

Quercetin inhibits Ca^{2+} uptake but not Ca^{2+} release by sarcoplasmic reticulum in skinned muscle fibers

(Ca^{2+} , Mg^{2+} -ATPase/ATP synthesis/muscle contraction/caffeine)

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ABSTRACT Quercetin inhibited Ca^{2+} -dependent ATP hydrolysis, ATP-dependent Ca^{2+} uptake, chelator-induced [ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid] Ca^{2+} release, and ATP synthesis coupled to Ca^{2+} release in isolated vesicles of sarcoplasmic reticulum. Use of this inhibitor permitted evaluation of whether Ca^{2+} release from sarcoplasmic reticulum *in situ* occurs through a reversal of the uptake pathway. Release of Ca^{2+} from the sarcoplasmic reticulum of skinned muscle fibers can be detected by the measurement of tension in the fiber. If the sarcoplasmic reticulum of these preparations is first allowed to accumulate Ca^{2+} tension development may be induced by the addition of Ca^{2+} itself or of caffeine to the bathing medium or by depolarization with Cl^- . The presence of quercetin during the loading phase inhibited Ca^{2+} uptake by sarcoplasmic reticulum *in situ*. When quercetin was added together with initiators of tension development, however, the rate of tension development was enhanced 4- to 7-fold and the relaxation rate of the fibers was greatly inhibited. These results suggest that quercetin had no effect on Ca^{2+} release in skinned fiber; its effect on Ca^{2+} reuptake could account for the apparent enhancement of the release rate and for the prolonged relaxation time. These observations rule out reversal of the Ca^{2+} pump as the mechanism of Ca^{2+} release *in situ*.

The contraction/relaxation cycle of muscle is mediated through changes in Ca^{2+} concentration within muscle cells (1). Muscle contraction is elicited by Ca^{2+} released from the sarcoplasmic reticulum into the myofilament space, whereas muscle relaxation results from sequestration of Ca^{2+} in the same membrane (1, 2). It is well established that the Ca^{2+} , Mg^{2+} -ATPase (ATP phosphohydrolase, EC 3.6.1.3) of the sarcoplasmic reticulum is the enzyme that catalyzes Ca^{2+} transport at the expense of ATP hydrolysis, and the enzymatic reactions of Ca^{2+} transport are understood in general terms (3,4). On the other hand, the process of Ca^{2+} release from the sarcoplasmic reticulum is not understood. Various kinds of stimuli applied directly to sarcoplasmic reticulum have been found to be effective in inducing Ca^{2+} release. Ca^{2+} will induce Ca^{2+} release, increasing permeability of the membrane through an interaction on the cytoplasmic surface (2, 5-9). Caffeine is also effective in Ca^{2+} release and its mode of action has been shown to be intimately related to the Ca^{2+} -induced Ca^{2+} release mechanism (2). Chloride-induced Ca^{2+} release is believed to result from a change in electrical potential across the membranes (10-13).

It has been shown that the entire process of Ca^{2+} transport can be reversed (14,15), and that ATP synthesis is coupled to Ca^{2+} efflux. The high ratio of ATP synthesized per Ca^{2+} released suggests that most of the Ca^{2+} leaves the vesicles through the pump system (15). This raises the question of whether the Ca^{2+} pump of the sarcoplasmic reticulum is directly involved

in the Ca^{2+} release process. Although in many respects the calcium release process is completely different from the characteristics of pump reversal (2), it has been suggested (16) that a common carrier may be involved in both processes.

We have discovered that quercetin, a potent inhibitor of the ATPase of sarcoplasmic reticulum (17,18), inhibits forward and backward reactions of ATP-dependent Ca^{2+} transport. These observations have provided us with the opportunity to examine involvement of the Ca^{2+} , Mg^{2+} -ATPase of sarcoplasmic reticulum in the release of Ca^{2+} under physiological conditions. Accordingly, we have studied the effects of quercetin on Ca^{2+} uptake and release in skinned muscle fibers. The results provide evidence that Ca^{2+} release *in vivo* does not occur through reversal of Ca^{2+} uptake and that Ca^{2+} uptake and release do not involve a common carrier, suggesting that there are independent pathways for the two processes.

