Excitation of Skinned Muscle Fibers by Imposed Ion Gradients

II. Influence of Quercetin and ATP Removal on the Ca\textsuperscript{2+}-insensitive Component of Stimulated \textsuperscript{45}Ca Efflux

ELIZABETH W. STEPHENSON

From the Department of Physiology, University of Medicine and Dentistry of New Jersey—New Jersey Medical School, Newark, New Jersey 07103

ABSTRACT Ionic gradients imposed by choline Cl replacement of K methanesulfonate (Mes) at constant [K][Cl] product stimulate \textsuperscript{45}Ca efflux from skinned muscle fibers; a small, sustained Ca\textsuperscript{2+}-insensitive efflux component, observed in EGTA, appears to grade a much larger Ca\textsuperscript{2+}-dependent component responsible for contractile activation and is likely to reflect intermediate steps in excitation-contraction coupling. The present studies examined ATP-related effects on the Ca\textsuperscript{2+}-insensitive stimulation. \textsuperscript{45}Ca efflux was measured on segments of frog semitendinosus muscle skinned by microdissection, with isometric force monitored continuously. The Ca\textsuperscript{2+}-insensitive component was potentiated by quercetin, a flavonoid thought to inhibit the sarcoplasmic reticulum (SR) Ca pump by stabilizing a phosphorylated intermediate. Quercetin increased the stimulated net \textsuperscript{45}Ca release in the absence of EGTA, as expected from inhibition of reaccumulation, but its effectiveness in EGTA indicated potentiation of unidirectional efflux as such. Quercetin also increased unstimulated (control) \textsuperscript{45}Ca efflux in EGTA, to a smaller extent; potentiation appeared to be a function of efflux, with stimulation above control loss increased ~2.6-fold. ATP removal before stimulation, which led to rigor force and increased stiffness, prevented all quercetin effects in EGTA. ATP removal by itself inhibited ionic stimulation of the Ca\textsuperscript{2+}-insensitive component, with little residual increase above the parallel control loss. Addition of the nonhydrolyzable ATP analogue AMP-PCP ([adenylyl-β,γ-methylene]diphosphate) (0.8 mM) after ATP removal gave similar results to ATP-free solution, which suggests that adenine nucleotide binding alone does not support stimulation by choline Cl. These results imply a fundamental role for ATP in the excitation of skinned fibers by imposed diffusion potentials; they also suggest that ATP regulates the SR Ca efflux channel, in a manner that could provide the positive feedback in Ca\textsuperscript{2+}-dependent Ca release.
INTRODUCTION

Ionic substitutions that produce an electrically negative diffusion potential are known to stimulate Ca release in skinned fibers from skeletal muscle, although the detailed mechanisms are not fully understood and indeed may not be the same under all conditions. In the preceding article (Stephenson, 1985b), it was shown that $^{45}$Ca efflux is stimulated vigorously when Cl replaces the relatively impermeant methanesulfonate (Mes) anion; the very large stimulation of release at constant [K] appears to include an osmotic component with low Ca$^{2+}$ dependence, while simultaneous substitution of the relatively impermeant choline cation for K, keeping the [K][Cl] product constant, stimulates a highly Ca$^{2+}$-dependent $^{45}$Ca release. The Ca$^{2+}$ dependence is not absolute, however; unlike previous results with KCl replacement of [K] propionate (Stephenson, 1978), a small, sustained Ca$^{2+}$-insensitive component is detectable when stimulation by the choline Cl diffusion potential is initiated in the presence of 5 mM EGTA. This component appears to grade the much larger Ca$^{2+}$-dependent $^{45}$Ca efflux that clearly is required to provide sufficient myoplasmic Ca for troponin saturation and account for the developed force, and it is likely to reflect intermediate steps in the excitation-contraction coupling process.

The Ca$^{2+}$-insensitive component could provide valuable information on the control of the stimulated Ca efflux channel of the sarcoplasmic reticulum (SR). The present studies on effects of the flavonoid quercetin and of ATP removal indicate that this release component is ATP dependent, and suggest that choline Cl stimulation is mediated by several steps that are influenced by nucleotide binding and/or phosphorylation. On quantitative grounds, the released $^{45}$Ca derives largely from the SR, but the immediate site of ion gradient stimulation could be T-tubules, the SR, or both (Stephenson, 1985b), which implies several possible targets of ATP binding or phosphorylation. A prime candidate is the final SR channel that mediates this stimulated efflux. It is of interest that Ca$^{2+}$-stimulated Ca release is mediated by a nucleotide-sensitive channel (e.g., Ogawa and Ebashi, 1976; Endo et al., 1981; Kakuta, 1984; Meissner, 1984). In the present studies, if the primary site of stimulation is the T-tubules or the T-SR junction, ATP or phosphorylation also could act on earlier steps in the coupling process, for example by regulation of ion channels (Kostyuk, 1984) or maintenance of a transmembrane potential by the Na/K pump (Lau et al., 1979).

Preliminary reports of this work have been presented (Stephenson, 1983, 1984).

