monitored in skinned fibers. The two phenomena show striking similarities, which suggests to us that a single mode of action of caffeine produces both effects. Both Ca release and inhibition of uptake are readily reversible (Figs. 1 and 6; references 4, 39, 45, 54), and with Ca^{2+} and ATP present, both processes are half-maximal with less than 2 mM caffeine and maximal at concentrations of 10 mM or less (Figs. 1 and 2, and references in Introduction). Both effects are reduced or eliminated by reducing the concentration of free ATP (Figs. 5 and 7; references 11, 36, 47, 51). In both

cases the action of caffeine decreases with very low external free Ca^{2+} concentrations (Fig. 3; references 22, 23, 36, 47).

A possible discrepancy arises when the sustained nature of the effect of caffeine on Ca uptake is compared with the transient nature of caffeine-induced Ca release (Figs. 6 and 9*A*, and references in Introduction). That this discrepancy can be eliminated is shown by the experiment of Fig. 9*C*, where prolonged tensions indicate that caffeine induced sustained Ca release from a fiber loaded with Ca oxalate. It is difficult to reconcile this result with the idea of a caffeine-triggered Ca^{2+} channel that becomes inactive in the presence of caffeine [22, 47]. One interpretation of the present data is that caffeine remains bound to a site on the SR membrane as long as it is present in the medium, activating a Ca^{2+} channel that allows Ca efflux as long as there is Ca available for release. The release may be transient when the fiber is loaded without oxalate (Fig. 9*A*) in part because the Ca content is much smaller (1 to 3 mM, compared with 20 to 170 mM) [45] and is rapidly depleted as Ca diffuses out of the fiber. This conclusion is supported by the observation (*not shown*, but similar to Fig. 1*A* of ref. 12) that under the conditions of Fig. 9*A*, washing out caffeine with R (5 mM EGTA) rather than W (0 EGTA) makes it impossible to obtain repeated tensions with caffeine unless the fiber is re-exposed to the loading medium. The wash medium W used in Fig. 9, on the other hand, has enough contaminating Ca in it to provide partial reloading. In intact fibers, evidence has been presented that refractoriness of caffeine tensions to a second challenge with caffeine can be altered by increasing the intracellular supply of Ca^{2+} available for refilling the SR [5].

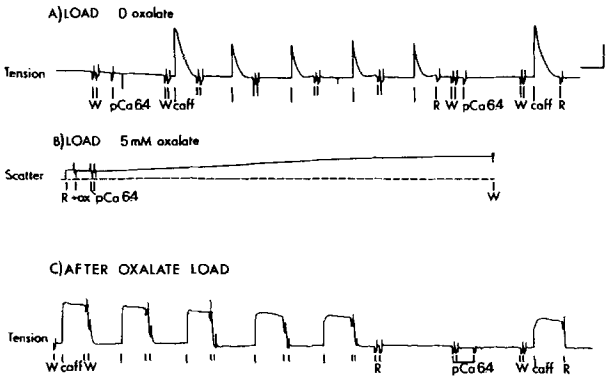

Fig. 9. Transient and sustained tensions evoked by caffeine. (*A*) Psoas fiber stretched to 150% of slack length in R was exposed to standard *p*Ca 6.4 loading medium for 2 min, preceded and followed by two washes with W (185 mM K propionate, 10 mM imidazole, pH 7.0, 2.5 mM Mg, 2.5 mM ATP) to remove EGTA. Transient tensions were evoked by addition of 5 mM caffeine to W, followed by two washes with W to remove caffeine (short vertical bars). After the 5th caffeine-induced tension, the fiber was re-exposed to the original loading medium before addition of caffeine. Calibration, 10 mg and 1 min. (*B*) Light-scattering record from same fiber during exposure to standard *p*Ca 6.4 loading medium in the presence of 5 mM oxalate, showing the accumulation of Ca oxalate to a plateau level over a period of 18.5 min. Time course is continuous with that of Fig. 9*C*. (*C*) Sustained tensions evoked by addition of 5 mM caffeine following maximal loading with Ca oxalate shown in *B*. Same sequence of solutions as in *A*, after removal of the first loading medium

Caffeine-Insensitive SR Membranes

A second factor that must contribute to the transient nature of caffeine-evoked Ca release in the absence of oxalate is suggested by experiments with isolated SR vesicles [22, 37, 39, 47]. In these experiments, released Ca was re-accumulated by the SR in an ATP-dependent process, and Su and Hasselbach [47] as well as Ohnishi [39] and Ogawa and Ebashi [38] showed that subsequently the SR was refractory to further additions of caffeine. Su and Hasselbach proposed that Ca might be released from a group of vesicles that are sensitive to caffeine, to be re-accumulated by vesicles that are not sensitive to caffeine. In the skinned fiber, a caffeine-insensitive population of SR membranes, if they were not maximally loaded beforehand, could help restore tension to resting levels by re-accumulating part of the Ca released by caffeine. This contribution would be lost if the caffeine-insensitive SR were already maximally loaded, as in the oxalate experiment of Fig. 9*B*.