MATERIALS AND METHODS

Materials. ATP, ADP, hexokinase (type V), Tris, imidazole, and quercetin were obtained from Sigma. Quercetin was dissolved in 95% ethanol, and the final ethanol concentration was not more than 2% in the control and in the samples containing quercetin. Quercetin concentration was determined spectrophotometrically by using a molar extinction coefficient of 16,100 at 385 nm and pH 8.0 (19). $^{45}\text{CaCl}_2$ was purchased from New England Nuclear and [^{32}P]phosphate was obtained from Amersham. [γ - ^{32}P]ATP was synthesized from ADP and ^{32}P by photophosphorylation (20) and was purified by the method of Glynn and Chappell (21). The other chemicals were analytical reagent grade and all solutions were prepared with deionized, distilled water.

Isolation of Sarcoplasmic Reticulum Membranes. Vesicles were prepared from rabbit white skeletal muscle by the method of MacLennan (22).

Skinned Fiber Preparations. Skeletal muscle fibers from human or rabbit were obtained by the method of Wood (23). The muscle fibers were skinned chemically by the method of Wood *et al.* (24) and were devoid of sarcolemma and mitochondria but contained actively functioning sarcoplasmic reticulum and myofilaments. All the experiments reported were carried out with human gluteus muscle fibers; similar results were obtained when muscle fibers from rabbit were used.

Isometric Tension Measurements The experimental apparatus was essentially as described by Wood *et al.* (24). A single intact fiber was isolated from the skinned muscle, the two ends of the fiber were secured in Lucite clamps, and the isometric tension was recorded with a mechanoelectronic transducer

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Abbreviations: EGTA, ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid.

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(Grass Instrument, model FT O3C) and a Gould Brush 2200 recorder. The maximal isometric tension was determined by exposing the fiber to 10 μM free Ca^{2+} . The experiments were carried out at 22° C in a thermoregulated chamber.

Assays. ATPase activity was determined at 37° C as described (22). The ^{32}P released from [$\gamma\text{-}^{32}\text{P}$]ATP was extracted as phosphomolybdate with isobutanol/benzene, 1:1 (vol/vol), by the method of Avron (20) and measured by liquid scintillation counting. Ca^{2+} uptake was assayed as described (22) by the Millipore filtration method (25). Protein concentration was determined according to Lowry *et al.* (26) with bovine serum albumin as a standard. The concentration of free Ca^{2+} in the Ca^{2+} /ethylene glycol bis(β -aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA) buffers was calculated by assuming an association constant of $2 \times 10^6 \text{ M}^{-1}$ at pH 7.0.

RESULTS

Quercetin Inhibition of ATPase Activity and Ca^{2+} Uptake in Isolated Sarcoplasmic Reticulum Vesicles. Quercetin, a flavonoid, has been reported to inhibit the activity of enzymes involved in energy conversion reactions (17-19, 27-29). Quercetin inhibited Ca^{2+} -dependent ATPase activity and Ca^{2+} uptake by sarcoplasmic reticulum membranes (Table 1). In these experiments, half-maximal inhibition was obtained with about 10 μM quercetin. The effective range of quercetin concentration, however, increased with increasing protein concentration (not shown).

Effect of Quercetin on the Reversal of Ca^{2+} Pump. It has been shown (14,15) that, when sarcoplasmic reticulum vesicles previously loaded with Ca^{2+} are incubated in medium containing ADP, P_i , Mg^{2+} , and EGTA, stored Ca^{2+} is released to the medium and Ca^{2+} efflux is coupled to ATP synthesis. Under the proper conditions for reversal of the Ca^{2+} pump, quercetin inhibited both ATP synthesis and Ca^{2+} release (Fig. 1). Some Ca^{2+} release was not coupled to ATP synthesis and was resistant to quercetin. Thus, the Ca^{2+} released per ATP synthesized was 2.3 rather than 2.0 as anticipated for perfect coupling.