METHODS

The general methods of tissue preparation, $^{45}$Ca loading and analysis, and washout protocols were as described in the preceding article. The rinsing procedure, after $^{45}$Ca loading and before pretreatment and stimulating or control washes, always included three preliminary rinses in 0.1 mM EGTA, K propionate solution (∼10 s each), followed by a 90-100-s rinse in 0.1 mM EGTA, KMes solution, and a 10-s rinse in 0.01 mM EGTA, KMes solution. The three preliminary rinses removed contaminant $^{45}$Ca and permitted the routine assay of the long ATP-free and control (5 mM ATP) KMes rinses in order to monitor $^{45}$Ca loss before the efflux washes during pretreatment and stimulation by choline Cl replacement of KMes in the ATP-free and control solutions (see Results). In the AMP-
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PCP ([adenyl-β,γ-methylene]diphosphate) studies (and concurrent ATP experiments), the efflux protocol was modified slightly: AMP-PCP was added in the pretreatment wash, which was prolonged from 10 to 15 s to promote equilibration, and the number of efflux washes was reduced from five to four, maintaining the same total wash time after stimulation. Residual $^{45}$Ca after efflux measurement was extracted routinely in normal (5 mM ATP) solution with 0.05% Triton X-100 and 5 mM CaEGTA, which permitted normalization of the rigor force developed in ATP-free solutions to the maximum active tension in the same fiber.

The solutions used for stimulation at constant [K][Cl] product were identical with those described in the preceding article, except for the following experimental modifications. Quercetin was added to choline Cl or control KMes washout solutions warmed to room temperature, by a 1:100 dilution of a stock solution of 3.47 mg/ml in 95% ethanol, giving a final concentration of 100 μM quercetin. The warming and dilution were made just long enough before use for thermal equilibration because quercetin solubility in aqueous solution is temperature dependent and limited even at 19°C. The wash solutions were monitored visually in the dissecting microscope during the fiber transfers through the wells, and early experiments with noticeable precipitation were rejected. Control experiments showed that the ethanol vehicle alone had no significant effect on efflux; the difference in total fraction $^{45}$Ca lost times 100 between paired segments from the same fiber with or without the same ethanol addition to normal solutions was $-0.08 \pm 0.66$ (6) in unstimulated (control) segments and $+0.53 \pm 0.82$ (6) in stimulated, pretreated segments. The ATP-free solutions contained 0.110 or 0.115 mM MgSO$_4$ in order to keep free Mg$^{2+}$ approximately constant at the level in the normal solutions with 5 mM ATP and 3 mM Mg (Stephenson, 1981b). Similarly, the ATP-free solutions with added 0.800 mM AMP-PCP contained 0.480 mM MgSO$_4$, which would give 0.118 mM Mg$^{2+}$ from binding to AMP-PCP with an assumed apparent stability constant of $7 \times 10^5$ M$^{-1}$ at pH 7.0; this $K_{app}$ is slightly larger than the $K_{app}$ calculated from the free Mg$^{2+}$ measured by an ion-selective electrode at pH 6.8 and at similar ionic strength, $5.7 \times 10^3$ (Meissnner, 1984), and is smaller than the (pH-dependent) $K_{app}$ determined at pH 7.4 and at similar ionic strength, $12.9 \times 10^3$ (Yount et al., 1971). The value assumed was biased toward a minimal $K_{app}$ (only 0.134 mM free Mg$^{2+}$ is calculated from the cited value at pH 6.8 with higher competitive [H$^+$]) in order to avoid an appreciable increase in free Mg$^{2+}$, which might obscure any ability of AMP-PCP to substitute for ATP (see Results). A possible reduction in free Mg$^{2+}$, on the other hand, should have either no effect or the opposite effect, since reduction to even 4 μM has little influence on unstimulated $^{45}$Ca efflux in the presence of EGTA and strongly stimulates $^{45}$Ca efflux in its absence (Stephenson, 1981b, c).

Quercetin, low-Ca$^{2+}$ ATP, and AMP-PCP were obtained from Sigma Chemical Co., St. Louis, MO. The AMP-PCP and a lot obtained for comparison from another supplier (Boehringer Mannheim, Indianapolis, IN) ran as identical single spots, distinct from ATP, on polyethyleneimino-cellulose chromatography in phosphate buffer. Other reagents were as described in the preceding paper (Stephenson, 1985b).

RESULTS

Quercetin Potentiation of Stimulation by Depolarizing Ion Gradients

The bioflavonoid quercetin inhibits Ca uptake and the Ca$^{2+}$- and Mg$^{2+}$-dependent ATPase activity of mammalian SR (Shoshan et al., 1980; Shoshan and MacLennan, 1981); similar actions on bullfrog SR have been described recently (Kurebayashi and Ogawa, 1985). The effect of quercetin on stimulation
by depolarizing ion gradients was examined initially in order to verify and exploit its ability to separate the net $^{45}$Ca flux into unidirectional components by blocking the primary influx pathway via the Ca pump. If this were the sole effect, stimulation of $^{45}$Ca release in the absence of EGTA would be increased (as inferred from force responses of chemically skinned rabbit fibers to other stimuli [Shoshan et al., 1980]), but stimulation in the presence of EGTA, where reaccumulation already is minimized by very low external [Ca$^{2+}$], should be unchanged. Fig. 1 shows the effects of 100 µM quercetin on the time course of stimulated $^{45}$Ca efflux when drug and ion substitution were applied simultaneously in the absence of EGTA; the addition of 5 mM EGTA was delayed to the final 10–15 s of washout. As would be expected from inhibition of any immediate reaccumulation of released $^{45}$Ca, quercetin increased the stimulated net $^{45}$Ca efflux in the absence of EGTA, by a factor of 1.5 in the initial wash.

