Mechanisms of $P_i$ regulation of the skeletal muscle SR $Ca^{2+}$ release channel

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Balog, Edward M., Bradley R. Fruen, Patricia K. Kane, and Charles F. Louis. Mechanisms of $P_i$, regulation of the skeletal muscle SR $Ca^{2+}$ release channel. Am. J. Physiol. Cell Physiol. 278: C601–C611, 2000.—Inorganic phosphate ($P_i$) accumulates in the fibers of actively working muscle where it acts at various sites to modulate contraction. To characterize the role of $P_i$ as a regulator of the sarcoplasmic reticulum (SR) calcium ($Ca^{2+}$) release channel, we examined the action of $P_i$ on purified SR $Ca^{2+}$ release channels, isolated SR vesicles, and skinned skeletal muscle fibers. In single channel studies, addition of $P_i$ to the cis chamber increased single channel open probability ($P_o$: 0.079 ± 0.020 in 0 $P_i$, 0.157 ± 0.034 in 20 mM $P_i$) by decreasing mean channel closed time; mean channel open times were unaffected. In contrast, the ATP analog, $\beta$-$\gamma$-methyleneadenosine 5'-triphosphate (AMP-PCP), enhanced $P_o$ by increasing single channel open time and decreasing channel closed time. $P_i$ stimulation of $[3H]$ryanodine binding by SR vesicles was similar at all concentrations of AMP-PCP, suggesting $P_i$ and adenine nucleotides act via independent sites. In skinned muscle fibers, 40 mM $P_i$ enhanced $Ca^{2+}$-induced $Ca^{2+}$ release, suggesting an in situ stimulation of the release channel by high concentrations of $P_i$. Our results support the hypothesis that $P_i$ may be an important endogenous modulator of the skeletal muscle SR $Ca^{2+}$ release channel under fatiguing conditions in vivo, acting via a mechanism distinct from adenine nucleotides.

Skeletal muscle contraction is triggered by the release of calcium ($Ca^{2+}$) from the sarcoplasmic reticulum (SR) that is mediated by the high conductance $Ca^{2+}$ release channel/ryanodine receptor (RyR1) complex. The SR $Ca^{2+}$ release channel is regulated in a complex fashion by numerous endogenous effectors, many of which are metabolites (19). It is well established that physiological concentrations of ATP enhance, whereas $Mg^{2+}$ and low pH inhibit, RyR1 channel opening (19). In a previous report, potential effects of $P_i$ on RyR1 channels were also examined (11). This work demonstrated that physiological concentration of $P_i$ stimulated both $[3H]$ryanodine binding by and $45Ca^{2+}$ release from SR vesicles, as well as RyR1 single channel open probability ($P_o$), and suggested that $P_i$ may be an important physiological modulator of the skeletal muscle SR $Ca^{2+}$ release channel in vivo. Although $P_i$ can enhance the $Ca^{2+}$-induced opening of the RyR1 channel, the mechanism by which this stimulation occurs is unclear. Xiang and Kentish (39) reported that, in cardiac trabecula, both ADP and $P_i$ stimulated $Ca^{2+}$-induced $Ca^{2+}$ release (CICR), and, when present in combination, the stimulatory effect was more than additive. Consequently, these authors proposed that both ADP and $P_i$ enhance cardiac RyR (RyR2) channel opening via the adenine nucleotide binding site, and, when present in concert, simultaneously bind to the site and mimic the potent stimulation by ATP.

In contrast to these stimulatory effects of $P_i$, Fryer and co-workers (12, 24) suggested from their work on pooled muscle fibers that $P_i$ could reduce SR $Ca^{2+}$ release by entering the SR and complexing with $Ca^{2+}$ to form a calcium phosphate precipitate. The formation of such a precipitate within the lumen of the SR would diminish release by decreasing the available free $Ca^{2+}$. However, an alternative explanation would be that $P_i$ inhibits RyR1 channel opening via interaction with the luminal side of the channel. Here, we test this hypothesis by examining the single channel properties of the RyR1 in planar lipid bilayers.

Finally, the opening of the RyR1 channel is modulated by a number of associated proteins, including triadin (13), FKBP12 (1), and calsequestrin (15). Purification of SR membrane fractions or RyR1 channels results, to varying degrees, in the loss of these proteins, and this has been linked to altered channel gating and may change the sensitivity to physiological regulators (1, 9, 34). Therefore, it is important to determine the effects of putative physiological modulators on regula-
Condition of the Ca$^{2+}$ release channel in a more intact preparation. Although Stienen et al. (32), using permeabilized frog skeletal muscle fibers, found larger caffeine contracture in the presence of 15 mM P$_i$, than in the absence of P$_i$, the difference was attributed to a slowed rate of Ca$^{2+}$ sequestration by the SR Ca$^{2+}$ ATPase rather than any direct action on the RyR1. Therefore, it remains to be demonstrated that P$_i$ enhances SR Ca$^{2+}$ release channel opening in a more intact mammalian skeletal muscle preparation.

We have now further characterized the postulated P$_i$ regulatory site(s) in the RyR1, and in particular, tested the hypothesis of Xiang and Kentish (39) that P$_i$ modulates RyR opening by P$_i$ in the adenine nucleotide binding site. In addition, because the concentrations of other metabolites and ions also change when intracellular P$_i$ levels rise, we examined the stimulation of RyR1 channel opening by P$_i$ in the presence of a number of other important muscle metabolites. The results reported here, from experiments using single RyR1 channels incorporated into planar lipid bilayers, [H]ryanodine binding to SR vesicles, saponin-permeabilized skeletal muscle fibers, and mechanically peeled muscle fibers, support the hypothesis that P$_i$ is an important endogenous modulator of the RyR1 channel that acts independently of the RyR1 adenine nucleotide binding site.

