Effects of caffeine and adenine nucleotides on Ca^{2+} release by the sarcoplasmic reticulum in saponin-permeabilized frog skeletal muscle fibres

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1. The effect of caffeine and adenine nucleotides on the sarcoplasmic reticulum (SR) Ca^{2+} release mechanism was investigated in permeabilized frog skeletal muscle fibres. Caffeine was rapidly applied and the resulting release of Ca^{2+} from the SR detected using fura-2 fluorescence. Decreasing the [ATP] from 5 to 0·1 mM reduced the caffeine-induced Ca^{2+} transient by 89 ± 1·4 % (mean ± S.E.M., $n = 16$), while SR Ca^{2+} uptake was unaffected.

2. The dependence of caffeine-induced Ca^{2+} release on cytosolic [ATP] was used to study the relative ability of other structurally related compounds to substitute for, or compete with, ATP at the adenine nucleotide binding site. It was found that AMP, ADP and the non-hydrolysable analogue adenylyl imidodiphosphate (AMP-PNP) partially substituted for ATP, although none was as potent in facilitating the Ca^{2+}-releasing action of caffeine.

3. Adenosine reversibly inhibited caffeine-induced Ca^{2+} release, without affecting SR Ca^{2+} uptake. Five millimolar adenosine markedly reduced the amplitude of the caffeine-induced Ca^{2+} transient by 64 ± 4 % (mean ± S.E.M., $n = 11$). The degree of inhibition was dependent upon the cytosolic [ATP], suggesting that adenosine may act as a competitive antagonist at the adenine nucleotide binding site.

4. These data show that (i) the sensitivity of the *in situ* SR Ca^{2+} channel to caffeine activation is strongly dependent upon the cytosolic [ATP], (ii) the number of phosphates attached to the 5′ carbon of the ribose ring influences the efficacy of the ligand, and (iii) removal of a single phosphate group transforms AMP from a partial agonist, to adenosine, which acts as a competitive antagonist under these conditions.

Our current understanding of the mechanisms underlying physiological and pharmacological regulation of the sarcoplasmic reticulum (SR) Ca^{2+} channel is mostly derived from experiments on membrane vesicles or purified channels reconstituted into planar lipid bilayers (for reviews see Coronado *et al.* 1994; Sitsapesan *et al.* 1995; Franzini-Armstrong & Protasi, 1997). It has been shown that a rise in [Ca^{2+}] at the cytosolic face of the channel increases the open probability (P_o) by increasing the frequency of opening without affecting the duration (Smith *et al.* 1986). Millimolar levels of caffeine and adenine nucleotides also activate the SR Ca^{2+} channel, although the underlying mechanisms differ. In skeletal muscle, physiological levels of ATP increase P_o in the absence or presence of Ca^{2+} (Smith *et al.* 1986; Rousseau *et al.* 1988). This effect of ATP is shared by other adenine nucleotides and is characterized by increases in both the frequency and duration of channel openings. In contrast, low levels of caffeine (< 2 mM) appear to sensitize the channel to cytosolic Ca^{2+}. Increases in P_o occur only in the presence of Ca^{2+} and the frequency of channel openings increases without change in the mean open lifetime. Higher concentrations of caffeine induce a Ca^{2+}-independent activation of the channel, with increases in both the frequency and duration of channel openings (Rousseau *et al.* 1988; McGarry & Williams, 1994; Sitsapesan & Williams, 1995). These differences in activation characteristics, combined with data from competitive binding studies, have led to the conclusion that caffeine and adenine nucleotides act at distinct sites on the SR Ca^{2+} channel (Rousseau *et al.* 1988; McGarry & Williams, 1994).

A limited amount of evidence also suggests that the sensitivity of the SR Ca^{2+} channel to activation by caffeine may depend upon occupancy of the adenine nucleotide site (Rousseau *et al.* 1988). Such an effect might have important consequences in studies involving skinned or intact muscle preparations, where caffeine is widely used to assess the Ca^{2+} content of the SR or to investigate other aspects of the excitation–contraction process. However, studies on isolated channels or membrane vesicles are often conducted under ionic conditions that differ markedly from the *in vivo* state. Furthermore, recent work suggests that the activity of the SR Ca^{2+} channel is influenced by a number of associated

proteins including FK506 binding protein (Mayrleitner *et al.* 1994; Brillantes *et al.* 1994), triadin (Brandt *et al.* 1992) and calsequestrin (Ikemoto *et al.* 1989). Purification of SR Ca^{2+} channels or membrane fractions results in loss of these proteins to varying degrees, and this has been linked to alterations in channel gating characteristics, or the sensitivity to physiological or pharmacological activators (Mayrleitner *et al.* 1994; Brillantes *et al.* 1994; Wagenknecht *et al.* 1996; Franzini-Armstrong & Protasi, 1997). It is not clear, therefore, to what extent data obtained on isolated Ca^{2+} channels or SR vesicles can be applied quantitatively to more complete systems such as skinned fibres or intact muscle. Indeed, a recent study on skinned skeletal muscle fibres found that the Ca^{2+}-releasing action of caffeine was largely independent of the cytosolic [ATP] (Stienen *et al.* 1993).

In the present study, we have investigated the effects of caffeine and adenine nucleotides on the SR Ca^{2+}-release process in saponin-permeabilized skeletal muscle fibres. In this preparation, the SR remains *in situ* and the ionic conditions closely match those in intact fibres. Caffeine was rapidly applied and the resulting release of Ca^{2+} from the SR detected using fura-2 fluorescence. It was found that decreasing the [ATP] from 5 to 0·1 mM reduced the amplitude of the caffeine-induced Ca^{2+} transient by almost 90%, without affecting SR Ca^{2+} uptake. The ATP dependence of caffeine-induced Ca^{2+} release was then used to study the relative efficacy of other structurally related compounds to substitute for, or compete with, ATP at the adenine nucleotide binding site. It was found that the non-hydrolysable analogue adenylyl imidodiphosphate (AMP-PNP), ADP and AMP partially substituted for ATP, although none was as effective in facilitating the Ca^{2+}-releasing action of caffeine. However, adenosine induced a concentration-dependent inhibition of the caffeine-induced Ca^{2+} release which was dependent on the cytosolic [ATP]. These results are discussed in relation to previous studies addressing the effects of adenine nucleotides and caffeine on the SR Ca^{2+} channel. Possible difficulties associated with the use of caffeine to assess SR Ca^{2+} content are also considered.

METHODS

Preparation

Frogs (*Rana temporaria*) were killed by stunning and decerebration. The sartorius muscle was removed rapidly and placed in 'relaxing' solution approximating to the intracellular milieu (see below). Small muscle fibre bundles (100–150 μm diameter) were then attached between an isometric tension transducer (SensoNor, Norway) and a fixed support using monofilament snares (diameter 30 μm) within stainless steel tubes (inside diameter 100 μm; Goodfellow Metals, Cambridge, UK). Small bundles of muscle fibres were used because it was found that the perturbation associated with rapid caffeine application produced deterioration in single fibres. Preparations were permeabilized by exposure to an ATP-containing solution with 10 μg ml^{-1} saponin for 10–15 min. Frog skeletal muscle fibres contain roughly equal amounts of RyR1 and RyR3 (α and β) isoforms of the ryanodine receptor (Franzini-Armstrong & Protasi, 1997). We assume, therefore, that the results of this study reflect the average behaviour of the α- and β-isoforms of the ryanodine receptor in the *in situ* state, under conditions commonly used in skinned muscle studies.