Effect of Quercetin on Ca^{2+} Uptake by Sarcoplasmic Reticulum in Skinned Muscle Fibers. The inactivation of the Ca^{2+} -ATPase by quercetin and the inhibition, thereby, of ATP-driven Ca^{2+} uptake and the reversal of this process can be used to test the relationship between the reversal of the Ca^{2+} pump and Ca^{2+} release in muscle fibers. The effect of quercetin on Ca^{2+} movement between the sarcoplasmic reticulum and the myofilament space was studied in skinned muscle fibers by using isometric force as an indicator of Ca^{2+} release from the sarcoplasmic reticulum. In the experiment described in Fig. 2, the sarcoplasmic reticulum in the fiber was loaded with Ca^{2+} in the absence or presence of quercetin at the concentration

Table 1. Effect of quercetin on ATPase and Ca^{2+} uptake activities of isolated sarcoplasmic reticulum

Quercetin	ATPase activity, $\mu\text{mol}/\text{mg protein}$	Ca^{2+} uptake, $\mu\text{mol}/\text{mg protein}$
None	12.47	1.454
7 μM	9.30	1.279
15 μM	3.25	0.518
30 μM	0.63	0.087

ATPase and Ca^{2+} uptake activities were assayed for 4 min. The reaction mixture for ATPase activity contained 20 mM Tris-HCl at pH 7.5, 100 mM KCl, 5 mM MgCl_2 , 5 mM [$\gamma\text{-}^{32}\text{P}$]ATP (containing $1.5 \times 10^5 \text{ cpm}/\mu\text{mol}$), 0.5 mM EGTA, 0.5 mM CaCl_2 , and sarcoplasmic reticulum at 40 $\mu\text{g}/\text{ml}$. Conditions for Ca^{2+} uptake were the same as for ATPase activity, except that 5 mM K oxalate was added to the reaction mixtures, unlabeled ATP was used, and $^{45}\text{CaCl}_2$ was added to a specific activity of $5 \times 10^6 \text{ cpm}/\mu\text{mol}$.

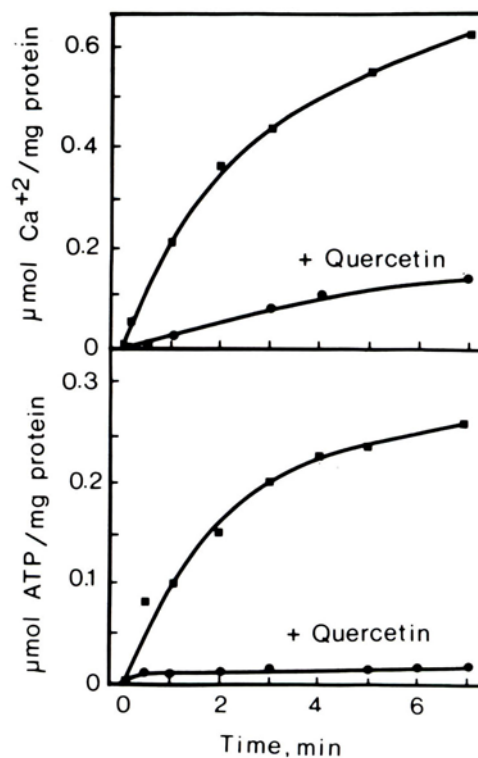


FIG. 1. Inhibition of reversal of the Ca^{2+} pump by quercetin. Ca^{2+} release (Upper) and ATP synthesis (Lower) were measured with Ca^{2+} -loaded vesicles. The loading solution for Ca^{2+} uptake was as described in Table 1 except that ^{45}Ca , to a specific activity of $10^7 \text{ cpm}/\mu\text{mol}$, was added only to the vesicles used for measurement of Ca^{2+} release. After 30 min at 22° C the vesicles were centrifuged at $80,000 \times g$ for 20 min and the pellets were resuspended in 20 mM Tris-HCl, pH 7.5/100 mM KCl and used immediately. ATP synthesis and Ca^{2+} release were assayed in a medium containing 20 mM Tris maleate at pH 6.5, 2 mM EGTA, 6 mM phosphate, 20 mM MgCl_2 , 20 mM glucose, 0.5 mM ADP, 10 units of hexokinase per ml, and 100 μg of loaded vesicles per ml ^{32}P ($8.8 \times 10^6 \text{ cpm}/\mu\text{mol}$) was added when ATP synthesis was measured. [^{32}P] Phosphate was extracted as described (20) and the glucose 6-phosphate formed in the reaction was measured by liquid scintillation counting. Ca^{2+} release was determined from the amount of ^{45}Ca retained in the filter (25). The quercetin concentration was 200 μM .