However, quercetin also potentiated the stimulation of the Ca$^{2+}$-insensitive component of $^{45}$Ca efflux when drug and ion substitution were applied in the presence of 5 mM EGTA, as shown in the upper part of Fig. 2. The unstimulated (control) efflux in 5 mM EGTA, shown in the lower part of Fig. 2, was increased to a smaller extent. This direct effect of quercetin on efflux could not be
differentiated in the original force measurements of Shoshan et al. (1980), but stimulation in EGTA under various conditions was later reported for SR vesicles (Kirino and Shimizu, 1982; Kim et al., 1983).

The extent of the quercetin effect and the differences between conditions are summarized in Fig. 3 by the cumulative $^{45}$Ca loss during the 1-min washout period. The absolute increase in release was largest with delayed EGTA addition, but the relative increase was largest in the pretreated, stimulated response. The

![Graph](image)

**Figure 2.** The effect of quercetin on stimulated (top) and control (bottom) $^{45}$Ca efflux to the bath in the presence of 5 mM EGTA. Efflux time during pretreatment with 5 mM EGTA is shown as a negative value relative to the time of choline Cl stimulation or identical protocol without choline Cl replacement of KMes. Dashed curves show stimulated and control efflux without quercetin; solid curves show efflux under the same conditions with 100 µM quercetin added to the solutions at time zero (with the stimulus).

mean stimulation above the appropriate control in this response was increased by a factor of 2.3 or 2.9, compared with a factor of 1.4 or 1.7 in the control response in EGTA (Figs. 3 and 5), which implies that quercetin potentiated the stimulated efflux preferentially.

A detailed analysis of the dependence of potentiation of efflux on the stimulated efflux itself would require more doubly paired data (stimulated and control, with and without quercetin, on segments from the same fiber) than were
available. However, a positive relationship between the amount of potentiation and the unpotentiated efflux was supported by a simple analysis of paired data on segments from the same fiber, in the presence and absence of quercetin, under either unstimulated (control) or stimulated conditions in EGTA. The total fractional $^{45}$Ca loss, $F_T$, was assumed to consist of a constant quercetin-insensitive component, $F_0$, and a variable quercetin-sensitive component, $F_p$. If $F_p$ in quercetin is proportional to $F_p$ in its absence by a potentiation factor $a$, these relations reduce to

$$F_T - F_p = F_T^a - F_p^a = F_T^a - aF_p$$

and

$$F_T^a - F_T = F_T(a - 1) + F_o(1 - a).$$
In Fig. 4, the increment in $^{45}$Ca release produced by quercetin is plotted against the release in its absence under the same conditions for each pair of segments from the same fiber. The data could be fit by a linear regression curve (15 pairs, $r = 0.623$, $P < 0.02$) with a slope corresponding to a potentiation factor of 2.1, and an intercept corresponding to a quercetin-insensitive component of 2.1% cumulative release. This potentiation factor is smaller than the mean ratios of 2.3 and 2.9 for the stimulated release above control (see above), which give the potentiation factor for the stimulated component directly, and indeed most unstimulated points fell below the regression line and most stimulated points fell above it in the paired data. The relationship is likely to be more complex than implied by this simplified treatment. It should be emphasized that the approximate linearity of this plot reflects a potentiation ratio for a single concentration of quercetin, rather than the order of Ca efflux dependence itself (which is within the cumulative efflux terms).

ATP Dependence of Quercetin Potentiation

Quercetin inhibition of the Ca pump of the SR is thought to involve stabilization of a phosphorylated intermediate of the ATPase (Shoshan and MacLennan, 1981; Kurebayashi and Ogawa, 1985), as proposed for its effect on other ion transport systems (see Kuriki and Racker, 1976). If quercetin potentiation of the Ca$^{++}$-insensitive $^{45}$Ca efflux also depended on a phosphorylation mechanism,
ATP should be required. This hypothesis was tested in a series of experiments in which fibers were exposed to ATP-free solution for 110–120 s before stimulation or control conditions and during efflux measurement.

Exogenous ATP was omitted from the solutions, with no additional agent to remove endogenously formed ATP. The development of rigor force and "stiffness" during continuous isometric force measurement was used to assess the fall in [ATP] within the fiber. A typical tension record following exposure to ATP-free solution is shown in Fig. 5. After a definite lag, rigor tension developed rather slowly to a steady plateau level maintained subsequently. This rigor tension was normalized to maximal active force in the same fiber, determined after efflux during extraction of residual \(^{45}\)Ca into Triton X-100 solution containing 5 mM ATP and 5 mM CaEGTA. In addition, force artifacts were produced during the lag and plateau when the fiber was moved across the solution-air interface to promote mixing. These artifacts were noticeably larger after force development in ATP-free solution than in the preceding ATP solutions, reflecting increased stiffness (change in force/change in length) in the rigor state. The mean fractional rigor force and its time course and the relative artifact size are summarized in Table 1. In two separate series, the mean fractional rigor force was 0.22 or 0.24 maximal force, and the mean relative "stiffness" was 1.35 or 1.46. These observations indicated that myofilament space [ATP] had fallen to very low levels at least 40 s (60 s in the second series) before stimulation.