Apparatus

The apparatus for simultaneous measurement of tension and SR Ca^{2+} release has been described previously (Duke & Steele, 1998*a*). Briefly, the mounted preparation was lowered within 5 μm of the bottom of a shallow bath with a coverslip base. A Perspex column (5 mm diameter) was lowered close to the surface of the muscle to minimize the volume of the solution above the preparation. Throughout the experimental protocols, preparations were perfused at 0·8 ml min^{-1} via a narrow duct (200 μm diameter) passing through the centre of the column. Waste solution was collected continuously at the column edge. The volume of solution between the coverslip and the base of the column (i.e. the effective bath volume) was approximately 6 μl. The basic perfusing solution was changed using a series of valves positioned above the column. Using this method, the solution within the bath could be exchanged within 10–15 s. The comparatively slow solution exchange reflects the mixing of solutions in the tubing between the valves and the column. Solutions containing caffeine were rapidly applied (20 ml min^{-1}) for 2 s duration via a narrow plastic tube connected to the injection duct at the column base. The higher flow rate and the smaller dead space allowed a more rapid exchange of solutions within the bath. Previous measurements based on quench of indo-1 fluorescence by caffeine (O'Neill *et al.* 1990) under similar conditions have shown that the caffeine concentration within the bath typically increased to 50% of the concentration injected within 8–10 ms (Smith & Steele, 1993).

The bath was placed on the stage of an S200 Nikon Diaphot inverted microscope. The muscle was viewed via a ×20 Fluor objective (Nikon CF Fluor, NA 0·75) and the length was increased to approximately 20% above slack length. However, in control experiments, we have found that length does not have a direct effect on SR Ca^{2+} release. The preparation was alternately illuminated with light of wavelengths 340 nm and 380 nm at 50 Hz frequency using a spinning wheel spectrophotometer (Cairn Research, Faversham, Kent, UK). The average [Ca^{2+}] within the visual field containing the preparation was indicated by the ratio of light intensities emitted at $>$ 500 nm. Light emitted from areas of the field not occupied by the muscle image was reduced using a variable rectangular diaphragm on the side port of the microscope.

Solution composition and Ca^{2+} detection

All chemicals were purchased from Sigma unless otherwise stated. In all experiments, the ionic composition of the solution was adjusted to maintain the [Ca^{2+}], [Mg^{2+}], [Na^{+}], [K^{+}] and pH constant. In brief, for most experiments a basic solution was prepared containing KCl (60 mM), Hepes (25 mM), EGTA (0·2 mM) phosphocreatine (10 mM) and fura-2 (5 μM). $MgCl_2$ and $CaCl_2$ were added (from 1 M BDH stock) to produce free concentrations of 1·4 mM and 100 nM respectively. This solution was then split and ATP or other adenine nucleotides added to each fraction. The amount of additional Mg^{2+} added to each solution to compensate for the variable amount of binding to adenine nucleotides was calculated using a computer program and binding constants as previously described (Fabiato & Fabiato, 1979; Miller & Smith, 1984). Corrections for ionic strength, details of pH measurement, allowance for EGTA purity and the principles of the calculations are described in Fabiato & Fabiato (1979). In some experiments, the Mg^{2+} levels were also checked using Mg fura-2. As some nucleotides were added as disodium salts, an equivalent amount of NaCl was

added to other solutions to maintain a constant level of Na^+. The pH was adjusted to 7·0 by addition of KOH. Following this procedure, the total concentration of K^+ and Na^+ was 40 and 90 mM, respectively. The Cl^- concentration varied between 76 and 108 mM. In control experiments, it was found that differences in [Cl^-] within this range had no apparent effect on SR function or fura-2 fluorescence. In some experiments, azide (5 mM) was added to inhibit possible mitochondrial activity. However, 5 mM azide had no apparent effect on the results obtained. In some experiments involving AMP or ADP, the myokinase inhibitor 0·1 mM diadenosine pentaphosphate was also included. However, there was no significant difference between results obtained in the absence or presence of diadenosine pentaphosphate. All experiments were done at room temperature (22–24 °C). Details of the experimental set-up and methods relating to Ca^{2+} measurement in skinned skeletal muscle preparations have been described elsewhere (Duke & Steele, 1998*a*).

Data recording and analysis

In all experiments, the ratio and individual wavelength intensities and the isometric tension signals were low-pass filtered (−3 dB at 30 Hz) and digitized for later analysis using an IBM-compatible 80486 computer with a Data Translation 2801A card.

RESULTS

Protocol to assess the effects of ATP on caffeine-induced Ca^{2+} release

Figure 1 shows simultaneous recordings of the 340 nm/380 nm fluorescence ratio (lower panel) and isometric tension (upper panel) from a saponin-permeabilized fibre bundle. In this experiment the preparation was perfused constantly with solutions containing 100 nM Ca^{2+}, and caffeine (20 mM) was briefly applied for 1·5 s at 2 min intervals. Each caffeine application resulted in a transient increase in the fluorescence ratio due to SR Ca^{2+} release, and an associated tension response. Application of caffeine for 1·5 s was sufficient to produce responses of maximal amplitude under control conditions, i.e. a more prolonged application did not increase the amplitude of the Ca^{2+} or tension transients. We have shown previously that (i) the descending phase of the Ca^{2+} transient following brief caffeine application is dependent upon re-accumulation of Ca^{2+} by the SR and (ii) the rate of rise of the Ca^{2+} transient appears to be limited by the inward diffusion of caffeine (Duke & Steele, 1998*a*). Hence, the rate of decline of the Ca^{2+} transient can be used to assess changes in net Ca^{2+} uptake (see below), while the amplitude can be used as an index of Ca^{2+} release.

Increasing the Ca^{2+} loading period (i.e. the interval between caffeine applications) from 2 to 4 min had no effect on the amplitude of the Ca^{2+} and tension transients. This shows that at a bathing [Ca^{2+}] of 100 nM, the SR Ca^{2+} content reaches a steady state within 2 min under these conditions. Therefore, in subsequent experiments, caffeine was applied at 4 min intervals and the level of adenine nucleotides (or related compounds) altered 2 min after a caffeine application. In most circumstances (see below), this procedure ensured that caffeine-induced Ca^{2+} release was triggered at the same SR Ca^{2+} content during each intervention.

Effects of cytosolic ATP on caffeine-induced Ca^{2+} release

Figure 2*A* shows simultaneous records of isometric tension (upper panel), the 340 nm/380 nm fluorescence ratio (middle panel) and the individual 340 nm and 380 nm fluorescence signals (lower panel). Caffeine was briefly applied at 4 min intervals in the presence of 5 mM ATP, until reproducible steady-state Ca^{2+} and tension transients were obtained. After the second steady-state response, a further 2 min was allowed to fully load the SR with Ca^{2+}.