METHODS

[H]ryanodine Binding

Isolation of SR vesicles. Skeletal muscle SR vesicles were prepared from porcine longissimus dorsi muscle as previously described (11). Briefly, muscle was homogenized in 0.1 M NaCl, 5 mM Tris maleate buffer (pH 6.8), and the membranes that pelleted between 2,600 and 10,000 g were extracted in 0.6 M KCl, 20 mM Tris (pH 6.8), centrifuged at 100,000 g, and then resuspended in 0.3 M sucrose, 0.1 M KCl, and 5 mM Tris (pH 6.8). All isolation buffers contained a mixture of protease inhibitors (11). SR vesicles were flash-frozen in liquid nitrogen and stored at −70°C.

[H]ryanodine binding. SR vesicles (0.2 mg/ml) were incubated at 36°C in media containing 150 mM potassium gluconate, 20 mM PIPES, pH 7.1, 100 nM [H]ryanodine, and a calcium acetate-EGTA buffer set to give a free Ca$^{2+}$ concentration ([Ca$^{2+}$]) of 10 µM (2). After 90 min, SR vesicles were collected on Whatman GF/B filters and washed with 8 ml ice-cold 100 mM potassium gluconate. Estimates of maximum [H]ryanodine binding capacity of each SR vesicle preparation were determined in media containing 150 mM potassium gluconate, 10 mM ATP, and 10 µM Ca$^{2+}$. Nonspecific binding was measured in the presence of 20 µM nonradioactive ryanodine.

Single Channel Studies

SR Ca$^{2+}$ release channel protein was purified from SR membrane vesicles as previously described (25). Muller-Rudin planar lipid bilayers were formed by painting a lipid mixture (phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine in a 5:3:2 ratio by wt, 50 mg/ml dissolved in n-decane) across a 250-µm aperture in a Delrin cup. The cis chamber was connected to the head stage input of an Axoclamp 200B patch-clamp amplifier (Axon Instruments, Foster City, CA). The trans chamber was held at virtual ground.

Data were filtered at 2 kHz with an eight-pole Bessel filter, recorded at 4.5 kHz, and stored on a Jazz disk drive (Iomega, Roy, Utah). Recording solution consisted of symmetrical 200 mM KCl, 20 mM MOPS, pH 7.2, and 1 mM EGTA. The P$_i$ concentration [P$_i$] was raised by adding small aliquots of concentrated KH$_2$PO$_4$. The free [Ca$^{2+}$] in the cis chamber was maintained at 10 µM by adding small aliquots of concentrated CaCl$_2$ (2). Single channel data were collected using a pulsing protocol in which the potential was held at 0 mV for 4 s between steps of 2-s duration to +70 mV (CLAMPPEX program, pCLAMP software, Axon Instruments). Only those channels that had a conductance of at least 700 pS were used (26). Single channel P$_i$ was calculated from at least 50 2-s sweeps using FETCHAN and PSTAT analysis programs (pCLAMP software). When two channels were present in the bilayer, indicated by current amplitudes of twice the expected magnitude, P$_o$ was estimated as the average P$_o$ of the two channels, calculated as $P_{o\text{level}} + (P_{o\text{level}} + 2)s/2$. Bilayers in which three channels had incorporated were dealt with similarly; recordings were not made from bilayers containing more than three channels. Open and closed dwell-time histograms were created using 0.2 ms bins. Individual lifetimes were fitted using the PSTAT analysis program to a probability density function (PDF) by the method of maximal likelihood according to the equation

$$f(t) = a_1(1/\tau_1)\exp(-t/\tau_1) + \ldots + a_n(1/\tau_n)\exp(-t/\tau_n)$$  (1)

where $a$ is the area or the fractional contribution for that component to the area under the curve, $\tau$ is the time constant, estimate of the mean dwell time of that component, and $t$ is the dwell time. A missed events correction was applied and a likelihood ratio test was used to compare fits up to three exponentials by testing the difference in the log (likelihood) against the chi-squared distribution at the 1% level.

CICR in Saponin-Permeabilized Skeletal Muscle Fibers

The methods used in these experiments were adapted from those described in detail by Endo and Iino (7). Female New Zealand white rabbits were euthanized with an overdose of pentobarbital in accordance with the University of Minnesota animal use guidelines. The adductor magnus muscle was removed and placed in ice-cold G2 relaxing solution (for solution composition see Table 1). Segments of single muscle fibers (60–90 µm diameter, 2–5 mm long) were carefully isolated under a dissection microscope in G2 relaxing solution using fine-tipped forceps and surgical scissors. Care was taken not to stretch the fibers. One end of the fiber segment was attached to an isometric force transducer (Cambridge model 300H; Cambridge, MA) and the other end was attached to a fixed post. Fiber attachment was made by tweezers. All experiments were performed at room temperature (20–22°C). Fiber bathing solutions could be changed in <2 s by moving the experimental chamber that was mounted on the stage of an inverted microscope (Nikon). The chamber consisted of a spring-mounted stainless steel plate with three wells containing fiber bathing solutions. The bottom of each well was sealed with a glass coverslip that allowed the mounted fiber to be transilluminated for viewing. The output of the force transducer was displayed on a digital oscilloscope ( Nicolet model 310) and stored on a computer disk.