The effects of ATP removal on quercetin potentiation of the Ca\(^{2+}\)-insensitive \(^{45}\)Ca release are summarized in Fig. 6. The small potentiation of control (unstimulated) efflux was prevented by ATP removal; the mean release in quercetin, slightly but not significantly reduced, did not exceed the appropriate control release in ATP-free solution only (Fig. 8), which was slightly larger than in ATP (see below), and the difference between paired segments from the same fiber (\(n = 5\)) was not significant in ATP-free solution. The larger potentiation by
quercetin of the stimulated Ca\textsuperscript{2+}-insensitive release was abolished in the absence of exogenous ATP (Fig. 6), and release in the presence of quercetin did not exceed that in its absence (compare Fig. 8). These results showed that quercetin potentiation of Ca\textsuperscript{2+}-insensitive \textsuperscript{45}Ca efflux was completely ATP dependent under both control and stimulatory conditions, and were consistent with a mechanism of quercetin action involving binding or phosphorylation, as suggested by the studies on the SR Ca\textsuperscript{2+}- and Mg\textsuperscript{2+}-dependent ATPase.

**ATP Dependence of the Stimulated Ca\textsuperscript{2+}-insensitive Efflux**

The effects of ATP removal on quercetin potentiation suggested that efflux stimulation per se required ATP, because the mean stimulated \textsuperscript{45}Ca release in quercetin solution without ATP was smaller than in normal stimulating solution without quercetin (Fig. 6). This possibility was examined directly in matched experiments comparing stimulated and control loss in the presence or absence of ATP. The time course of Ca\textsuperscript{2+}-insensitive \textsuperscript{45}Ca efflux under these conditions is shown in Fig. 7 and the cumulative \textsuperscript{45}Ca release during the washout period is summarized in Fig. 8. The substantial efflux increment above control produced by choline Cl stimulation in the presence of 5 mM ATP was nearly abolished in the absence of ATP, with only a small increase detectable later in washout (Fig. 7). The cumulative effect of the small increase was a barely significant stimulation above control loss in ATP-free solution, compared with the large stimulation of \textsuperscript{45}Ca release in normal ATP in these fibers (Fig. 8). Mean control loss was very slightly but not significantly larger during the matching efflux period in ATP-free solution.

During the long conditioning period before stimulation, \textsuperscript{45}Ca loss into the ATP-free KMes rinse solutions was significantly larger than in normal ATP, as

**TABLE I**

| Rigor Effects After ATP Removal |
|--------------------------------|
|                              | Series I       | Series II      |
| Steady force (P/P_o)\textsuperscript{*} | 0.22±0.01 (16) | 0.24±0.02 (14) |
| Delay to force rise (s)        | 45.2±2.6 (18)  | 39.0±1.4 (14)  |
| Rise time to P (s)             | 15.6±0.06 (18) | 14.8±0.8 (14)  |
| Relative stiffness (A'/A)\textsuperscript{2} | 1.35±0.06 (18) | (a) 1.46±0.08 (14) |
|                               | (b) 1.55±0.06 (14) |

Values shown are means ± SEM (n) for the experimental group with ATP removal alone (series I) and a later experimental group with AMP-PCP added to ATP-free solutions after rigor development (series II). See text for details.

\textsuperscript{*} Ratio of the steady tension developed in rigor to the maximum tension later developed by the same fiber segment in the presence of 5 mM ATP, 5 mM CaEGTA, 0.05% Triton X-100 extraction solution.

\textsuperscript{2} The ratio of tension artifacts engendered by fiber movement through the aqueous-air interface, after and before rigor force development, was used to indicate rigor stiffness relative to the relaxed state. In series II, the upper value (a) refers to the ATP-free (rinse) solution and the lower value (b) refers to the ATP-free solution with 800 \textmu M AMP-PCP; the ratio of ATP-free to AMP-PCP artifacts (in the same fiber) was 0.96 ± 0.06 (14).
shown in Table IIA. The increase raised the possibility that inhibition of stimulation after ATP removal was due to a lower initial SR $^{45}$Ca content. If this were the case, stimulated $^{45}$Ca release would have been smaller in individual fibers that had lost more $^{45}$Ca in ATP-free solution preceding stimulation. However, as shown in Table IIB, release in ATP-free solution after and before stimulation had a good linear correlation with a large, positive, rather than negative, slope. This evaluation ruled out the explanation that lower SR $^{45}$Ca at the time of stimulation was causative, and suggested instead that the ATP-removal procedure promoted some transient efflux process that varied between fibers and had a residual effect early in the post-stimulus efflux period (also see Fig. 7).

This effective increase in background flux in ATP-free solution obscured any decrease in the component of the unstimulated release that is potentiated by quercetin, which might be small. However, the clear inhibition of choline Cl stimulation by ATP removal could be attributed to a specific action on a step or steps in the stimulation pathway.