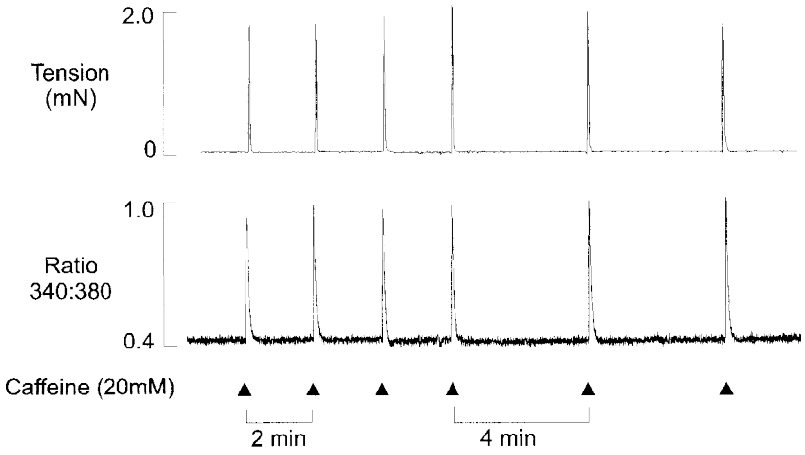

Figure 1. Steady-state caffeine-induced responses at 2 and 4 min loading periods

Simultaneous records of the 340 nm/380 nm fluorescence ratio (lower panel) and isometric tension (upper panel) from a saponin-permeabilized muscle fibre bundle in the presence of 100 nM bathing Ca^{2+}. Caffeine (20 mM) was briefly applied (for 1·5 s) at 2 min intervals as indicated. Each application resulted in a transient increase in the fluorescence ratio and a corresponding tension response. After 4 control responses, the time between caffeine applications was increased from 2 to 4 min.

The [ATP] in the perfusing solution was then reduced from 5 mM to 0·1 mM, and in this example, the subsequent caffeine-induced Ca^{2+} transient was almost completely abolished. On average, the caffeine-induced response was reduced by $89 \pm 1{\cdot}4\%$ (mean ± S.E.M., $n = 16$) in the presence of 0·1 mM ATP.

Resting tension was unaffected by the reduction in [ATP], suggesting that 0·1 mM ATP was sufficient to prevent the formation of rigor bridges under these conditions. We have shown previously that complete ATP withdrawal results in a slow maintained release of Ca^{2+} from the SR which appears to reflect passive Ca^{2+} efflux via a leak pathway (Steele & Duke, 1997; Duke & Steele, 1998*a*). The fact that the $[Ca^{2+}]$ did not rise after [ATP] was decreased to 0·1 mM confirms that sufficient substrate was present to support the activity of the Ca^{2+} pump, preventing passive loss of Ca^{2+} prior to caffeine application.

Withdrawal of ATP was associated with an increase in the 340 and 380 nm fluorescence signals. This suggests that the millimolar levels of ATP normally present in the cytosol quench fura-2 fluorescence. However, neither the 340 nm/380 nm ratio (middle panel) nor the apparent K_d of the dye for Ca^{2+} was affected by the change in [ATP] (data not shown). Therefore, the changes in emitted fluorescence can be used to monitor the rate of change of [ATP] within the preparation following a solution change, without affecting the Ca^{2+} signal.

Relationship between [ATP] and caffeine-induced Ca^{2+} release

Figure 2*B* shows superimposed caffeine-induced Ca^{2+} transients at a range of [ATP]. The protocol used was similar to that in Fig. 1, such that each release was induced at a constant SR Ca^{2+} load. Reducing the [ATP] from 5 to 0·1 mM produced a concentration-dependent decrease in the caffeine-induced Ca^{2+} transient. The superimposed (Fig. 2*B*) and normalized responses (inset) demonstrate that the time course of the Ca^{2+} transient was unaffected by the decrease in [ATP]. The lack of effect on the descending phase of the Ca^{2+} transient is consistent with previous findings that decreasing the [ATP] from millimolar levels to 100 μM does not influence Ca^{2+} re-uptake by the SR (Stienen *et al.* 1993).

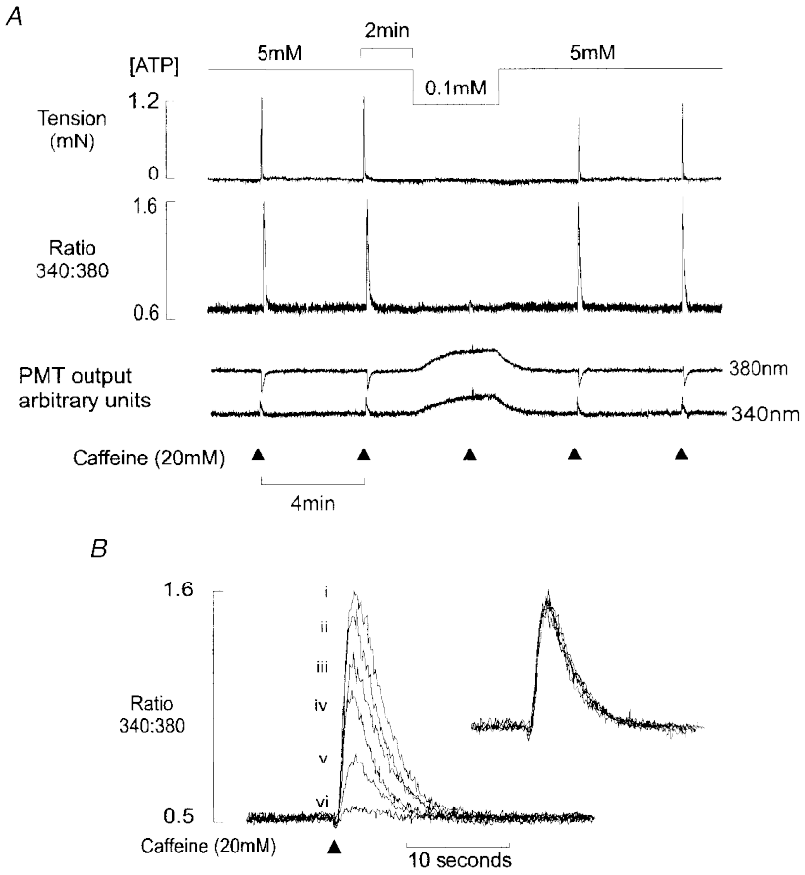

Figure 2. The effect of cytosolic [ATP] on caffeine-induced Ca^{2+} release

A, simultaneous records of the isometric tension (upper panel), the 340 nm/380 nm fluorescence ratio (middle panel) and the 340 and 380 nm fluorescence signals are shown. Reduction in the bathing [ATP] caused a marked decrease in the caffeine-induced Ca^{2+} transient. The decrease in [ATP] was associated with a parallel increase in the 340 nm and 380 nm signals, with no effect on the ratio. *B*, superimposed caffeine-induced Ca^{2+} transients in the presence of [ATP] (mM): (i) 5, (ii) 4, (iii) 3, (iv) 2, (v) 1 and (vi) 0·1. Inset shows normalized responses. All data are from the same preparation.

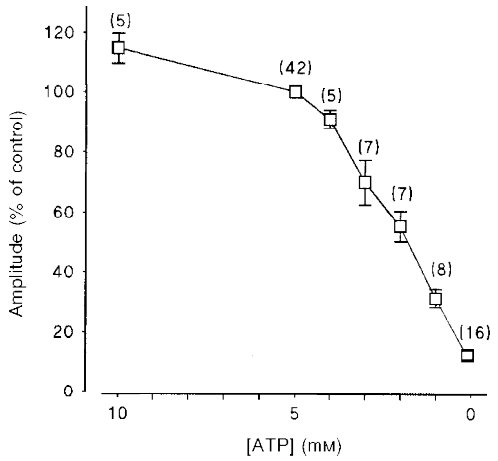

Figure 3. Cumulative data showing the relationship between the cytosolic [ATP] and the amplitude of the caffeine-induced Ca^{2+} transient

Each point is the mean (± S.E.M.) expressed as a percentage of the control response obtained in the presence of 5 mM ATP using the protocol shown in Fig. 2*A*. The number of preparations is indicated in parentheses.

