The Interactions of ATP, ADP, and Inorganic Phosphate with the Sheep Cardiac Ryanodine Receptor

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ABSTRACT The effects of ATP, ADP, and inorganic phosphate (P_i) on the gating of native sheep cardiac ryanodine receptor channels incorporated into planar phospholipid bilayers were investigated. We demonstrate that ATP and ADP can activate the channel by Ca^{2+}-dependent and Ca^{2+}-independent mechanisms. ATP and ADP appear to compete for the same site/s on the cardiac ryanodine receptor, and in the presence of cytosolic Ca^{2+} both agents tend to inactivate the channel at supramaximal concentrations. Our results reveal that ATP not only has a greater affinity for the adenine nucleotide site/s than ADP, but also has a greater efficacy. The EC_{50} value for channel activation is ~0.2 mM for ATP compared to 1.2 mM for ADP. Most interesting is the fact that, even in the presence of cytosolic Ca^{2+}, ADP cannot activate the channel much above an open probability (P_o) of 0.5, and therefore acts as a partial agonist at the adenine nucleotide binding site on the channel. We demonstrate that P_i also increases P_o in a concentration and Ca^{2+}-dependent manner, but unlike ATP and ADP, has no effect in the absence of activating cytosolic [Ca^{2+}]. We demonstrate that P_i does not interact with the adenine nucleotide site/s but binds to a distinct domain on the channel to produce an increase in P_o.

INTRODUCTION

Early work by Fabiato and Fabiato (1978) demonstrated that adenine nucleotides could stimulate Ca^{2+}-induced Ca^{2+} release (CICR) in cardiac muscle. Subsequently, Smith et al. (1986) demonstrated that physiological levels of ATP directly activated skeletal muscle ryanodine receptors (RyR) incorporated into planar phospholipid bilayers. The nonhydrolyzable analog of ATP, AMP-PCP, also increased P_o, indicating that the effect of ATP was not the result of phosphorylation of the channel or a closely associated protein. Surprisingly, very little is known about the mechanisms involved in ATP activation of the cardiac ryanodine receptor. It is thought that ATP binds to a site on the cardiac ryanodine receptor that is distinct from the caffeine site (McGarry and Williams, 1994) and present evidence suggests that ATP, adenosine, α,β-methylene-ATP, cyclic ADP-ribose (cADPR), ADP-ribose, and βNAD^+ bind to a common site on the sheep cardiac ryanodine receptor channel to elicit an increase in P_o (McGarry and Williams, 1994; Sitsapesan et al., 1995).

The levels of many adenine nucleotides, and correspondingly the level of P_i, change by large amounts during myocardial ischemia (Allen and Orchard, 1987) and therefore it is important to evaluate if and how any of these agents affect the gating of the cardiac RyR channel. Myocardial ischemia leads to marked changes in intracellular Ca^{2+} handling and a rapid reduction in contractile force. It has been proposed that the increased levels of P_i and ADP that occur during ischemia contribute to the contractile failure. Both P_i and ADP have been shown to decrease the Ca^{2+} content of the SR in skinned isolated cardiac muscle preparations, but there is disagreement over the precise mechanisms involved (Smith and Steele, 1992; Steele et al., 1995, 1996; Xiang and Kentish, 1995). Fruen et al. (1994a,b) reported that P_i stimulated ryanodine binding in skeletal, but not cardiac, SR membrane fractions, thereby inferring that P_i can increase the P_o of skeletal, but not cardiac, RyR. The possibility that P_i could reduce cardiac SR Ca^{2+} content by activating cardiac RyR was therefore dismissed. However, differences between the effects of caffeine-induced and Ca^{2+}-induced release of Ca^{2+} from the SR led to the suggestion that P_i may in fact directly activate the cardiac RyR (Xiang and Kentish, 1995). It was also reported that ADP potentiated, rather than reduced, CICR and found that P_i enhanced this effect. They suggested that ADP and P_i exerted these effects by binding to the adenine nucleotide binding site on the cardiac RyR.

We have therefore examined the mechanism by which ATP activates the cardiac RyR and tested the hypothesis that ADP and P_i may activate the channel by binding to the adenine nucleotide site. We find that adenine nucleotides induce a complex mode of gating. Our results indicate that ADP and ATP share a common binding site on the channel, but that ADP is a partial agonist at this site. In contrast to the report by Fruen et al. (1994a) we also demonstrate that P_i does activate the cardiac RyR. P_i does not appear to bind to the adenine nucleotide binding site, but interacts with the cardiac RyR by a different mechanism.