The muscle fiber sarcolemma was permeabilized by incubating the fiber for 30 min in G2 relaxing solution containing 50 µM saponin. Saponin permeabilizes the sarcolemma but leaves the SR intact (6). The fiber was rinsed in G2 relaxing solution to remove the saponin, then transferred to a depletion solution containing 25 mM caffeine and 0 ATP to empty...
the SR of Ca\textsuperscript{2+}. The fiber was then viewed at \( \times 600 \) and the sarcomere length was set to 2.8 \( \mu \)m with the use of a calibrated eyepiece micrometer. The longer than optimal sarcomere length has been reported to slow the rundown of the fibers (7). The CICR protocol consisted of three basic steps: 1) loading the SR to a fixed level, 2) CICR in the absence of ATP to prevent SR Ca\textsuperscript{2+} uptake, and 3) assay the Ca\textsuperscript{2+} remaining in the SR via a maximal caffeine contracture. Because Pi, or other CICR modulators were present only in the release solution and the assay occurred under standard conditions, the size of the assay caffeine contracture was proportional to the amount of Ca\textsuperscript{2+} remaining in the SR after the CICR step. Loading the SR with Ca\textsuperscript{2+} was accomplished by incubating the fiber in the pCa 6.6 loading solution for 2 min. Loading was quickly stopped by moving the fiber to a relaxing solution containing 10 mM EGTA (G10) for 1 min. The fiber was then transferred to the rigor solution to remove ATP and prevent uptake of Ca\textsuperscript{2+} by the SR during the CICR step. However, removal of ATP from permeabilized fibers is slow (7), therefore the fiber was maintained in the rigor solution for 2 min, the rigor solution was changed, and the fiber incubated for an additional 2 min. Then Mg\textsuperscript{2+}, a potent inhibitor of CICR, was removed by incubating the fiber in 1 min in the Mg\textsuperscript{2+}-free prerelease solution. CICR was then induced by moving the fiber to the release solution [pCa\textsuperscript{2+} 4.5, pH 7.0, \( \pm \) P\textsubscript{i}, or \( \pm \) Mg\textsuperscript{2+}], a nonhydrolyzable ATP analog, or Mg\textsuperscript{2+} for 5–30 s. Moving the fiber to the stop solution containing 10 mM Mg\textsuperscript{2+} for 1 min then rapidly terminated CICR. ATP was then restored and the Mg\textsuperscript{2+} concentration lowered by moving the fiber to the preassay solution. The fiber was incubated in the preassay solution for 2 min, moved to fresh preassay solution, and incubated for an additional 2 min. The amount of Ca\textsuperscript{2+} remaining in the SR after the CICR step was determined by moving the fiber to the 25 mM caffeine assay solution. Finally, the fiber was returned to the G2 relaxing solution. Experimental trials were bracketed by controls in which the fiber was taken through each of the solutions except the CICR solution. Any rundown between controls was corrected for by assuming a linear relationship between the number of contractions and the extent of the rundown. The time-tension integral of the caffeine contractures, normalized to the initial control (no CICR), was used as an indicator of the amount of Ca\textsuperscript{2+} remaining in the SR after the CICR step. The area of the caffeine contractures were integrated using the Clampfit analysis program (pCLAMP software). The half time (t\textsubscript{1/2}) was determined from the equation: 

\[ y = \frac{1}{1 + ax} \]

where \( y \) was the amount of Ca\textsuperscript{2+} remaining in the SR after CICR and \( x \) was the duration of CICR.

Inhibition of Caffeine-Induced Ca\textsuperscript{2+} Release by Prior P\textsubscript{i} Incubation

Fibers were mechanically peeled, then mounted as described above. After depleting the SR of Ca\textsuperscript{2+}, fibers were rinsed in G2 relaxing solution and loaded with Ca\textsuperscript{2+} for 2 min. Loading was quickly stopped by placing fibers in the 10 mM EGTA relaxing solution for 30 s. Fibers were then moved to the standard rigor solution or a rigor solution containing 40 mM P\textsubscript{i}. The total time in the rigor solution was 30 s, which consisted of 0, 10, 20, or 30 s in the standard rigor solution and the remainder of the time in the P\textsubscript{i}-containing rigor solution. ATP was restored to the fibers by incubation in the preassay solution for 30 s. The releasable Ca\textsuperscript{2+} in the SR was then assayed via a maximal caffeine contracture. The area of the ensuing force contracture was used as a measure of the amount of released Ca\textsuperscript{2+}.

Statistics. Results are expressed as means ± SE. Comparisons between groups were made using Student’s t-test. The effects of increasing concentration of P\textsubscript{i} was tested using a one-way ANOVA. The level of significance was set at \( P < 0.05 \).

RESULTS

Effects of P\textsubscript{i} on Single Channel P\textsubscript{o}

It has previously been reported that 10 mM P\textsubscript{i} increased the single channel P\textsubscript{o} of purified RyR1 channels (11). Here, we examined the [P\textsubscript{i}] dependence of P\textsubscript{o} and determined the specificity of P\textsubscript{i} action by comparing the effects of myoplasmic vs. SR luminal P\textsubscript{i}. In addition, to begin to characterize the putative P\textsubscript{i} regulatory site(s), we compared the effects of P\textsubscript{i} with that of AMP-PCP. When added to the cis (cytoplasmic) chamber, P\textsubscript{i} significantly (\( P < 0.05 \)) increased single channel P\textsubscript{o} in a concentration-dependent manner (Fig. 1). Thus, in the experiment shown in Fig. 1A, single channel P\textsubscript{o} increased from 0.03 (Fig. 1A, top) in the absence of P\textsubscript{i} to 0.08 and 0.16 in the presence of 15 (Fig. 1A, middle) and 40 mM (Fig. 1A, bottom) cis P\textsubscript{i}, respectively. The summarized data (Fig. 1B) indicate that P\textsubscript{i} increased from 0.079 ± 0.020 in the absence of P\textsubscript{i} to 0.117 ± 0.020, 0.244 ± 0.033, and 0.215 ± 0.048 in the presence of 10 mM, 40 mM, and 100 mM cis P\textsubscript{i}, respectively. In contrast to the effect of cis P\textsubscript{i}, the addition of up to 100 mM P\textsubscript{i} to the trans chamber did not significantly alter single channel P\textsubscript{o} (Fig. 1B). Thus, in the absence of P\textsubscript{i}, the summarized data indicate the single channel P\textsubscript{o} was 0.013 ± 0.006, and in the presence of 100 mM trans, P\textsubscript{i} single channel P\textsubscript{o} was 0.036 ± 0.016 (Fig. 1B; not significantly different, \( P > 0.05 \)).
In agreement with the previously described effect of Pi on RyR2 (16), kinetic analysis of single RyR1 channel data revealed that cis Pi enhanced single channel Po by increasing the frequency of channel opening through a significant (P < 0.05) decrease in the mean closed time of the channel, with very little effect on the mean open time (Fig. 2B). In the experiment illustrated in Fig. 2C, in both the absence and presence of 40 mM Pi, both the open and closed dwell-time histograms could be fit with a PDF composed of the sum of two exponentials. Whereas Pi had little effect on the open time constants, Pi shortened both closed time constants and increased the proportion of closing to the shorter closed state.