indicated. Then the fiber was washed[§] and the tension response was elicited by the addition of 10 mM caffeine. The amplitude of the caffeine-induced tension was plotted against quercetin concentration in the loading step. Quercetin decreased the tension amplitude, presumably because it inhibited prior Ca^{2+} accumulation by the sarcoplasmic reticulum. The range of quercetin concentrations required to bring about inhibition of tension development varied somewhat from fiber to fiber but was usually lower than that presented in Fig. 2 (see Fig. 3, where inhibition of relaxation and presumably of Ca^{2+} reuptake was complete at 100 μM quercetin).

Effect of Quercetin on Caffeine-Induced Ca^{2+} Release from Sarcoplasmic Reticulum. In order to test the effect of quercetin on Ca^{2+} release, quercetin was added to the caffeine solution. Fig. 3 shows the force spikes obtained with or without quercetin in the caffeine solution. In caffeine solution without quercetin, the force increased to a maximum and then declined due to calcium reuptake by sarcoplasmic reticulum. With ad-

[§] Quercetin can be removed by washing in aqueous solution. This is shown by the ability of the washed fiber to reload Ca^{2+} and to redevelop tension if the fiber is washed after exposure to quercetin.

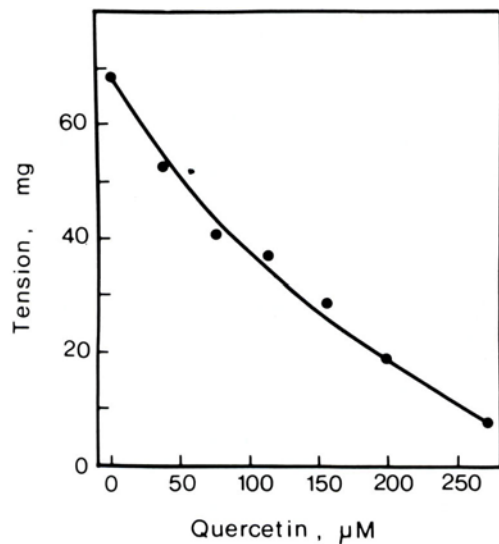


FIG. 2. Effect of quercetin on Ca^{2+} loading by sarcoplasmic reticulum in skinned, single fiber (diameter, $75 \mu\text{m}$). The sarcoplasmic reticulum was loaded with Ca^{2+} by exposing the fiber for 30 sec to a wash solution (5 mM imidazole/2.5 mM ATP/2.5 mM MgO/185 mM K propionate, pH 7.0) containing 5 mM EGTA, $0.158 \mu\text{M}$ free Ca^{2+} , and the indicated concentrations of quercetin. The fiber was then rinsed twice with wash solution. To elicit a tension response, wash solution containing 10 mM caffeine was added. The fiber was relaxed with wash solution containing 5 mM EGTA followed by wash solution containing 40 mM caffeine (to empty the vesicles) and then rinsed twice with wash solution. The fiber was then reloaded with Ca^{2+} as described above. Usually, 6-10 cycles of Ca^{2+} uptake and release were obtained with a fiber and the maximum tension decreased only by about 10% during the course of the experiments.