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METHODS

Preparation of SR membrane vesicles and planar lipid bilayer methods

SR membrane vesicles were prepared from sheep cardiac muscle as previously described by Sitsapesan et al. (1991b). Heavy SR membrane

vesicles were frozen rapidly in liquid nitrogen and stored at −80°C. Vesicles were fused with planar phosphatidylethanolamine lipid bilayers as previously described (Sitsapesan et al., 1991b). The vesicles fused in a fixed orientation such that the *cis* chamber corresponded to the cytosolic space and the *trans* chamber to the SR lumen. The *trans* chamber was held at ground and the *cis* chamber held at potentials relative to ground. After fusion the *cis* chamber was perfused with 250 mM HEPES, 125 mM Tris, 10 μM free Ca^{2+} buffered with EGTA and $CaCl_2$, pH 7.2, and the *trans* chamber was perfused with a solution containing 250 mM glutamic acid, 10 mM HEPES, pH 7.2 with $Ca(OH)_2$ (free $[Ca^{2+}]$ ~50 mM). The free $[Ca^{2+}]$ and pH of the solutions were measured at 23°C using a calcium electrode (Orion 93-20) and Ross-type pH electrode (Orion 81-55) as described previously in detail (Sitsapesan et al., 1991b). The free $[Ca^{2+}]$ of the cytosolic solutions was maintained at 10 ± 2 μM with 1 mM EGTA and 0.95 mM total $CaCl_2$. Additions of ADP (≤50 mM), P_i (KH_2PO_4, ≤100 mM) and ATP (≤5 mM), did not alter the free $[Ca^{2+}]$ of the solution. To investigate the effects of 10 and 20 mM ATP, the *cis* chamber was perfused with a solution containing 1 mM EGTA and 2.3 mM $CaCl_2$ to maintain the free $[Ca^{2+}]$ at 10 ± 2 μM. Subnanomolar $[Ca^{2+}]$ were obtained by additions of EGTA (12 mM) and the free $[Ca^{2+}]$ was calculated using the computer program EQCAL (Biosoft, Cambridge, UK). ATP, ADP, and P_i were obtained from Sigma (Poole, UK).

The ATP used in the experiments was 99% pure (assessed by Sigma by HPLC), and to determine whether degradation of ATP occurred during the experiments we carried out control experiments in which the [ATP] of our solutions was measured using the luciferin-luciferase reaction first described by Stanley and Williams (1969). For the control experiments, SR vesicles were fused with the bilayers and the *cis* and *trans* chambers were perfused with the recording solutions as described above. ATP (1 mM) was added to the *cis* chamber and the levels of ATP were measured immediately after addition to the chamber and at 3 and 9 min (the longest duration of an ATP experiment) later. After 3 and 9 min the [ATP] was 102.3 ± 3.9% [mean value ± standard error of the mean (mean ± SE $n = 4$)] and 101.0% ± 2.8% (mean ± SE; $n = 4$) of the initial values, respectively. Therefore, there is no detectable degradation of ATP during the experiments. The mean ± SE is given where $n \geq 4$. For $n = 3$, standard deviation (SD) is given.

Data acquisition and analysis

Single-channel recordings were displayed on an oscilloscope and recorded on Digital Audio Tape (DAT). All steady-state recordings were carried out at 0 mV. Under these conditions, current flow through the cardiac RyR was in the luminal-to-cytosolic direction. Current recordings were filtered at 1 kHz and digitized at 2 kHz. Channel open probability (P_o) and the lifetimes of open and closed events were monitored by 50% threshold analysis. Channel P_o values were obtained from 3 min of steady-state recording. Lifetime analysis was carried out only when a single channel incorporated into the bilayer. Events <1 ms in duration were not fully resolved and were excluded from lifetime analysis. Lifetimes accumulated from ~3-min steady-state recordings were stored in sequential files and displayed in noncumulative histograms. Individual lifetimes were fitted to a probability density function (pdf) by the method of maximum likelihood (Colquhoun and Sigworth, 1983) according to the equation: $f(t) = a_1(1/\tau_1)\exp(-t/\tau_1) + \ldots + a_n(1/\tau_n)\exp(-t/\tau_n)$ with areas a and time constants τ. A missed events correction was applied as described by Colquhoun and Sigworth (1983). A likelihood ratio test (Blatz and Magleby, 1986) was used to compare fits to up to four exponentials by testing twice the difference in $\log_e$ (likelihood) against the chi-squared distribution at the 1% level. Single-channel current amplitudes were measured from digitized data using manually controlled cursors.