In a manner similar to that previously reported for ATP (16, 27, 28), the ATP analog AMP-PCP also increased the single channel Po (Fig. 2A, c–d). In the experiment illustrated in Fig. 2, 2 mM AMP-PCP increased single channel Po from 0.040 to 0.310. However, in contrast to Pi, AMP-PCP increased Po by increasing the mean open time and decreasing the mean closed time. Dwell-time analysis confirmed the effects of AMP-PCP on channel kinetics. AMP-PCP increased both the open time constants and increased the proportion of opening to the longer open state. AMP-PCP also decreased the duration of both the closed time constants while increasing the proportion closings to the shorter closed state. These differences in the effects of Pi and AMP-PCP on the single channel kinetics indicate that Pi and adenine nucleotides enhance single channel Po via different mechanisms.

Modulation of Ryanodine Binding by Pi in the Presence of Other Putative Physiological Effectors of Skeletal Muscle CICR

Pi stimulation of SR vesicle ryanodine binding was examined to further characterize the putative Pi binding site(s) and to determine the interaction of Pi with other effectors of the RyR1. AMP-PCP increased [3H]ryanodine binding to SR vesicles in a concentration-dependent manner (Fig. 3); however, AMP-PCP concentrations >3 mM resulted in a slight decrease in ryanodine binding. AMP (2 mM) enhanced ryanodine binding at AMP-PCP concentrations of 0.1 mM or lower, but had no effect on binding at concentrations >0.1 mM, suggesting that AMP-PCP and AMP likely act at a common adenine nucleotide binding site on the RyR1. To test the hypothesis that Pi may also act at the adenine nucleotide binding site, the AMP-PCP dependence of ryanodine binding was determined in the presence of 20 mM Pi. It is clear (Fig. 3) that, in contrast to AMP, the stimulation of ryanodine binding by 20 mM Pi was similar at all concentrations of AMP-PCP tested, indicating that Pi and AMP-PCP likely act at independent sites to stimulate [3H]ryanodine binding to SR vesicles.

Pi has a pH of 6.75 and, therefore, at physiological pH, exists as a combination of both the mono- and diprotonated species. To determine whether Pi interactions with RyR1 channels may be affected by physiological changes in pH, the Pi dependence of [3H]ryanodine binding to SR vesicles was determined at pH 7.1 and
6.5. Pi stimulated ryanodine binding in a concentration-dependent manner at both pH 7.1 and 6.5; however, the binding was decreased at all concentrations of Pi at pH 6.5 (Fig. 4). The inset to Fig. 4 presents the same data normalized as the percent stimulation of [3H]ryanodine binding indicated [Pi] buffered to either pH 7.1 (○) or pH 6.5 (●). Data represent means ± SE for 3 experiments performed in duplicate.

Mg2+ inhibition and Pi stimulation of ryanodine binding. Mg2+-dependent inhibition of ryanodine binding was shifted to higher Mg2+ concentrations by Pi (Fig. 5). Thus the half-inhibitory concentration of Mg2+ was

Fig. 2. Comparison of effects of 40 mM Pi and 2 mM β,γ-methyleneadenosine 5′-triphosphate (AMP-PCP) on single channel parameters. A: single channel recordings before (a and c) and after addition of 40 mM Pi (b) or 2 mM AMP-PCP (d) to cis chamber. a: P0 = 0.03, open time (OT) = 0.485 ms, closed time (CT) = 15.488 ms; b: P0 = 0.16, OT = 0.624, CT = 3.262; c: P0 = 0.040, OT = 0.873 ms, CT = 33.900 ms; d: P0 = 0.310, OT = 2.600 ms, CT = 2.949 ms. Calibration bars correspond to 30 pA and 200 ms or 20 ms for expanded time scale. B: dependence of frequency of channel opening (●), open (○), and closed times (●) on Pi concentration. Data represent means ± SE for 3 experiments performed in duplicate.

Fig. 3. Concentration-dependent stimulation of [3H]ryanodine binding to skeletal muscle SR vesicles by AMP-PCP in presence of other putative modulators of Ca2+ release (CICR). SR vesicle [3H]ryanodine binding was determined as described in METHODS in a media containing indicated concentrations of AMP-PCP (●), and either 20 mM Pi (●), or 2 mM AMP (●). Data represent means ± SE for 3 experiments performed in duplicate.
significantly greater in the presence of 20 mM Pi than in its absence (1.31 ± 0.13 vs. 0.63 ± 0.04 mM, respectively).