Accumulated data showing the relationship between cytosolic [ATP] and the amplitude of the caffeine-induced Ca^{2+} transient are given in Fig. 3. The abscissa indicates the [ATP] and the ordinate, the mean steady-state amplitude of caffeine-induced Ca^{2+} transients, expressed as a percentage of control values obtained in the presence of 5 mM ATP. It is apparent that caffeine-induced Ca^{2+} release decreased markedly as the [ATP] was reduced below 5 mM. However, increasing the [ATP] from 5 to 10 mM only increased Ca^{2+} release by a further 14·6 ± 5% (mean ± S.E.M., $n = 5$).

Substitution of ATP with other nucleotides

As ATP facilitates caffeine-induced Ca^{2+} release from the SR, the ability of other structurally related nucleotides to substitute for ATP was investigated. Again, steady-state responses were obtained in the presence of 5 mM ATP and a further 2 min period was allowed to fully load the SR. However, the solution was then changed to one containing 0·1 mM ATP and a 4·9 mM concentration of the substituted nucleotide. ATP was retained at 0·1 mM in the perfusing solution to support the activity of the SR Ca^{2+} pump and myofilaments. The cumulative data (Fig. 4) show the mean control Ca^{2+} transient amplitudes obtained in the presence of 5 mM ATP or 0·1 mM ATP. Also shown are responses obtained in the presence of 0·1 mM ATP in combination with 4·9 mM AMP-PNP, ADP, AMP, adenosine or CTP. A substantial caffeine-induced release was observed in the presence of the non-hydrolysable ATP analogue AMP-PNP. The adenine nucleotides ADP and AMP also facilitated caffeine-induced Ca^{2+} release, although none of these compounds was as effective as 5 mM ATP alone. Interpretation of the results obtained with ADP was complicated by the finding that the introduction of the compound resulted in a slow ryanodine-insensitive efflux of Ca^{2+} from the SR, which probably resulted from reversal of the SR Ca^{2+} pump (not shown). However, the loss of Ca^{2+} from the SR prior to addition of caffeine was limited due to the slow rate of efflux on addition of ADP (see Discussion). CTP and adenosine failed to facilitate the action of caffeine. Indeed, the mean amplitudes of responses obtained in the presence of 4·9 mM adenosine or CTP (and 0·1 mM ATP) were significantly smaller than responses obtained in the presence of 0·1 mM ATP alone. This suggests that CTP, and to a greater extent adenosine, inhibit caffeine-induced Ca^{2+} release under these conditions. Furthermore, it was found that complete substitution of 5 mM ATP with 5 mM

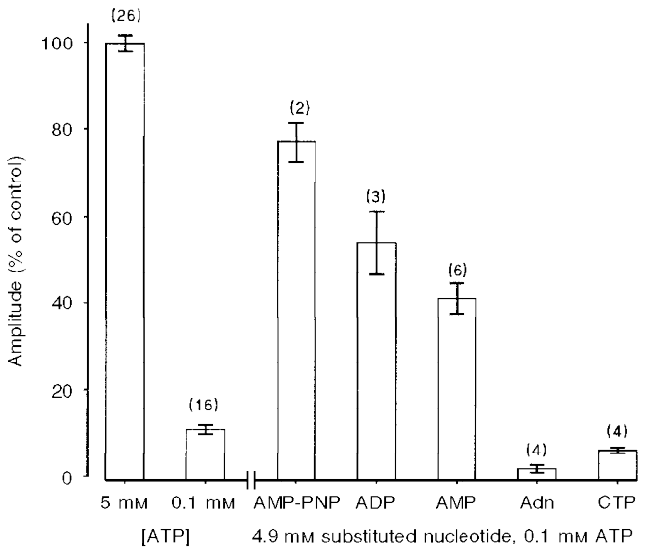

Figure 4. Effect of substituting 5 mM ATP with selected nucleotides

Each point is the mean ± S.E.M. expressed as a percentage of the control response obtained in the presence of 5 mM ATP using the protocol shown in Fig. 2*A*. Mean Ca^{2+} transient amplitude in the presence of 5 mM ATP or 0·1 mM ATP is shown. Responses obtained in the presence of 0·1 mM ATP and 4·9 mM AMP-PNP, ADP, AMP, adenosine (Adn) or CTP are also given. Responses in the presence of adenosine and CTP are significantly smaller than those obtained in the presence of 0·1 mM ATP alone ($P < 0·05$). The number of preparations is indicated in parentheses.

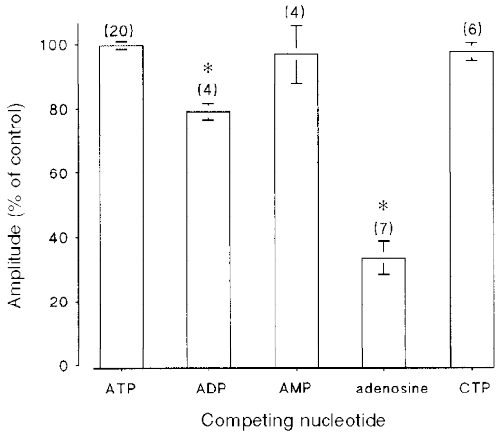

Figure 5. Effect of 5 mM nucleotide in the presence of 5 mM ATP
Each point is the mean ± S.E.M. expressed as a percentage of the control response obtained in the presence of 5 mM ATP alone, using the protocol shown in Fig. 2*A*. Mean Ca^{2+} transient amplitude in the presence of 5 mM ATP or 5 mM ATP in combination with 5 mM ADP, AMP, adenosine or CTP. * Responses significantly different ($P < 0{\cdot}05$) from those obtained in the presence of 5 mM ATP. The number of preparations is indicated in parentheses.

adenosine consistently abolished caffeine-induced Ca^{2+} release ($n = 4$, data not shown).

Competition between adenine nucleotides at the SR Ca^{2+} channel

One possible explanation for the inhibitory action of adenosine and CTP is that these compounds bind to the adenine nucleotide site on the SR Ca^{2+} channel, but do not induce activation or facilitate the action of caffeine. The possibility of competitive antagonism at the ATP binding site was investigated by application of caffeine in the presence of equal concentrations of ATP and other nucleotides. Figure 5 shows accumulated data illustrating the effect of 5 mM concentrations of selected nucleotides on the amplitude of the caffeine-induced response, in the presence of 5 mM ATP. All responses are expressed relative to the mean Ca^{2+} transient amplitude in the presence of 5 mM ATP alone. Again, a 2 min period was allowed to fully load the SR before introduction of 5 mM nucleotide in the continued presence of 5 mM ATP (not shown). Under these conditions, AMP and CTP produced only a small decrease in the caffeine-induced Ca^{2+} transient, which was not significant. A more pronounced decrease in the amplitude of the response occurred in the presence of ADP, although this may partially reflect a decrease in SR Ca^{2+} content due to the Ca^{2+} efflux which occurred prior to application of caffeine. However, 5 mM adenosine markedly reduced the amplitude of the caffeine-induced Ca^{2+} transient to 36 ± 4% (mean ± S.E.M., $n = 11$) of controls.