**Figure 6.** The effect of ATP removal on quercetin potentiation of $^{45}$Ca release in EGTA. The bars indicate the percent $^{45}$Ca lost (± SEM) under control conditions (first set) or stimulated pretreated conditions (second set, hatched); the presence of quercetin is marked Q, and the absence of ATP is indicated by stippling (third bar in each set). Release in the absence of ATP without quercetin is shown in Fig. 7. Note that the ordinate is broken between 12 and 18% to accommodate the very large quercetin-potentiated stimulated release in the presence of ATP in these fibers.
Addition of AMP-PCP to ATP-free Solution

The dependence of the stimulated Ca\(^{2+}\)-insensitive \(^{45}\)Ca efflux on ATP could reflect a requirement for phosphorylation or only nucleotide binding. In order to differentiate these alternatives, the ATP-removal experiments were repeated with addition of the nonhydrolyzable analogue AMP-PCP before stimulation. At low concentrations, this analogue stimulates Ca release from rabbit SR vesicles by a mechanism thought to be related to "Ca\(^{2+}\)-induced Ca release" (Ogawa and Ebashi, 1976), and promotes \(^{45}\)Ca efflux from Ca\(^{2+}\)-sensitive release fractions.

![Figure 7](image_url)

**Figure 7.** The effect of ATP removal alone on choline Cl stimulation of \(^{45}\)Ca efflux in EGTA. The square symbols and solid curves correspond to unstimulated (control) efflux (± SEM) in 5 mM EGTA and the triangles and dashed curves to stimulated (pretreated) efflux. The upper part of the figure shows stimulation in the presence of ATP, the lower part stimulation in its absence.

(Meissner, 1984); it supports Ca\(^{2+}\)-induced and caffeine-stimulated Ca release in skinned Xenopus fibers (Endo et al., 1981; Kukuta, 1984).

The effect of 0.8 mM AMP-PCP on choline Cl stimulation of the Ca\(^{2+}\)-insensitive \(^{45}\)Ca release is summarized in Fig. 9. Since the control loss appeared high in the presence of the analogue, stimulation was compared with additional experiments in ATP run concurrently. The mean stimulation above the control release in ATP-free AMP-PCP solution was small and similar to that in ATP-free solution alone (Fig. 8), but not significant; however, the difference between paired segments from the same fiber was significant (\(P < 0.05\)). The correspond-
Figure 8. The effect of ATP removal on stimulation of the cumulative $^{45}\text{Ca}$ release in EGTA. The bars depict the percent $^{45}\text{Ca}$ lost ($\pm$ SEM) under control or stimulated pretreated conditions; hatched bars indicate stimulation, and stippled bars indicate the absence of ATP. The braces show significant differences between means.

The unstimulated (control) release in ATP-free AMP-PCP solution was significantly larger than in ATP, unlike the insignificant increase in ATP-free solution alone; however, this increase could not be attributed specifically to AMP-PCP because the effect of ATP removal per se, before AMP-PCP addition, was appreciably larger in this group of fibers. The additional $^{45}\text{Ca}$ lost in the ATP-

| Table II A |
| Effects of ATP Removal on Prestimulus $^{45}\text{Ca}$ Loss |

| Conditions | Percent lost in ATP-removal rinse (mean ± SEM) | Difference | Percent lost in pretreatment wash (mean ± SEM) | Difference |
|-----------|-----------------------------------------------|------------|-----------------------------------------------|------------|
| Series I  | Without ATP 9.06±0.61 (17) | +3.88* | 3.64±0.21 (18) | +1.77* |
|           | With ATP 5.18±0.33 (16)    |           | 1.87±0.10 (16) |           |
| Series II | Without ATP 15.72±1.36 (14) | +8.50* | 6.93±0.70 (14) | +3.91* |
|           | With ATP 7.22±1.58 (9)     |           | 3.02±0.35 (9)  |           |

Values shown are means ± SEM ($n$) pooled for pretreated (stimulated) and control fibers, which have the same prestimulus protocols. The ATP-removal rinse and corresponding ATP-containing rinse were ~90 s in duration. The pretreatment washes (5 mM EGTA) were 10 s in duration in series I and 15 s in duration in series II, where this wash had added AMP-PCP in the ATP-free solution. Series II results for the ATP-removal rinse also are shown separately because of the significantly larger loss in identical ATP-free solution in this group of fibers. *$P < 0.01$ (Student's t test).
TABLE II.B

Positive Correlation Between \(^{45}\)Ca Loss After Stimulation and Prior \(^{45}\)Ca Loss During ATP Removal*

| Conditions   | Percent lost in ATP-removal rinse | Percent lost after stimulation | Slope  | r   | P     |
|--------------|----------------------------------|-------------------------------|--------|-----|-------|
| Series I     |                                  |                               |        |     |       |
| Without ATP  | 10.12±1.19 (8)                   | 8.73±0.62 (8)                 | +0.45  | 0.865 | <0.01 |
| With ATP     | 5.16±0.60 (7)                    | 12.32±0.81 (7)                | +0.14  | 0.107 | NS    |
| Series II    |                                  |                               |        |     |       |
| Without ATP  | 15.99±2.32 (7)                   | 12.38±1.43 (7)                | +0.58  | 0.948 | <0.01 |
| With ATP     | 7.83±0.95 (5)                    | 11.79±0.98 (5)                | +0.31  | 0.295 | NS    |