dition of $50 \mu\text{M}$ quercetin, the rate of tension development increased and relaxation was inhibited. Increasing the quercetin concentration to $100 \mu\text{M}$ caused a further increase in the rate of tension development and completely prevented the relaxation of the fiber. The inhibitory effect of quercetin on the relaxation of the fiber was consistent with the concept that relaxation is due to reaccumulation of calcium by the sarcoplasmic reticulum. The transient nature of the tension may thus reflect the dynamic equilibrium between uptake and release of Ca^{2+} by the sarcoplasmic reticulum and may explain the positive effect of quercetin on the rate of tension development (Table 2). Addition of 100-200 μM quercetin alone to Ca^{2+} -loaded fibers caused a slow increase in tension (about 0.5 mg/sec after an initial lag). This increased tension probably resulted from passive leakage of Ca^{2+} from sarcoplasmic re-

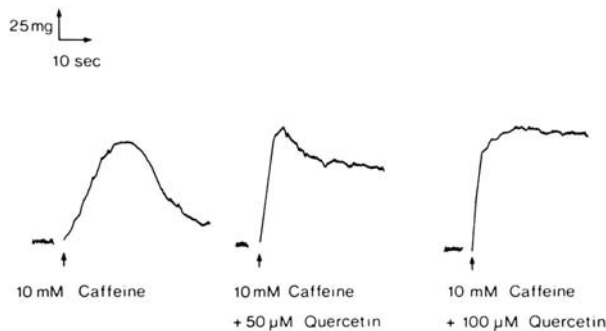


FIG. 3. Effect of quercetin on the response of a skinned fiber to caffeine. The experiments were carried out as described in Fig. 2, except that the fiber diameter was $62 \mu\text{m}$ and, where indicated, quercetin was added to the wash solution containing 10 mM caffeine.

Table 2. Effect of quercetin on level and rate of change of caffeine-induced tension

Quercetin, μM	Tension	
	mg	mg/sec
0	101.3	7.0
50	100	17.5
100	103.8	25.6
150	106.8	33.8
200	112.2	45.0

The experiments were performed as described in Fig. 2, except that quercetin, at the indicated concentrations, was present in the wash solution containing 10 mM caffeine. The chart speed was increased to determine the rates of tension development more precisely. The rates were calculated from the linear part of the responses, which was about 80% of the maximal tension.

ticulum when the Ca^{2+} uptake system was inhibited by quercetin. Quercetin had no effect on the activity of the contractile proteins themselves because the tension developed by direct exposure of an unloaded fiber to $10 \mu\text{M}$ free Ca^{2+} was not affected by quercetin (data not shown). In addition, the effects of quercetin described above were completely reversible when the fiber was washed (see legend to Fig. 2).

Effect of Quercetin on Tension Development Induced by Chloride and Calcium. Muscle fiber contraction can be induced by various calcium release stimuli (6-8,10-13). In order to induce Ca^{2+} efflux from the loaded sarcoplasmic reticulum by depolarization of the internal membrane, K propionate was replaced by KCl. We found that high concentrations of free Mg^{2+} inhibited the chloride-induced Ca^{2+} release, in confirmation of a previous report (13). Accordingly, the MgCl_2 concentration was reduced from 2.5 mM to 0.5 mM; the effect of quercetin on the chloride-induced tension development was similar to that found with caffeine (Table 3). Quercetin also increased the rate of tension development induced by the addition of Ca^{2+} to the bathing medium. In this case the MgCl_2 concentration in the bathing solution was also reduced (30). It seems, therefore, that the effect of quercetin on tension development was not dependent on the specific stimulus used.

DISCUSSION

The mechanism that initiates Ca^{2+} release and the molecular mechanism of Ca^{2+} release from sarcoplasmic reticulum are still not understood. Our study, directed toward the molecular mechanism of Ca^{2+} release, concerned the relationship between

Table 3. Effect of quercetin on the Ca^{2+} release induced by various stimuli

Ca^{2+} release induced by	Tension		Relaxation, mg/sec
	mg	mg/sec	
Caffeine	64	17	10
Caffeine + quercetin	90	64	1
KCl	46	20	7
KCl + quercetin	62	64	>1
Calcium	38	22	28
Calcium + quercetin	50	68	2