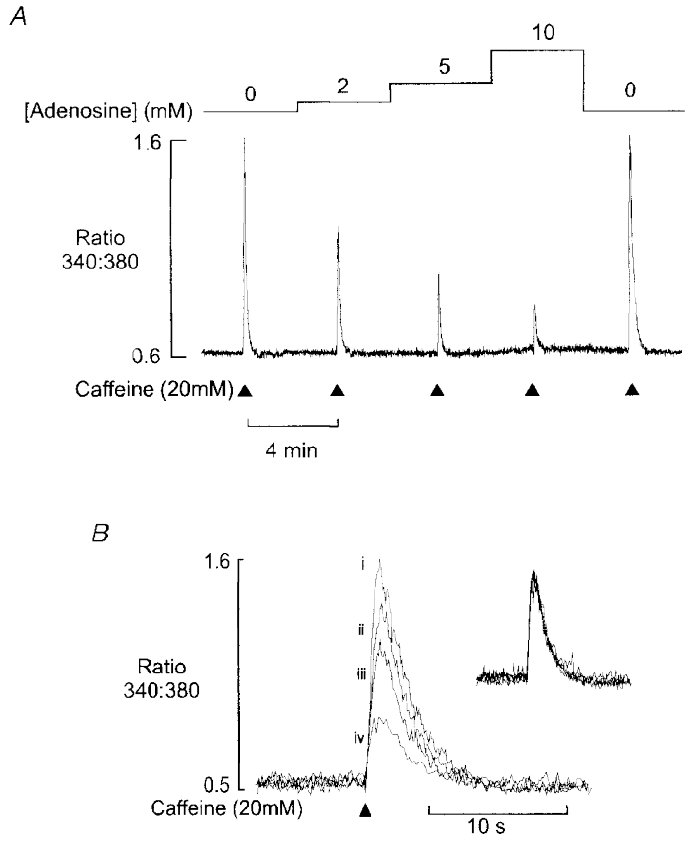

Figure 6. Effect of adenosine on caffeine-induced Ca^{2+} transient
A, continuous record of the 340 nm/380 nm fluorescence ratio from a saponin-permeabilized fibre in the presence of 100 nM Ca^{2+} and 5 mM ATP. Caffeine (20 mM) was briefly applied at 4 min intervals as indicated. Following an initial steady-state control response, the adenosine concentration was increased in a stepwise manner from 2 to 10 mM. This resulted in a corresponding decrease in the amplitude of the caffeine-induced Ca^{2+} transient. Removal of adenosine resulted in a rapid return to control levels. *B*, superimposed Ca^{2+} transients from the same preparation, in the presence of (i) 0, (ii) 2, (iii) 5 and (iv) 10 mM adenosine. Normalized responses (inset) show that the time course of the Ca^{2+} transient was unaffected.

The effect of adenosine on caffeine-induced Ca^{2+} release

The inhibitory effect of adenosine on caffeine-induced Ca^{2+} release was investigated in more detail, as shown in Fig. 6*A*. This record shows caffeine-induced Ca^{2+} transients obtained in a saponin-treated preparation in the constant presence of 5 mM ATP. Caffeine was briefly applied at 4 min intervals, and after an initial control Ca^{2+} response, the adenosine concentration was increased in a stepwise manner over the range 2–10 mM. The progressive increase in adenosine concentration resulted in corresponding decreases in the amplitude of the caffeine-induced Ca^{2+} transient, and this effect was fully and rapidly reversible. The fact that the introduction of adenosine was not associated with a release of Ca^{2+} suggests that the decline in the SR Ca^{2+} transient reflects inhibition of the release process, rather than a decrease in the SR Ca^{2+} content. Figure 6*B* shows superimposed Ca^{2+} transients from the same preparation in the absence (i) and presence (ii–iv) of increasing adenosine levels. These data, and the normalized responses (inset), show that adenosine reduces the amplitude of the caffeine-induced Ca^{2+} transient without affecting the time course of the response. This suggests that adenosine reduces the amount of released Ca^{2+}, without affecting the kinetics of the release process, or the activity of the SR Ca^{2+} pump. Figure 7*A* shows accumulated data illustrating the concentration dependence of the inhibitory action of adenosine on caffeine-induced Ca^{2+} release, in the presence of 5 mM ATP. On average, 10 mM adenosine reduced the amplitude of the response by 81·2 ± 3·46% (mean ± S.E.M., $n = 5$).

On balance, the data suggest that adenosine inhibits caffeine-induced Ca^{2+} release indirectly by antagonizing the binding of ATP to the adenine nucleotide site on the SR Ca^{2+} channel rather than direct inhibition of the caffeine-induced release process. This possibility is further supported by experiments involving the application of adenosine in the presence of differing levels of ATP. If adenosine acts by competitive antagonism of ATP binding to the SR Ca^{2+} channel, then the relationship shown in Fig. 3 suggests that the degree of inhibition should be more pronounced at 5 mM ATP than in the presence of 10 mM ATP. This was confirmed in experiments which showed that 5 mM adenosine reduced the caffeine-induced Ca^{2+} transient by 64 ± 4% (mean ± S.E.M., $n = 11$) in the presence of 5 mM ATP. However, in the presence of 10 mM ATP, the transient was reduced by only 20 ± 5·19% (mean ± S.E.M., $n = 4$) of the control response (Fig. 7*B*).

DISCUSSION

Effects of ATP on caffeine-induced Ca^{2+} release in skinned muscle preparations

The present study has demonstrated that in permeabilized muscle fibres, with an intact SR, the caffeine-sensitive efflux pathway is markedly influenced by cytosolic ATP. Decreasing the [ATP] from 10 to 0·1 mM reduced the amount of Ca^{2+} released in response to 20 mM caffeine. This is consistent with previous studies on heavy SR vesicles from rabbit skeletal muscle, where caffeine-activated $^{45}Ca^{2+}$ efflux was potentiated by addition of millimolar levels of ATP to the bathing medium. Similar results were obtained in SR Ca^{2+} channels incorporated into lipid bilayers, where the increase in P_o induced by 20 mM caffeine was more than doubled by addition of 2 mM ATP (Rousseau *et al.* 1988).