* Values shown are means ± SEM (n) for pretreated (stimulated) fibers only, and the linear regressions of total \(^{45}\)Ca loss after stimulation on the loss in the same fiber during the ATP-removal rinse (see Table IIA). The positive slopes indicate greater, not lesser, loss after stimulation in individual fibers with greater prestimulus loss during ATP removal (and slightly lower \(^{45}\)Ca content at the time of stimulation), although stimulation (above control) was strongly inhibited after ATP removal.

removal rinse (above that in ATP) in this second series of experiments was 2.2 times that in the first series, the same ratio as the additional \(^{45}\)Ca lost in the pretreatment wash, which contained AMP-PCP in the second series (Table IIA). As in the case of ATP removal alone, the effective increase in background

**FIGURE 9.** The effect of AMP-PCP in ATP-free solution on stimulation of the cumulative \(^{45}\)Ca release in EGTA. The bars depict the percent \(^{45}\)Ca lost (± SEM) under control or stimulated conditions in the presence of ATP (left set) or 800 \(\mu\)M AMP-PCP, added to ATP-free solution ~15 s before stimulation (right set, stippled). The small bar at the right shows the difference between stimulated and control release in AMP-PCP in paired segments from the same fiber. Braces indicate differences between means. See text for details.
prevented determination of the ability of AMP-PCP to support the quercetin-sensitive component of control efflux.

Stimulation in the presence of ATP (Fig. 9) was highly significant even with a smaller number of concurrent experiments, and within the range and variability of the earlier series (see Figs. 3 and 8). Therefore, the similarity of the results in AMP-PCP to those in ATP-free solution alone suggested that nucleotide binding could not support the stimulation of the Ca\(^{2+}\)-insensitive efflux component by choline Cl. This would imply a requirement for phosphorylation at a site crucial in the excitation-contraction coupling process in this system.

**DISCUSSION**

*Effect of Quercetin on \(^{45}\)Ca Movement*

In the absence of EGTA, the increase in stimulated \(^{45}\)Ca efflux and cumulative release produced by 100 µM quercetin (Figs. 1 and 3) could be attributed to quercetin inhibition of backflux (influx) through the active Ca transport system, as assumed by Shoshan et al. (1980). However, this mechanism cannot account for the substantial potentiation of stimulation and significant increase in control loss at the very low external [Ca\(^{2+}\)] in 5 mM EGTA (Figs. 2, 3, and 6). Thus, quercetin must increase unidirectional efflux, and in particular potentiate its stimulation by choline Cl. Recent studies on SR vesicles also suggest incomplete influx inhibition and direct effects on efflux (Kurino and Shimizu, 1982; Kim et al., 1983; Watras et al., 1983; Kurebayashi and Ogawa, 1985). The evidence for direct efflux potentiation makes it necessary to re-evaluate inferences based on the assumption that quercetin potentiates net release only by influx inhibition, for example that pump carriers uncoupled from ATPase activity could not function in Ca release (Shoshan et al., 1980).

The potentiation of Ca\(^{2+}\)-insensitive efflux by quercetin does not reflect a nonspecific increase in Ca permeability, in agreement with recent studies on bullfrog SR vesicles (Kurebayashi and Ogawa, 1985). Not only was ATP required, but the quercetin effect was not simply additive; the increment in stimulated \(^{45}\)Ca release, above control loss, was substantially larger than the increment in control loss (Figs. 3 and 6). The preferential increase suggests that the rate or extent of quercetin potentiation depends on stimulation, i.e., on Ca movement through the relevant efflux channel. This idea is supported by a positive linear correlation between the increment in cumulative release produced by quercetin and the release in the absence of quercetin in paired fibers under control or stimulated conditions (in 5 mM EGTA) (Fig. 4). This difference treatment of the data at a single quercetin level would not directly reflect the order with respect to efflux, but rather an increased constant in a regulatory mechanism. However, the mean potentiation ratio for stimulated release in EGTA, above control loss, is larger than the potentiation factor given by the linear regression slope and the factor is probably not a simple ratio of constants. Such an effect would be exaggerated at the much higher efflux rates in the absence of EGTA. Quercetin did greatly increase the large initial stimulated \(^{45}\)Ca efflux in the absence of EGTA (Fig. 1), compared with the small correspond-
ing initial efflux in EGTA (Fig. 2), but the contribution of backflux inhibition could not be evaluated. While the increment in $^{45}$Ca release under control conditions in EGTA is relatively small, this potentiation has the important implication that quercetin acts on a final SR Ca efflux channel rather than on ionic stimulation per se. Recent studies on SR vesicles dissociated from T-tubular vesicles support this conclusion (Ikemoto et al., 1984).