RESULTS

Effects of ATP on the cardiac RyR

As has been previously reported for the skeletal RyR (Smith et al., 1985) we found that ATP modulated the gating of the cardiac channel but did not cause any observable changes in conduction (data not shown). Fig. 1 (*top*) illustrates the effects of ATP on the gating of a representative cardiac RyR incorporated into a bilayer. In the presence of 10 μM cytosolic Ca^{2+}, 100 μM cytosolic ATP increased open

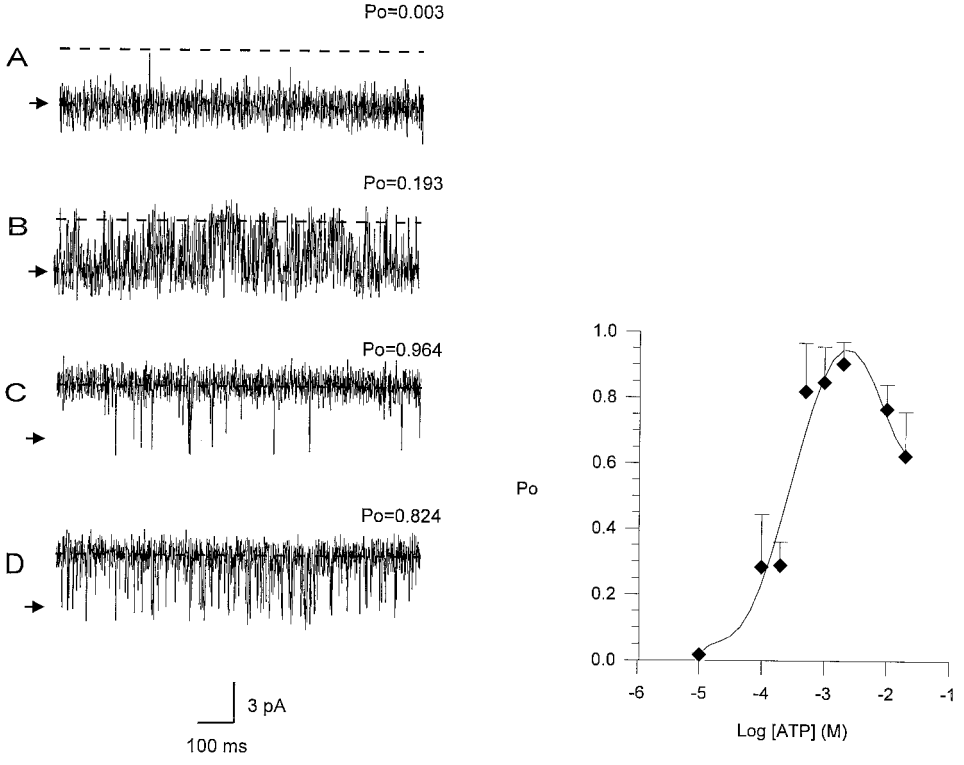

FIGURE 1 (*Top*) Activation of a representative single cardiac RyR by ATP. In (*A*) the channel is activated solely by 10 μM cytosolic Ca^{2+}. Subsequent additions of 100 μM (*B*), 2 mM (*C*), and 10 mM ATP (*D*) to the cytosolic solution are shown. The holding potential was 0 mV. The dotted lines indicate the open channel level and the arrows indicate the zero current level. Channel open probability is indicated above each trace. (*Bottom*) The effects of cytosolic ATP on the P_o of the sheep cardiac channel. The experiments were carried out in the presence of 10 μM cytosolic Ca^{2+}. The mean and standard error of four observations are shown.

probability (P_o) from 0.052 ± 0.019 (mean ± SE; $n = 11$) to 0.284 ± 0.161 (mean ± SE; $n = 4$). Peak P_o values were obtained in the presence of 2 mM ATP, which almost fully opened the channel, as shown in trace *C* (P_o = 0.905 ± 0.066; mean ± SE, $n = 4$). Further increases in [ATP] resulted in a decline in P_o, as can be observed in Fig. 1 (*top, trace D*). At 20 mM ATP the P_o was 0.626 ± 0.135 (mean ± SE; $n = 4$).

Fig. 1 (*bottom*) illustrates the relationship between the cytosolic [ATP] and the P_o of the sheep cardiac RyR in the presence of 10 μM cytosolic Ca^{2+}. Half maximal activation of the channel occurred at 0.22 mM ATP and the Hill coefficient was 1.5. This indicates that at least two molecules of ATP will bind to the channel to cause maximal activation.