The present results, however, differ from previous work on saponin-permeabilized skeletal muscle fibres from the toad (Stienen *et al.* 1993). In this preparation, the amount of Ca^{2+}

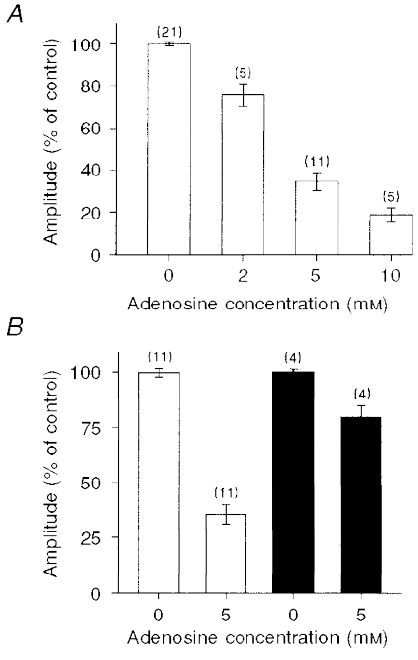

Figure 7. Interactions between caffeine and adenosine

A, relationship between adenosine concentration and caffeine-induced release. Mean (± S.E.M.) amplitude of the caffeine-induced Ca^{2+} transient in the absence and presence of 2, 5 or 10 mM adenosine. All responses were obtained in the presence of 5 mM ATP using the protocol shown in Fig. 6*A*. The number of preparations is indicated in parentheses. *B*, ATP dependence of adenosine-induced release inhibition. Each point is the mean (± S.E.M.) amplitude of the caffeine-induced Ca^{2+} transient in the presence or absence of 5 mM adenosine, at 5 (□) or 10 mM (■) ATP. All results are expressed relative to the mean response in the presence of 5 mM ATP, 0 mM adenosine. The number of preparations is indicated in parentheses.

released by caffeine appeared relatively unaffected by a large decrease in [ATP] from 5 mM to 10 μM. Indeed, it was reported that 5 mM caffeine released more than 90% of the total SR Ca^{2+} content, in the complete absence of ATP. However, the onset of Ca^{2+} release was delayed at low levels of ATP, and it was concluded that the rate of Ca^{2+} efflux was reduced. This contrasts with the present study, where decreasing the [ATP] from 10 mM to 100 μM markedly reduced the amplitude of the caffeine-induced Ca^{2+} transient, without effect on the time course of Ca^{2+} release (Fig. 2*B*).

This apparent discrepancy might be explained by differences in the experimental techniques and protocols as follows. (i) Stienen and colleagues used tension as an indirect index of SR Ca^{2+} release. Low levels of ATP have pronounced effects on myofilament Ca^{2+} sensitivity and peak force, which complicated the interpretation of the results. (ii) Responses obtained at low levels of ATP may also be influenced by changes in the activity of the SR Ca^{2+} pump. Previous work on skinned fibres has shown that re-uptake of Ca^{2+} by the SR can attenuate the rise in cytosolic [Ca^{2+}] following caffeine application (Makabe *et al.* 1996; Steele & Duke, 1997). Therefore, at low levels of ATP, where the rate of Ca^{2+} uptake is reduced (see below), a given amount of Ca^{2+} released from the SR would be expected to cause a larger Ca^{2+} and tension transient. (iii) When ATP is absent completely, a low level of channel activation combined with efflux via the Ca^{2+} leak pathway will fully deplete the SR given sufficient time. Hence, a large fraction of total SR Ca^{2+} may be released, even if caffeine is much less effective. (iv) If SR Ca^{2+} uptake is impaired, local accumulation of Ca^{2+} at the surface of the SR might also result in a progressive increase in the Ca^{2+} efflux rate, due to Ca^{2+}-induced Ca^{2+} release. Such an effect could explain the rise in tension after a very prolonged (6 s) delay, as observed by Stienen and colleagues. In the present study, complications associated with the effects of ATP on the myofilaments were avoided by direct measurement of SR Ca^{2+} release. We have also limited our study to ATP levels above that to support the activity of the SR Ca^{2+}-ATPase (~100 μM). Under these conditions, a clear relationship between caffeine-induced Ca^{2+} release and cytosolic [ATP] is observed.

Although not addressed in detail, one other interesting aspect of this study is that altering the cytosolic [ATP] over the range 0·1–5 mM had no effect on the SR Ca^{2+} content. Experiments on isolated SR vesicles suggest that increasing the [ATP] would be expected to progressively activate the SR Ca^{2+} channel (Smith *et al.* 1986). In skinned fibres, this should result in a release of Ca^{2+} from the SR and a decrease in the SR Ca^{2+} content. However, the present data suggest that under the conditions of this study, changes in the cytosolic [ATP] do not have major direct effects on the activity of the *in situ* SR Ca^{2+} channel. Instead, the occupancy of the adenine nucleotide site by ATP or other agonists appears to have a marked influence on the physiological release process (Owen *et al.* 1996) and the sensitivity of the SR Ca^{2+} channel to activation by drugs such as caffeine.

The marked dependence of caffeine-induced Ca^{2+} release on cytosolic [ATP] may have implications for experiments on intact and skinned skeletal muscle, where the amplitude of the Ca^{2+} or tension transient is commonly used as an index of SR Ca^{2+} content. The present results suggest that any intervention causing a local decrease in [ATP] at the junctional surface of the SR will reduce the fraction of the total Ca^{2+} released by caffeine. Such an effect might be misinterpreted as a decrease in SR Ca^{2+} content. The hydrolysis products substitute partially at the adenine nucleotide binding site (see below), limiting this potential difficulty. However, ADP releases Ca^{2+} from the SR via a ryanodine-insensitive efflux pathway which might further complicate interpretation of the data in circumstances where ATP is weakly buffered.

Interaction between caffeine and other nucleotides

A number of difficulties were encountered in experiments involving ADP which complicate the interpretation of the results. First, introduction of ADP was associated with a slow Ca^{2+} release from the SR. ADP-induced Ca^{2+} release was found to be insensitive to Ruthenium Red or ryanodine, but was abolished by the SR Ca^{2+} pump inhibitor cyclopiazonic acid (not shown). This is consistent with ADP-induced efflux via the SR Ca^{2+} pump (Hasselbach, 1978). If significant SR Ca^{2+} efflux occurs prior to addition of caffeine, a smaller release will occur and the effect of ADP and its ability to substitute for ATP may be underestimated. However, we estimate that this factor is unlikely to influence the Ca^{2+} transient by more than 10–20% because the slow nature of the ADP-induced Ca^{2+} release allowed caffeine to be applied before significant loss of SR Ca^{2+} occurred. A second potential difficulty is that a fraction of the ADP diffusing into the preparation will be converted to ATP via the creatine phosphokinase (CPK) or the adenylate kinase reactions. Inhibition of the myokinase with diadenosine pentaphosphate had no apparent effect on the responses obtained, suggesting that synthesis via this pathway is not of major importance under these conditions. However, it was not possible to study the effects of ADP in the absence of creatine phosphate (CrP) because it was found that SR Ca^{2+} uptake and release mechanisms were markedly impaired in the absence of CrP (Korge *et al.* 1993; Duke & Steele, 1998*b*). Despite this, it seems unlikely that the CPK reaction could reduce the ADP levels in the vicinity of the SR Ca^{2+} channel significantly for the following reasons. (i) ADP is free to diffuse from the effectively infinite volume of surrounding solution into the muscle. (ii) Any ATP produced is free to diffuse out of the muscle. (iii) The fact that ADP-induced reversal of the SR Ca^{2+} pump occurs in the presence of CrP demonstrates that high levels of ADP are present in the vicinity of the SR. (iv) Ca^{2+} transients are not observed in the presence of solutions containing millimolar ADP with CrP, but lacking ATP (not

shown). This suggests that insufficient ATP is produced from ADP to support the activity of the SR Ca^{2+} pump and the release mechanism. Thus, it seems reasonable to assume that the effect of adding ADP to the perfusing solution is not a secondary consequence of ATP synthesis. However, these uncertainties remain a limitation of the method.