**Implications of Quercetin Potentiation**

Quercetin potentiation of $^{45}$Ca efflux in EGTA seems entirely ATP dependent. No significant increase in either control or stimulated $^{45}$Ca release was observed after ATP removal (Figs. 6 and 8), unlike quercetin stimulation in rabbit SR vesicles under different loading and release conditions (Kim et al., 1983). Quercetin stimulation of bullfrog SR vesicles also is sensitive to ATP concentration (Kurebayashi and Ogawa, 1985). Since quercetin inhibition of isolated SR ATPase and Ca uptake rates is associated with stabilization of a phosphorylated intermediate of the ATPase (Shoshan and MacLennan, 1981; Kurebayashi and Ogawa, 1985), as is quercetin interaction with Na/K-ATPase (Kuriki and Racker, 1976), the ATP requirement for its action on efflux suggests that the efflux pathway is affected by a similar type of mechanism.

The Ca pump moiety itself can transport Ca in modes uncoupled from net ATP hydrolysis or synthesis (e.g., Chiesi and Wen, 1983; Gerdes and Moller, 1983), and is in fact the only completely identified SR pathway for Ca efflux. Alternative transport modes of the Ca pump could provide direct and energetically efficient dual control of net Ca release (Stephenson, 1981b), and this possibility should not be ruled out. Among arguments against such a mechanism, however, the uncoupled pathway in SR vesicles has been observed under highly specialized conditions, and SR Ca release channels do not appear to co-purify with pump fractions, although Meissner (1984) has noted the interesting possibility of modified pumps.

In addition, the specificity of quercetin interactions is uncertain. It acts at least on other transport ATPase proteins, also with stabilization of a phosphorylated intermediate (see Kuriki and Racker, 1976), so modification of a target protein other than the Ca$^{2+}$- and Mg$^{2+}$-dependent ATPase might underly the present findings. The T-tubular Na/K-ATPase is not a likely target; quercetin inhibition of the Na/K-ATPase system is slow, reduced at high protein concentration, and apparently unaccompanied by inhibition of coupled ion translocation (Kuriki and Racker, 1976), while the quercetin potentiation of Ca release is rapid. The SR membrane contains tightly bound calmodulin (Chiesi and Carafoli, 1982), and several recently isolated SR proteins that undergo phosphorylation dependent on calmodulin and/or Ca$^{2+}$ have been considered as possible regulators of Ca release (Campbell and MacLennan, 1982; Chiesi and Carafoli, 1983); depending on the chemical basis of quercetin action, these are possible targets for the potentiation of efflux.

With the plausible assumption that quercetin stabilizes or promotes phosphorylation or nucleotide binding, the quercetin potentiation and its ATP requirement provide evidence that an SR channel mediating both unstimulated and stimulated Ca efflux is regulated by phosphorylation (or binding). Since the
present observations were made in the presence of EGTA, the dependence of the amount of potentiation on Ca efflux itself would be consistent with a Ca²⁺-regulated phosphorylation (or binding) site closely associated with the Ca efflux channel and partially sequestered from rapid buffering by EGTA. Subsequent promotion of Ca efflux by phosphorylation (or binding) would be consistent with the known dependence of Ca²⁺-induced Ca release and Ca²⁺-stimulated SR efflux channels on nucleotide binding (see Kakuta, 1984; Meissner, 1984). It is important to note that an interaction between promotion of phosphorylation (or binding) by local Ca and promotion of Ca efflux by phosphorylation (or binding) forms a reciprocal relationship that could provide the positive feedback inherent in Ca²⁺-dependent or -stimulated Ca release. If this hypothesis is correct, the nature of the potentiation observed in the Ca²⁺-insensitive efflux can explain the large amount of positive feedback with choline Cl stimulation in the absence of EGTA (Fig. 1) and the grading of the stimulated Ca²⁺-dependent efflux by the Ca²⁺-insensitive component, described in the preceding article (Stephenson, 1985b).

**ATP Dependence of Stimulated Efflux**

The inhibition of choline Cl stimulation by ATP removal alone is consistent with the interpretation that phosphorylation or binding potentiates the SR channel mediating the stimulated efflux, although this may not be the sole mechanism (see discussion below). In ATP-free solution, stimulation (without quercetin) fell approximately threefold, leaving only a small component resistant to both ATP removal and EGTA (Figs. 7 and 8). The basis of the barely detectable residual stimulation is unclear; possibilities are (a) a small endogenous ATP concentration, (b) a small residual osmotic component, and (c) a separate ATP-insensitive SR membrane effect, such as altered surface charge (Caswell and Brandt, 1981).

Detection of a small decrease in control release that would mirror the small quercetin potentiation in ATP was compromised by a transient increase originating during ATP removal. ⁴⁶Ca loss increased significantly during the ATP-removal rinse (0.1 mM EGTA) and pretreatment efflux wash (Table II A and Fig. 7); the basis of this increase was not investigated, but it might result from T-tubule depolarization (see below). With the larger effect in the later studies on AMP-PCP (Table II A), control loss remained significantly higher during the post-stimulus period (Fig. 9), which probably reflects elution at the increased EGTA of previously released ⁴⁶Ca. In view of the large transient increase during ATP removal, the absence of a demonstrable decrease in control loss during the post-stimulus time period in ATP-free solution (Fig. 8) does not rule out a small ATP-dependent component under normal conditions. It is plausible to postulate that ATP removal, like quercetin potentiation, acts on a final efflux pathway that mediates unstimulated and stimulated release.