Experimental conditions were as in Table 2, except that for chloride-induced Ca^{2+} release, K propionate was replaced by KCl and 2.5 mM MgO was replaced by 0.5 mM MgCl_2 in the wash solution. For the Ca^{2+} -induced Ca^{2+} release, Ca/EGTA solutions were added to the wash solution in which MgO was replaced by 0.5 mM MgCl_2 . The free Ca^{2+} concentration was $0.2 \mu\text{M}$ and this concentration had no effect on the unloaded fiber. The same results with caffeine-induced Ca^{2+} release were obtained when the caffeine solution contained 0.5 mM MgCl_2 . The quercetin concentration was $200 \mu\text{M}$ and the fiber diameter was $62 \mu\text{m}$.

Ca²⁺ uptake and Ca²⁺ release and used quercetin as an inhibitor of the Ca²⁺ ATPase. The present findings show that quercetin inhibits Ca²⁺ ATPase and Ca²⁺ uptake activities of the sarcoplasmic reticulum in isolated membranes and in skinned fibers (Table 1; Fig. 2).

One Ca²⁺ release mechanism is that represented by the reversal of ATP-dependent Ca²⁺ uptake. We have shown, by using isolated sarcoplasmic reticulum (Fig. 1), that quercetin inhibits Ca²⁺ release and ATP synthesis concomitantly. Thus, if Ca²⁺ release in the muscle cells, under physiological conditions, were due to reversal of the Ca²⁺ pump, one would expect that quercetin would be an inhibitor of the physiologically relevant Ca²⁺ release. Analysis of the effect of quercetin on Ca²⁺ release from the sarcoplasmic reticulum in skinned fibers shows that Ca²⁺ release is not affected by quercetin (Fig. 3; Table 2).

The Ca²⁺ release mechanisms induced by caffeine, Ca²⁺, or Cl⁻ are believed to be different from the reversal of the Ca²⁺ pump because the characteristics of pump reversal are different from those of Ca²⁺ release elicited by the various inducers. For example, external free Ca²⁺ inhibits pump reversal (14, 31) and stimulates Ca²⁺ release. Moreover, external free Mg²⁺ is required for pump reversal but has an inhibitory effect on Ca²⁺ release (30). In addition, the requirement for ADP and phosphate for reversal of the Ca²⁺ pump and its inhibition by ATP (14, 31) are exactly the opposite of the conditions required for Ca²⁺ release (2). It is possible, however, that the carrier for the pump might be uncoupled from the ATP-splitting system under certain conditions and act as a carrier for Ca²⁺ release. This suggestion is inconsistent with the results in Table 2 and Fig. 3 which indicate that ATPase is active in transporting Ca²⁺ during the period of Ca²⁺ release. This conclusion is deduced from the marked increase in the rate of Ca²⁺ release under conditions such that the Ca²⁺ ATPase was inactive. This increase in the rate of Ca²⁺ release is a reflection of the dynamic equilibrium between the uptake and release of Ca²⁺ under conditions which elicited Ca²⁺ release; inhibition of Ca²⁺ uptake by quercetin results in an apparent stimulation in the rate of Ca²⁺ release. Ogawa and Ebashi (16) found that the ATP analogue AMPOPCP inhibited the Ca²⁺ pump and at the same time enhanced Ca²⁺-induced Ca²⁺ release, both with similar affinities. They suggested that a common carrier is active in both the release and the uptake of Ca²⁺. Their results, like our results with quercetin, can be explained, however, by an inhibition of Ca²⁺ uptake which results in a change in the equilibrium between the release and uptake of Ca²⁺, thereby leading to an apparent stimulation of Ca²⁺ release.

The use of skinned muscle fibers for the study of Ca²⁺ release for sarcoplasmic reticulum has limitations because connections between the surface membrane and the sarcoplasmic reticulum are disrupted and electrical stimulation no longer elicits Ca²⁺ release. We have examined the effect of quercetin on whole muscle fibers that were electrically excitable and found no inhibition of the electrically stimulated twitches. In these experiments we could not prove that quercetin was penetrating to intracellular sites but we could deduce that it did not act at the cell surface to prevent excitation-contraction coupling.

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