An increase in intracellular [Pi] is associated with a decrease in cellular phosphocreatine and an increase in creatine and lactate concentrations. Therefore, the interaction of Pi with the effect of these metabolites on SR vesicle [3H]ryanodine binding was also examined. Physiological concentrations of lactate (30 mM), creatine phosphate (30 mM), and creatine (30 mM) had no effect on [3H]ryanodine binding either in the absence or presence of 20 mM Pi (Fig. 6).

Modulation of CICR in Saponin-Permeabilized Muscle Fibers by Pi

Although the data presented previously (10, 11) demonstrated that Pi can modulate the opening of RyR1 channels, it is not clear whether Pi can regulate SR Ca\(^{2+}\) release in more intact systems. Therefore, the rate of CICR was determined in permeabilized fibers by the previously described protocol (7). We report here that high concentrations of Pi, similar to those observed in severe muscle fatigue, enhanced CICR in saponin-permeabilized rabbit skeletal muscle fibers. Thus, in Fig. 7A, a maximal caffeine contracture, which empties all the Ca\(^{2+}\) remaining in the SR, is compared following 5 s of CICR either in the absence (top) or presence (bottom) of 40 mM Pi. After 5 s of CICR in the absence of Pi, 86% of the loaded Ca\(^{2+}\) remained in the SR, whereas after 5 s of CICR in the presence of 40 mM Pi, 37% of the loaded Ca\(^{2+}\) remained in the SR.

Figure 7B summarizes the effects of Pi, AMP-PCP, and Mg\(^{2+}\) on CICR in saponin-permeabilized skeletal muscle fibers. Pi enhanced CICR in permeabilized muscle fibers in a concentration-dependent manner (Fig. 7Ba).

<table>
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<th>[Mg] (mM)</th>
<th>0</th>
<th>0.1</th>
<th>1</th>
<th>10</th>
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<tr>
<td>Ryarodine Bound (% Bmax)</td>
<td>100</td>
<td>75</td>
<td>50</td>
<td>25</td>
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Thus, the t\(_{1/2}\) for CICR decreased from 30 ± 6 s in the absence of Pi to 21 ± 5 s and 10 ± 2 s in the presence of 20 and 40 mM Pi, respectively (Table 2). The decrease in t\(_{1/2}\) was significant at the P < 0.05 level only at the higher Pi concentration. However, as pointed out by Stienen et al. (31), Pi does not diffuse freely into skinned muscle fibers; consequently, the stimulation of CICR by Pi may be slightly underestimated, particularly at short release durations. AMP-PCP, which enhances SR Ca\(^{2+}\) release channel opening (18), also stimulated, in a concentration-dependent manner, CICR in the permeabilized fibers (Table 2, Fig. 7Bb). In contrast, Mg\(^{2+}\) which inhibits channel opening (27), slowed fiber CICR (Table 2, Fig. 7Bc). Indeed, in two of the five fibers studied, Mg\(^{2+}\) slowed CICR to such an extent that accurate t\(_{1/2}\) values could not be ascertained. To determine whether the Pi stimulation of CICR occurs under more physiological adenine nucleotide and Mg\(^{2+}\) concentrations, the amount of Ca\(^{2+}\) remaining in the SR after 10 s of CICR in solutions containing AMP-PCP (1 mM) and Mg\(^{2+}\) (1.5 mM) with and without Pi (40 mM) was compared. Pi significantly enhanced SR Ca\(^{2+}\) release under these conditions; 83 ± 5% of the loaded Ca\(^{2+}\) remained in the SR after CICR in the absence of Pi, whereas only 49 ± 5% of the Ca\(^{2+}\) remained after CICR in the presence of Pi (P < 0.05; n = 7). In addition, Pi also enhanced CICR at lower triggering Ca\(^{2+}\). After 10 s of CICR at pCa 6.0, 82 ± 12% of the loaded Ca\(^{2+}\) remained in the SR. When 40 mM Pi,

![Figure 6](https://example.com/fig6.png)
was included in the CICR solution, only 25 ± 6% of the loaded Ca\(^{2+}\) remained in the SR after 10 s of CICR (P < 0.05, n = 6).

### Inhibition of Caffeine-Induced Ca\(^{2+}\) Release by Prior Pi Incubation in Mechanically Peeled Fibers

Previous reports suggest that Pi may have antithetical effects on SR Ca\(^{2+}\) release, depending on the timing of the Pi exposure in the experimental protocol. Fryer et al. (12) described an inhibition of SR Ca\(^{2+}\) release in mechanically peeled fibers when the fibers were exposed to Pi after the SR was loaded with Ca\(^{2+}\) but before release was initiated. They proposed that Pi enters the SR and forms a calcium phosphate precipitate that reduces the amount of free Ca\(^{2+}\) available for release (12, 24). The data in Fig. 8 are consistent with that proposal. After loading the SR with Ca\(^{2+}\), exposing fibers to a rigor solution containing 40 mM Pi for 10–30 s significantly (P < 0.05) reduced the amount of Ca\(^{2+}\) released upon subsequent exposure to caffeine. Figure 8A shows a typical experiment. It is clear that, as the duration of Pi exposure increased, the size of the caffeine contracture was reduced, indicating a reduction in the amount of released Ca\(^{2+}\). Figure 8B summarizes this data. Incubation of the fibers in the 40 mM Pi, rigor solution for 10, 20, or 30 s reduced the amount of Ca\(^{2+}\) released by 25 mM caffeine to 73 ± 10%, 52 ± 11%, and 18 ± 6% of control, respectively.