Figures 5 and 7 show a free ATP requirement for inhibition of Ca uptake by caffeine that is similar to that reported for caffeine-induced Ca release from SR vesicles [36, 47, 51]. Figure 7 suggests that lowering the ATP concentration causes a decrease in the population of caffeine-sensitive SR elements.

The existence of two membrane populations, one with and one without caffeine binding sites, is supported by the observation that only part of the accumulated Ca is released from isolated SR vesicles (*see* references in Introduction). Several of our experiments with skinned fibers and muscle homogenates provide further evidence for caffeine-sensitive and caffeine-insensitive membranes. First, even a maximally effective dose of caffeine does not completely inhibit Ca uptake, either in the skinned fiber (Figs. 1 and 2) or in the crude homogenate (Table 1). Second, much of the caffeine sensitivity is lost along with 80 to 90% of the original SR membranes during the preparation of isolated SR vesicles (Table 1). Third, in the presence of caffeine, the final steady-state capacity is reduced (Tables 1 and

3 and Fig. 6). This indicates that the action of caffeine cannot be one of simply reducing the rate of uptake throughout the SR, since when the rate of uptake is reduced by lowering the external Ca^{2+}, the capacity is unaffected (*see* Table 3). By analogy with the effect of lowering the oxalate concentration, which decreases both rate and capacity [45], we infer that some SR elements fail to form Ca oxalate precipitates in the presence of caffeine.

The fact that the rate of uptake under standard loading conditions is reduced by caffeine to exactly the same extent as the capacity (Table 3) suggests that the elements that remain functional continue to fill at the same rate as in the absence of caffeine—in Table 3, 7% of the final volume per minute. In other words, they are completely unaffected by caffeine. For the reason outlined in the preceding paragraph, we favor the hypothesis that the loss of caffeine sensitivity that occurs when the membranes are isolated (Table 1) reflects selective removal of an SR membrane population that is enriched in caffeine binding sites. In the fiber preparations and in whole-muscle homogenates, the caffeine-insensitive elements might be the longitudinal SR, and the caffeine-sensitive ones the terminal cisternae [53]. However, it is worth noting that the "heavy" SR vesicle fractions used by Kim et al. [22] and by Su and Hasselbach [47], although probably enriched in membranes of the terminal cisternae, nevertheless showed evidence of containing caffeine-insensitive vesicles. This raises the possibility that the process of washing the membranes may remove or inactivate caffeine binding sites that are normally distributed among SR membranes in both regions of the sarcomere. A secondary effect of washing might also occur, such as reduced ability to respond to bound caffeine or a decrease in the binding of free ATP.

Primary Effect of Caffeine

Several laboratories have reported that caffeine does not inhibit the ATPase activity of SR membranes [20, 24, 47, 50, 51], and it does not appear to alter steady-state phosphorylation levels formed from ATP [38]. The data of Table 3 and the lack of an effect on the $K_{0.5}$ for Ca^{2+} (Table 2) are consistent with lack of an effect on ATP-dependent Ca influx, since the decrease in V_{max} is largely accounted for by the loss of capacity rather than by inhibition of turnover. On the other hand, Fig. 8 shows that the effect of caffeine on both rate and capacity can be imitated by use of an ionophore whose primary effect is to increase Ca efflux. Figure 2*B* shows that caffeine, as previously reported for Ca ionophores [16, 21, 45], loses much of its inhibitory effectiveness at low concentrations if it is added after Ca oxalate precipitates have begun to form throughout the SR. This may indicate that caffeine is more effective when the internal free Ca^{2+} concentration is high, as would be expected if caffeine enhances efflux by increasing membrane permeability to Ca. Experiments on skinned fibers [25] and with isolated SR vesicles in the presence of EDTA or EGTA (19, 22, 23, 34, 36] are consistent with this interpretation.

The data presented in this paper do not appear to us to add new evidence concerning whether or not caffeine-induced Ca release and Ca^{2+}-induced Ca release share a common pathway. Instead, we wish to emphasize two similarities between caffeine-induced Ca release and caffeine's inhibition of Ca uptake that seem most promising for future work on the mechanism of action of caffeine. The data of Fig. 3 (*see also* refs. 10 and 51) suggest that the inhibitory effect of caffeine requires a certain minimum concentration of Ca^{2+}. The effect of caffeine on Ca uptake thus in this respect finds a parallel with caffeine-induced Ca release, which several authors have shown to be enhanced by Ca^{2+} [10, 21–23, 34, 36, 47, 51]. Free ATP is another factor that enhances caffeine-induced Ca release [11, 36, 47, 51], and the analog AMP-PCP is also effective in this role [38]; both nucleotides have been reported to promote Ca efflux in the absence of caffeine as well [6, 10, 11, 33, 55]. Free ATP alone was without effect on net uptake in our experiments, possibly because influx was strongly activated by MgATP (*see* ref. 55). However, ATP clearly potentiated greatly the inhibition of net uptake by caffeine (Figs. 5 and 7), and one interpretation of Fig. 7 is that ATP somehow enhances caffeine binding to the SR. Since our experiments with ATP were done with only one or two concentrations of caffeine, they do not show whether ATP increases the affinity for caffeine in a fixed population of caffeine-sensitive membranes, or increases the maximal effect of caffeine by recruiting new elements into the caffeine-sensitive population.

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