This interpretation is consistent with the nucleotide dependence of Ca²⁺-stimulated SR channels and Ca²⁺-induced release in skinned fibers, noted above. An increase in control release with AMP-PCP addition would have supported this view, since AMP-PCP has some effect on Ca²⁺-sensitive release fractions of rabbit SR vesicles even in 1 mM EGTA (Meissner, 1984). No clear effect of
AMP-PCP as such on the Ca\textsuperscript{2+}-insensitive control efflux in ATP-free solution with 5 mM EGTA could be identified (see Results), but this can be attributed not only to the higher [EGTA], but to the presumably small contribution of a quercetin-sensitive component in the increased control flux in ATP-free solution as well as the large variability introduced by ATP removal, as discussed above.

The quercetin-sensitive component of the stimulated release is much larger, which should improve detection of AMP-PCP effects on the SR Ca efflux channel. However, 800 µM AMP-PCP is clearly unable to support choline Cl stimulation of the Ca\textsuperscript{2+}-insensitive component; the residual increase in release above the appropriate control loss was small and indistinguishable from that in ATP-free solution alone (Figs. 8 and 9). This ineffectiveness suggests that nucleotide binding may not substitute for phosphorylation in all the steps that mediate choline Cl stimulation. In view of the known effectiveness of AMP-PCP on the Ca\textsuperscript{2+}-sensitive SR Ca channel, there might be an earlier step in this stimulation pathway that requires phosphorylation.

Sites of ATP Dependence

ATP could influence the Ca\textsuperscript{2+}-insensitive stimulation at the final SR Ca efflux channel, at earlier steps in the stimulation pathway, or at both. Evidence discussed above supports direct regulation of the SR channel that mediates stimulated Ca efflux. Quercetin potentiation of unstimulated as well as stimulated efflux in EGTA is entirely ATP dependent. The increased potentiation with higher Ca efflux suggests an interaction between channel Ca\textsuperscript{2+} and nucleotide effects that has the elements of a positive feedback system and relates this channel to the Ca\textsuperscript{2+}-stimulated Ca efflux channel of the SR, which clearly has been shown to be nucleotide dependent (citations above). Thus, the present results at very low external Ca\textsuperscript{2+} also suggest that separation of Ca\textsuperscript{2+}-sensitive and -insensitive pathways can be quantitative and operational; the channels may be closely associated or modes of the same entity.

In addition, earlier steps such as the stimulation itself by imposed diffusion potentials could be inhibited after ATP removal. Inhibition of direct SR stimulation by diffusion potentials has no obvious a priori basis and seems unlikely. The SR of intact fibers has no resting transmembrane gradients for elemental Na, K, and Cl (Somlyo et al., 1977) that would require ATP-dependent transport; in skinned fibers, neither the permeant anion distribution before stimulation nor the permeant cation distribution during choline Cl stimulation suggests appreciable monovalent ion gradients across the SR (Stephenson, 1981d, 1985a, manuscript in preparation). On the other hand, there is considerable indirect evidence (see Stephenson, 1981a, 1985b) that negative diffusion potentials can stimulate the sealed polarized T-tubule system of skinned fibers, and recent studies on isolated triad preparations support this view (Ikemoto et al., 1984). T-tubule polarization gives a plausible basis for inhibition by Na\textsubscript{2}ATP removal, after sufficient dissipation of the cation gradients dependent on the tubular Na/K pump (Lau et al., 1979). The rate of dissipation is difficult to estimate precisely, but the fibers were pre-exposed to Na\textsubscript{2}ATP-free solution for 50–60 s after rigor development in order to minimize ATP levels at the SR. If one assumes a K flux
of ~1 pmol/cm²·s (Hodgkin and Horowicz, 1959; Eisenberg and Gage, 1969), and a T-tubular surface/volume ratio estimated for intact fibers of 69 μm⁻¹ (Mobley and Eisenberg, 1975), the unidirectional efflux (with zero backflux) initially could increase luminal [K⁺] by 0.7 mM/s. While this upper limit neglects backflux and the possibility of a smaller surface/volume ratio in sealed tubules, sufficient prior depolarization seems possible in 50–60 s to inhibit excitation by the choline Cl stimulus. The increased release during ATP removal (Table II A) might be due to such prior depolarization. The ineffectiveness of AMP-PCP is consistent with the requirement for nucleotide hydrolysis to energize Na/K transport that is implied by this mechanism of inhibition.

The combined data are consistent with the hypothesis that ATP influences the stimulated Ca²⁺-insensitive efflux both indirectly, through the steady state energy requirement for T-tubular polarization and excitability, and directly, through regulation of the SR Ca efflux channel. The indirect influence supports growing evidence that ionic diffusion potentials can stimulate Ca release in skinned fibers by a mechanism initiated at the T-tubules, thus requiring junctional transmission and activating the same SR channel as in intact fibers. The direct influence relates the channel that mediates this stimulated efflux to the Ca²⁺-stimulated SR channel, and furthermore suggests a positive feedback mechanism through which the small Ca²⁺-insensitive stimulated efflux can generate and control the large Ca²⁺-dependent efflux required for contractile activation.

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