The experiments involving substitution of ATP with other nucleotides showed that AMP-PNP, ADP and AMP also facilitate the action of caffeine on the SR Ca^{2+} channel, although none was as effective as ATP (Fig. 4). These results are consistent with data on isolated SR Ca^{2+} channels and vesicle preparations showing that the relative potency to induce activation of the Ca^{2+} channel is AMP-PNP > ADP > AMP (Morii & Tonomura, 1983; Meissner, 1984; Pessah *et al.* 1986). This structure–activity relationship of the *in situ* SR confirms previous suggestions that the number of phosphates attached to the 5′ carbon of the ribose ring determines the efficacy of the ligand. Failure of CTP to substitute for ATP is also consistent with previous studies showing that non-adenine triphosphates do not activate the skeletal SR Ca^{2+} channel (Meissner, 1984). Indeed, there was some evidence of a weak competitive antagonism when the [CTP] is much in excess of the [ATP] (Fig. 4) as has been reported in isolated SR (Morii & Tonomura, 1983).

Effects of adenosine

The effects of adenosine on the skeletal SR Ca^{2+} channel have not been characterized in detail previously. However, recent experiments on isolated channels have shown that adenosine is a potent activator of the cardiac SR Ca^{2+} channel (McGarry & Williams, 1994). It was found that both adenosine and caffeine maximally activated the channel in the presence of micromolar Ca^{2+}, although adenosine was more potent with an EC_{50} of approximately 0·75 mM. Application of 1 mM ATP to channels activated by 1 mM adenosine resulted in a decrease in P_o. This was interpreted as evidence that adenosine acts at the same site as ATP, but with greater potency. A disproportionate increase in P_o occurred when both caffeine and adenosine were introduced together, suggesting a synergistic interaction.

Based on these observations, adenosine might be expected to substitute for ATP at the adenine nucleotide binding site and facilitate the action of caffeine on skeletal muscle. However, adenosine failed to substitute for ATP or facilitate the Ca^{2+}-releasing action of caffeine (Fig. 4). Indeed, introduction of adenosine was associated with a marked concentration-dependent inhibition of caffeine-induced Ca^{2+} release in the presence of ATP (Figs 6 and 7*A*). The inhibitory effect of adenosine on caffeine-induced release was much less pronounced in the presence of 10 mM than 5 mM ATP. Given the relationship between caffeine-induced release and [ATP] (Fig. 3), this suggests that adenosine may competitively antagonize ATP binding to the SR Ca^{2+} channel.

The apparent difference between the present work and previous studies on isolated channels from cardiac muscle probably reflects intrinsic differences in the activation characteristics of cardiac and skeletal SR Ca^{2+} channels. It is known that RyR1 receptors are more sensitive to activation by adenine nucleotides (Meissner & Henderson, 1987). Experiments on rabbit skeletal muscle have also shown that the base compound adenine inhibits rather than potentiates caffeine-induced Ca^{2+} efflux in the presence of ATP (Rousseau *et al.* 1988). The present study suggests that in skeletal muscle (but not cardiac muscle) removal of a single phosphate group may reduce AMP from a moderately potent agonist at the adenine nucleotide site to adenosine, which acts a competitive antagonist.

The ability of adenosine to antagonize the action of ATP may be useful in determining whether other endogenous substances or drugs act at the adenine nucleotide binding site. However, further work is required to establish whether adenosine has similar effects in mammalian skeletal muscle. Frog skeletal muscle fibres contain roughly equal amounts of RyR1 and RyR3 (α and β) isoforms of the ryanodine receptor whereas mammalian muscle contains a much greater (although variable) proportion of RyR1 relative to RyR3 (Franzini-Armstrong & Protasi, 1997). Preliminary experiments suggest that in rat extensor digitorum longus muscle fibres, which contain virtually no RyR3, caffeine-induced Ca^{2+} release is similarly affected by cytosolic ATP and adenosine (not shown).

Physiological significance

Previous work on mechanically skinned rat skeletal muscle fibres has shown that the physiological, depolarization-induced Ca^{2+}-releasing process, is inhibited by a reduction in the cytosolic [ATP] (Owen *et al.* 1996). One possible explanation for this is that ATP directly facilitates the interaction between the t-tubule voltage sensor and the SR Ca^{2+} channel. However, the fact that caffeine-induced Ca^{2+} release is similarly affected by ATP, suggests that (i) caffeine-induced Ca^{2+} release and depolarization-induced Ca^{2+} release share a common pathway and (ii) ATP may influence the excitation–contraction coupling process by acting at a point subsequent to the initial voltage-dependent step. One possibility is that caffeine-induced Ca^{2+} release and depolarization-induced Ca^{2+} release both involve a component of positive feedback via Ca^{2+}-induced Ca^{2+} release, the sensitivity of which is modulated the cytosolic [ATP].

It has been suggested that a local reduction in [ATP] contributes to the rapid decline in SR Ca^{2+} release during the latter stages of fatigue by inhibiting the SR Ca^{2+} channel (Owen *et al.* 1996; Allen *et al.* 1997). One argument against this is that the ATP hydrolysis products may also bind to the adenine nucleotide binding site, thereby maintaining the sensitivity of the SR Ca^{2+} channel. However, this study has shown that the products of ATP breakdown are less potent than ATP in facilitating the action of caffeine in the

intact SR. Assuming that depolarization-induced Ca^{2+} release is similarly affected, a local decrease in [ATP] and accumulation of hydrolysis products would be expected to inhibit release.

Another interesting aspect of the present study is that adenosine was found to act as a competitive antagonist at the adenine nucleotide binding site. It has been shown that adenosine is released from intact frog skeletal muscle following stimulation (Rodrigo *et al.* 1998). There is also some evidence of a small rise in the average intracellular adenosine concentration following intense exercise or hypoxia in human skeletal muscle (Tullson *et al.* 1998). It has been argued that, although the mean fall in cytosolic [ATP] during fatigue is comparatively small (~20%), the local decrease in the vicinity of the SR Ca^{2+} channel might be much larger. Similarly, if local adenosine levels in the vicinity of the SR Ca^{2+} channel increased significantly during intense activity, the sensitivity of the SR Ca^{2+} channel would be further decreased by competitive antagonism at the adenine nucleotide binding site.

Conclusions

The results of this study demonstrate that caffeine-induced Ca^{2+} release exhibits a marked dependence on cytosolic [ATP] in saponin-permeabilized skeletal muscle fibres. Other adenine nucleotides partially substituted for ATP while adenosine inhibited caffeine-induced Ca^{2+} release in a manner consistent with competitive antagonism at the adenine nucleotide binding site. These data suggest that the number of phosphates attached to the 5′ carbon of the ribose ring determines the efficacy of the ligand and that removal of a single phosphate group transforms AMP from a partial agonist to adenosine, which acts as a competitive antagonist under these conditions.

Allen, D. G., Lannergren, J. & Westerblad, H. (1997). The role of ATP in the regulation of intracellular Ca^{2+} release in single fibres of mouse skeletal muscle. *Journal of Physiology* **498**, 587–600.

Brandt, N. R., Caswell, A. H., Brunschwig, J. P., Kang, J. J., Antoniu, B. & Ikemoto, N. (1992). Effects of anti-triadin antibody on calcium release from sarcoplasmic reticulum *FASEB Journal* **6**, A431.