This effect raises the possibility that the results shown in Fig. 7 were actually due to the precipitation of Ca\(^{2+}\) inside the SR by Pi, rather than an enhancement of CICR by Pi. However, because these experiments utilized mechanically peeled fibers where the sarcolemma is completely removed, Pi entry into the fiber and subsequent inhibition of caffeine-induced Ca\(^{2+}\) release may have been more rapid than would have occurred had saponin-permeabilized fibers been used. It has also been suggested that, in the presence of ADP, Pi could induce the SR Ca\(^{2+}\) pump to reverse, transport Ca\(^{2+}\) out of the SR, and synthesize ATP from ADP and Pi (30). Because CICR was performed while the fibers were in rigor, it is possible that all the ADP was not washed out of the fibers, and the addition of Pi may have induced the reversal of the SR Ca\(^{2+}\) pump. To exclude these two alternative interpretations of the data shown in Fig. 7, the effects of Pi on CICR were examined at nanomolar Ca\(^{2+}\) because low [Ca\(^{2+}\)] slows the rate of CICR.

### Table 2. Half time for SR Ca\(^{2+}\) release in saponin permeabilized skeletal muscle fibers in presence of modulators of RyR1 channel activity

<table>
<thead>
<tr>
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<th>(t_{1/2}), s</th>
<th>n</th>
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<tr>
<td>pCa 4.5</td>
<td>30 ± 6</td>
<td>12</td>
</tr>
<tr>
<td>pCa 4.5 + 20 mM Pi</td>
<td>21 ± 5</td>
<td>8</td>
</tr>
<tr>
<td>pCa 4.5 + 40 mM Pi</td>
<td>10 ± 2*</td>
<td>8</td>
</tr>
<tr>
<td>pCa 4.5 + 0.075 mM AMP-PCP</td>
<td>5 ± 2</td>
<td>4</td>
</tr>
<tr>
<td>pCa 4.5 + 1 mM AMP-PCP</td>
<td>4 ± 3*</td>
<td>4</td>
</tr>
<tr>
<td>pCa 4.5 + 2 mM Mg(^{2+})</td>
<td>&gt;100†</td>
<td>5</td>
</tr>
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</table>

Values are means ± SE; n, no. of fibers studied. AMP-PCP, \(\beta_{\gamma}\)-methylene adenosine 5’-triphosphate. *Significantly different from the pCa 4.5 value (P < 0.05). †Not tested.
ever, calcium phosphate formation within the SR and SR Ca\(^{2+}\) pump reversal should be independent of the [Ca\(^{2+}\)] in the release solution. Therefore, the [Ca\(^{2+}\)] in the release solution was lowered to pCa 7.5, and the amount of Ca\(^{2+}\) remaining in the SR after 30 s of CICR in the presence and absence of 40 mM Pi was determined (Fig. 7B, filled and open diamonds, respectively). Lowering the [Ca\(^{2+}\)] of the release solution to pCa 7.5 significantly increased the amount of Ca\(^{2+}\) remaining in the SR after 30 s of Ca\(^{2+}\) release. After 30 s of CICR at pCa 7.5 in the presence of 40 mM Pi, 73 ± 3% of the Ca\(^{2+}\) remained in the SR. However, after 30 s of CICR at pCa 4.5 in the presence of 40 mM Pi, only 28 ± 6% of the loaded Ca\(^{2+}\) remained in the SR (Fig. 7B, filled squares). If calcium phosphate precipitation within the SR or SR Ca\(^{2+}\) pump reversal were responsible for the reduction in SR Ca\(^{2+}\) after exposing the fiber to the Pi-containing release solution, the reduction in SR Ca\(^{2+}\) content after CICR should have been independent of the Ca\(^{2+}\) concentration of the release solution; this was not the case. In addition, Posterino and Fryer (24) reported the half time for the recovery of SR Ca\(^{2+}\) release after washout of Pi to be 35 s. In the experiments examining the stimulation of CICR by Pi, 5 min elapsed between Pi exposure and the assay of SR Ca\(^{2+}\) content. Therefore, if calcium phosphate did form within the SR of these fibers during the release step, very little if any would have remained during the assay step, further arguing against the involvement of calcium phosphate formation in these experiments. Therefore, the primary mechanism by which Pi, in the release solution reduces SR Ca\(^{2+}\) content is via its action on the RyR1 to stimulate CICR.

**DISCUSSION**

In working muscle, the concentration of Pi may rise to 30 mM or more (4, 5). Such concentrations of Pi have a profound effect on skeletal muscle function, stimulating metabolism (33) and decreasing force production by impairing cross-bridge function (8). However, the effects of Pi, on the skeletal muscle SR Ca\(^{2+}\) release channel have only recently been examined (10, 11). These studies suggested that in addition to its effects on cross-bridge function, Pi also modulates skeletal muscle contraction by enhancing the activity of the SR Ca\(^{2+}\) release channel. In the present study, Pi enhanced SR Ca\(^{2+}\) release channel activity in all three preparations used. Pi (20 mM) more than doubled single channel P\(_o\) (Fig. 1) and increased ryanodine binding twofold above control (Figs. 3 and 4). Pi (40 mM) also decreased the t\(_{1/2}\) for CICR in saponin-permeabilized muscle fibers to ~30% of control (Table 2 and Fig. 7).

**Characterization of the Putative Pi Binding Site**

The data presented in Fig. 1 suggests that Pi may act via a saturable site on the cytoplasmic aspect of the SR Ca\(^{2+}\) release channel. It is also clear from Fig. 1 that Pi has virtually no effect via the SR luminal side of the channel. This observation negates the possibility that the inhibition of SR Ca\(^{2+}\) release reported by Fryer and co-workers (12, 24) was due to direct effects of Pi on the luminal face of the channel. Therefore, we considered the possibility that Pi acts at the adenine nucleotide binding site on the cytoplasmic aspect of the release channel. However, our single channel and ryanodine-binding data do not support this proposal. Pi increased single channel P\(_o\) by increasing the frequency of opening and decreasing the mean closed time. In contrast to Pi, and consistent with the previously reported effects of ATP on RyR1 and RyR2 (16, 27, 28), AMP-PCP increased channel P\(_o\) by both decreasing the mean closed time and increasing the mean open time. Therefore, it appears that Pi and adenine nucleotides enhance P\(_o\) via different mechanisms and that their effects result from their interaction with distinct sites on the RyR1. The different sites of action of Pi and AMP-PCP on the RyR1 were confirmed by the ryanodine-binding experiment shown in Fig. 3. In the absence of AMP-PCP, the stimulation of ryanodine binding by 20 mM Pi was approximately one-half that observed in 2 mM AMP. However, unlike AMP, which acts via the adenine
nucleotide site and did not increase binding in the presence of maximally activating concentrations of AMP-PCP. P_i enhances ryanodine binding, to a similar extent, at all concentrations of AMP-PCP examined (Fig. 3). Therefore, P_i appears to increase ryanodine binding to the RyR1 via a site or sites other than the adenine nucleotide binding site.

The accumulation of P_i in the myoplasm is usually associated with a decline in intracellular pH, making the functional effects of P_i and H^+ difficult to separate. Because P_i has a pK_a near physiological pH, myoplasmic P_i exists as two ionic species, a monobasic form and a dibasic form. As pH decreases, the proportion of P_i existing as dibasic P_i decreases, whereas the proportion of P_i existing as monobasic P_i increases. Nosek et al. (22) suggested that the inhibition of force generation by the contractile proteins is a specific effect of the monobasic form of P_i rather than an effect of P_i in general. Therefore, we examined the possibility that a physiologically relevant decrease in pH may alter the P_i-induced stimulation of the RyR1 channel. The results presented in Fig. 4 indicate that P_i enhanced ryanodine binding to a similar extent at both pH 7.1 and 6.5, with no difference in the half-stimulatory concentration of P_i (Fig. 4, inset). Therefore, it appears that the physiological changes in pH may have no major effects on P_i modulation of the SR Ca^{2+} release channel.

Inorganic Phosphate Stimulation of CICR

A potential confounding factor in single channel and ryanodine-binding experiments is that removing the Ca^{2+} release channel from its environment in intact muscle, so that it does not interact with the physiological excitation-contraction coupling architecture, may alter the channel’s sensitivity to putative modulators. However, this drawback was overcome by studying CICR in saponin-permeabilized muscle fibers. Although CICR is not the primary trigger for SR Ca^{2+} release in skeletal muscle, it may play a secondary function in amplifying the initial Ca^{2+} release and is therefore a valuable technique in examining the regulation of the release channels in their native environment.

The stimulation of CICR by P_i in saponin-permeabilized skeletal muscle fibers is similar to the findings of Stienen et al. (32). They reported that caffeine contractures in saponin-permeabilized frog muscle fibers, after correcting for the P_i effects on the cross bridges, were larger in the presence of 15 mM P_i than in the absence of P_i. However, because the caffeine contractures were carried out in the presence of MgATP, SR Ca^{2+} release and uptake occurred simultaneously. Because P_i inhibits the SR Ca^{2+-}ATPase, it was not possible, in the study of Stienen et al. (32), to separate the effects of P_i on Ca^{2+} release and uptake. In the present study, however, CICR occurred in the absence of MgATP, and the amount of Ca^{2+} remaining in the SR was later assayed in the absence of P_i. Therefore, the effects of P_i on Ca^{2+} release and Ca^{2+} uptake were clearly isolated. The stimulation of CICR by P_i in these fibers strongly suggests that P_i is an important endogenous modulator of the SR Ca^{2+} release channel in situ.

Inhibition of Caffeine-Activated SR Ca^{2+} Release by Prior Exposure to P_i

In contrast to the stimulation of CICR when P_i was included in the release media (Fig. 7), if fibers were exposed to P_i after the SR was loaded with Ca^{2+}, the amount of Ca^{2+} released from the SR by maximal caffeine stimulation was reduced (Fig. 8). It should be noted that this decrease in caffeine-induced release was not due to SR Ca^{2+} release in a step between loading the SR and the induction of release, because the [Ca^{2+}] in each of these steps was below that required to trigger CICR. In addition, it has previously been demonstrated that, although P_i can enhance CICR from SR vesicles, it cannot trigger Ca^{2+} release at [Ca^{2+}] below 100 nM (11). Therefore, SR Ca^{2+} release could not have been initiated by P_i exposure in the rigor solution (free [Ca^{2+}] < 10 nM). Fryer et al. (12) suggested that this reduction in SR Ca^{2+} release is due to the entry of P_i into the SR. If the concentration of P_i rises high enough, the solubility product of calcium phosphate is exceeded and a calcium phosphate precipitate is formed, reducing the free Ca^{2+} in the SR available for release.

Although indirect evidence is accumulating in support of the hypothesis that P_i entry into the SR causes a decrease in releasable Ca^{2+} (12, 24, 37; Fig. 8), it is not clear that such a mechanism operates in vivo. However, this proposal is attractive for two main reasons. First, in fatigued skeletal muscle, the intracellular concentration of P_i does rise to concentrations that, if P_i equilibrated across the SR membrane, would be sufficient to exceed the solubility product of calcium phosphate (given that the luminal concentration of free Ca^{2+} is ~1 mM). Second, the ability of P_i to complex Ca^{2+} in the SR in vitro is well established, as P_i has been used extensively as a Ca^{2+}-precipitating anion in isolated SR vesicles (14).