Brillantes, A. M. B., Ondrias, K., Scott, A., Korbinsky, E., Ondriasova, E., Moschella, M. C., Jayaraman, T., Landers, M., Ehrlich, B. E. & Marks, A. R. (1994). Stabilization of calcium-release channel (ryanodine receptor) function by FK506-binding protein. *Cell* **77**, 513–523.

Coronado, R., Morrissette, J., Sukhareva, M. & Vaughan, D. M. (1994). Structure and function of ryanodine receptors. *American Journal of Physiology* **266**, C1485–1504.

Duke, A. M. & Steele, D. S. (1998*a*). Effects of cyclopiazonic acid on Ca^{2+} regulation by the sarcoplasmic reticulum in saponin permeabilized skeletal muscle fibres. *Pflügers Archiv* **436**, 104–111.

Duke, A. M. & Steele, D. S. (1998*b*). Effects of phosphocreatine on Ca^{2+} regulation by the sarcoplasmic reticulum in isolated mechanically skinned rat skeletal muscle fibres. *Journal of Physiology* **509.P**, 45*P*.

Fabiato, A. & Fabiato, F. (1979). Calculator programs for computing the composition of the solutions containing multiple metals and ligands used for experiments in skinned muscle cells. *Journal de Physiologie* **75**, 463–505.

Franzini-Armstrong, C. & Protasi, F. (1997). Ryanodine receptors of striated muscles: a complex channel capable of multiple interactions. *Physiological Reviews* **77**, 699–729.

Hasselbach, W. (1978). The reversibility of the sarcoplasmic calcium pump. *Biochimica et Biophysica Acta* **515**, 23–53.

Ikemoto, N., Ronjat, M., Meszaros, L. G. & Koshita, M. (1989). Postulated role of calsequestrin in the regulation of calcium release from the sarcoplasmic reticulum. *Biochemistry* **28**, 6764–6771.

Korge, P., Byrd, S. K. & Campbell, K. B. (1993). Functional coupling between sarcoplasmic reticulum bound creatine kinase and Ca^{2+}-ATPase. *European Journal of Biochemistry* **213**, 973–980.

McGarry, S. J. & Williams, A. J. (1994). Adenosine discriminates between the caffeine and adenine nucleotide sites on the sheep cardiac sarcoplasmic reticulum calcium release channel. *Journal of Membrane Biology* **137**, 169–177.

Makabe, M., Werner, O. & Fink, R. H. A. (1996). The contribution of the sarcoplasmic reticulum Ca^{2+}-transport ATPase to caffeine-induced Ca^{2+} transients of murine skinned skeletal muscle. *Pflügers Archiv* **423**, 717–726.

Mayrleitner, M., Timerman, A. P., Wiederrecht, G. & Fleischer, S. (1994). The calcium release channel of sarcoplasmic reticulum is modulated by FK-506 binding protein – effect of FKBP-12 on single channel activity of the skeletal muscle ryanodine receptor. *Cell Calcium* **15**, 99–108.

Meissner, G. (1984). Adenine nucleotide stimulation of Ca^{2+}-induced Ca^{2+} release in sarcoplasmic reticulum. *Journal of Biological Chemistry* **259**, 2365–2374.

Meissner, G. & Henderson, J. S. (1987). Rapid calcium release from cardiac sarcoplasmic reticulum vesicles is dependent on Ca^{2+} and is modulated by Mg^{2+}, adenine nucleotide, and calmodulin. *Journal of Biological Chemistry* **262**, 3065–3073.

Miller, D. J. & Smith, G. L. (1984). EGTA purity and the buffering of calcium ions in physiological solutions. *American Journal of Physiology* **246**, C160–166.

Morii, H. & Tonomura, Y. (1983). The gating behavior of a channel for Ca^{2+} induced Ca^{2+} release in fragmented sarcoplasmic-reticulum. *Journal of Biochemistry* **93**, 1271–1285.

O'Neill, S. C., Donoso, P. & Eisner, D. A. (1990). The role of $[Ca^{2+}]_i$ and $[Ca^{2+}]$ sensitization in the caffeine contracture of rat myocytes: measurement of $[Ca^{2+}]_i$ and $[caffeine]_i$. *Journal of Physiology* **425**, 55–70.

Owen, V. J., Lamb, G. D. & Stephenson, D. G. (1996). Effect of low [ATP] on depolarization-induced Ca^{2+} release in skeletal muscle fibres of the toad. *Journal of Physiology* **493**, 309–315.

Pessah, I. N., Stambuk, R. A. & Casida, J. E. (1986). Ca^{2+} activated ryanodine binding: Mechanisms of sensitivity and intensity modulation by Mg, caffeine and adenine nucleotides. *Molecular Pharmacology* **31**, 232–238.

Rodrigo, A., Cunha, A. & Sebastiao, M. (1998). Adenosine and adenine nucleotides are independently released from both nerve terminals and muscle fibres upon electrical stimulation of innervated skeletal muscle of the frog. *Pflügers Archiv* **424**, 503–510.

Rousseau, E., Ladine, J., Liu, Q. & Meissner, G. (1988). Activation of the Ca^{2+} release channel of skeletal muscle sarcoplasmic reticulum by caffeine and related compounds. *Archives of Biochemistry and Biophysics* **267**, 75–86.

Sitsapesan, R., McGarry, S. J. & Williams, A. J. (1995). Cyclic ADP-ribose, the ryanodine receptor and Ca^{2+} release. *Trends in Pharmacological Sciences* **16**, 386–390.

Sitsapesan, R. & Williams, A. J. (1995). Cyclic ADP-ribose activation of the sheep skeletal ryanodine receptor Ca^{2+}-release channel is dependent upon luminal Ca^{2+}. *British Journal of Pharmacology* **114**, P246.

Smith, G. L. & Miller, D. J. (1985). Potentiometric measurements of stoichiometric and apparent affinity constants of EGTA for protons and divalent ions including calcium. *Biochimica et Biophysica Acta* **839**, 287–299.

Smith, G. L. & Steele, D. S. (1993). Effects of 2,3 butanedione monoxime on sarcoplasmic reticulum of saponin treated rat cardiac muscle. *American Journal of Physiology* **265**, H1493–1500.

Smith, J. S., Coronado, R. & Meissner, G. (1986). Single channel measurements of the calcium release channel from skeletal muscle sarcoplasmic reticulum – activation by Ca^{2+} and ATP and modulation by Mg^{2+}. *Journal of General Physiology* **88**, 573–588.

Steele, D. S. & Duke, A. M. (1997). Effects of cyclopiazonic acid on caffeine-induced Ca^{2+} release in isolated saponin skinned frog skeletal muscle fibres. *Journal of Physiology* **501.P**, 144*P*.

Stienen, G. J. M., Grass, I. A. & Elzinga, G. (1993). Uptake and caffeine induced release of calcium in fast muscle fibres of *Xenopus laevis*: effects of MgATP and Pi. *American Journal of Physiology* **265**, C650–657.

Tullson, C. P., Bangsbo, J., Hellsten, Y. & Richter, E. A. (1998). IMP metabolism in human skeletal muscle after exhaustive exercise. *Journal of Applied Physiology* **87**, 146–152.

Wagenknecht, T., Grassucci, R., Berkowitz, J., Wiederrecht, G. J., Xin, H. B. & Fleischer, S. (1996). Cryoelectron microscopy resolves fk506-binding protein sites on the skeletal muscle ryanodine receptor. *Biophysical Journal* **70**, 1709–1715.

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