However, there are also problems with this hypothesis. The effect of P_i on SR Ca^{2+} release in vivo will depend on the distribution of P_i in various cellular compartments. Unfortunately, how P_i is distributed in fatigued skeletal muscle is not clear. In addition, it is questionable whether a calcium phosphate precipitate forms in the SR lumen of muscle fibers in vivo. The solubility product quoted by Fryer et al. (12) of 6 mM^2 was determined in an aqueous calcium phosphate solution. However, the composition of the SR lumen is a much more complex media, and the solubility product of calcium phosphate in such an environment is unknown. Indeed, Sorenson et al. (29) used light scattering by permeabilized skeletal muscle fibers to assess the ability of various anions to precipitate Ca^{2+} within the SR. Although oxalate, pyrophosphate, and, to a lesser extent, fluoride, all increased light scattering, suggesting the formation of an insoluble Ca^{2+} salt, P_i (up to 50 mM) had no effect on light scattering, indicating it did not precipitate as calcium phosphate in these fibers. The multiple forms of calcium phosphate, their different rates of formation, and their differing solubility...
ties, however, complicate the interpretation of this study. A further problem is that the pathway for $P_i$ entry into the SR is unknown (for detailed discussion, see Posterino and Fryer (24)). Finally, as pointed out by Westerblad and Allen (37), in repeatedly contracting skeletal muscle, the concentration of $P_i$ often increases well before SR Ca$^{2+}$ release is reduced. Coupled with the rapid onset of $P_i$ impairment of SR Ca$^{2+}$ release in skinned fibers (Fig. 8), this temporal discordance is difficult to explain in terms of calcium phosphate precipitation in the SR lumen. However, it is clear that $P_i$ does not decrease SR Ca$^{2+}$ release via interaction with the luminal side of the SR Ca$^{2+}$ release channel (Fig. 1). To address these issues and precisely define the role of $P_i$ in the regulation of SR Ca$^{2+}$ release in vivo will clearly require further study.

**Physiological Significance**

$P_i$ has been proposed to be a factor contributing to skeletal muscle fatigue (8), although the relationship between force production by muscle and intracellular $[P_i]$ is not a simple one. During high-intensity muscle contraction, intracellular $[P_i]$ may rise faster than force declines (21). This may be due, at least in part, to the multiple effects of $P_i$ on muscle function. Studies using skinned fibers to examine cross-bridge function have shown that the concentrations of $P_i$ examined here have direct action on the actomyosin cross bridges. $P_i$ decreases the maximal Ca$^{2+}$-activated force and decreases the Ca$^{2+}$ sensitivity of contraction, such that, in the presence of $P_i$, higher Ca$^{2+}$ concentrations are required to achieve the same submaximal level of contractile activation compared with contractions in the absence of $P_i$ (20). However, $P_i$ also enhances SR Ca$^{2+}$ release channel opening so that, in the absence of other changes, increased $P_i$ concentrations would result in a greater SR Ca$^{2+}$ release. This stimulation of SR Ca$^{2+}$ release may partially compensate for the $P_i$-induced decreased Ca$^{2+}$ sensitivity of the actomyosin cross bridges.

$P_i$ also appears to have multiple effects on SR Ca$^{2+}$ release. $P_i$ may decrease the amount of SR Ca$^{2+}$ available for release via two mechanisms. $P_i$ slows the SR Ca$^{2+}$-ATPase and may impair the ability of the ATPase to completely refill the SR in the interval between successive muscle contractions (5). As discussed above, $P_i$ may enter the SR to form a calcium phosphate precipitate and reduce the free Ca$^{2+}$ available for release (12, 24, 40). Regardless of the effects of $P_i$ on the amount of Ca$^{2+}$ in the SR available for release, the stimulation of the release channel by cytoplasmic $P_i$ would still occur.

In severely fatigued skeletal muscle, a significant fraction of the force decline has been attributed to reduced SR Ca$^{2+}$ release (35). This impairment has been hypothesized to be due, in part, to the depletion of certain metabolites (in particular, ATP) and the accumulation of others, such as Mg$^{2+}$ and H$^+$. Although the changes in the intracellular concentration of ATP and Mg$^{2+}$ appear to be rather small, it has been suggested that there is a significant compartmentalization of metabolites within the muscle fiber, such that the concentration of some metabolites, in the triadic region, could be significantly different from the “average” concentration in the muscle cytoplasm (17). However, the results presented in Figs. 3–5 suggest that $P_i$ would partially mitigate the deleterious effects, on the SR Ca$^{2+}$ release channel, of a decrease in ATP or an increase in Mg$^{2+}$ or H$^+$. Significantly, the stimulation of channel opening by $P_i$ occurs even when there are substantial changes in the concentration of several other metabolites associated with skeletal muscle contraction. However, it should be noted that other factors, such as damage to proteins and membranes via the activation of proteases, lipases (8), and the accumulation of reactive oxygen species (3), may also be involved in the decreased SR Ca$^{2+}$ release associated with skeletal muscle fatigue. The interaction of $P_i$ with these putative fatigue-inducing agents is unknown.

In conclusion, $P_i$ has been shown to enhance the opening of the SR Ca$^{2+}$ release channel in three different preparations, including purified single SR Ca$^{2+}$ release channels, SR vesicles, and the more intact permeabilized skeletal muscle fiber preparation. $P_i$ enhanced channel opening via its interaction with the cytoplasmic side of the channel at a site distinct from the adenine nucleotide regulatory site. In addition, $P_i$ enhanced SR Ca$^{2+}$ release channel activity in the presence of a number of important metabolites known to modulate channel function. These different approaches provide important new evidence regarding the potential role $P_i$ plays as an endogenous regulator of the skeletal muscle SR Ca$^{2+}$ release channel.

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