Lifetime analysis provides more information about the mechanisms involved in ATP activation of the channel. It is well documented that both the cardiac and skeletal isoforms of the RyR exhibit brief open events when activated by cytosolic Ca^{2+} alone (Smith et al., 1986; Ashley and Williams, 1990; Sitsapesan and Williams, 1994, 1995). Reported mean open lifetime values are generally between 0.5 and 1 ms and these values depend heavily on the resolution of the single channel events as the mean open time is very close to the minimum resolvable event time. Fig. 2 *A* shows the open and closed lifetime distributions together with pdfs of a typical single cardiac RyR activated by 10 μM cytosolic Ca^{2+}. The best fit to the open lifetime distribution is obtained with two exponentials, indicating that the channel has at least two open states. Ninety-seven percent of the events are <2 ms in duration. In contrast, the closed lifetimes are of longer duration and a triple exponential produces the best fit, indicating the presence of at least three closed states.

In the presence of 100 μM ATP we observed a large increase in the number of brief openings and only a slight increase in the mean open time from 0.94 ± 0.30 ms (mean ± SE; $n = 4$) to 2.31 ± 0.81 ms (mean ± SE; $n = 4$). Lifetime analysis demonstrates that in the presence of 100 μM ATP (Fig. 2, *A* and *B*) the duration of all three closed states decreases. We also observed an increase in the duration of the open lifetimes and a third longer component to the pdf. However, although ATP is modulating both the frequency and duration of the open events, clearly at this concentration the main factor causing the increase in channel P_o is the increase in the frequency of channel opening.

Increasing the [ATP] to 2 mM almost fully activates the channel and increases the mean open time to 70.16 ± 51.24 ms ($n = 4$). In Fig. 1 (*top, trace C*) it can be seen that at this concentration of ATP the channel is virtually always open. Only very brief closings occur and many of these events are so brief that they are not fully resolved. Fig. 2 *C* demonstrates the marked changes in the distributions of both open and closed lifetimes at 2 mM ATP. The open lifetime distribution is now fitted by a pdf with two exponential components with two long time constants. The very brief open time constant can no longer be detected and at optimum [ATP] it appears that the channel can only dwell in the two long open states. Interestingly, the loss of the shortest time constant from the open lifetime distribution is coupled to the loss of the longest time constant from the closed lifetime distribution. It therefore appears that the short open events are linked with the long closing events, and vice versa.

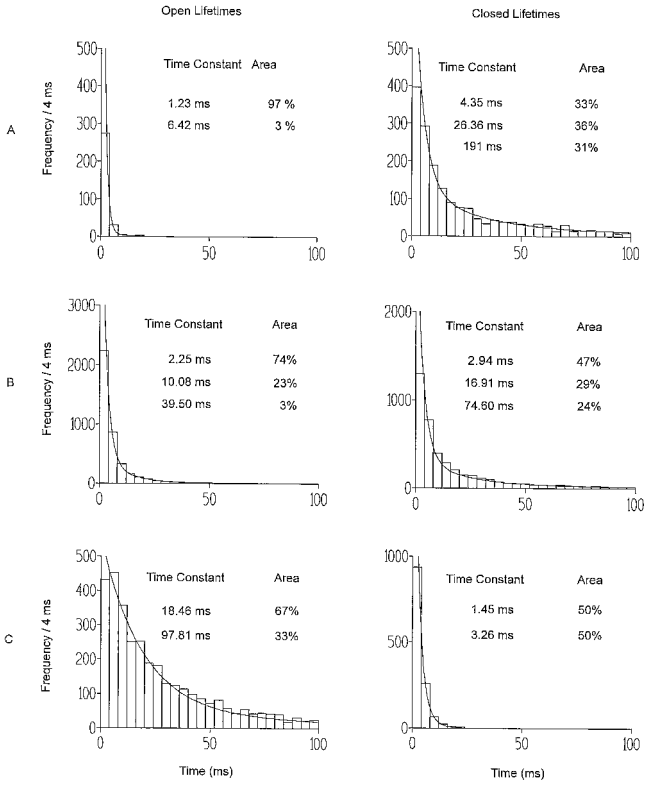

FIGURE 2 Effects of ATP on open (*left*) and closed (*right*) lifetime distributions and probability density functions (pdf). The time constants and percentage areas obtained from the best fit to the data are shown. Lifetimes were determined as described in Methods. In (*A*) the channel is activated by 10 μM cytosolic Ca^{2+} alone. In (*B*) and (*C*) the effects of 100 μM and 2 mM ATP are shown.

We have previously reported in abstract form that high concentrations of cytosolic Ca^{2+} or ATP can inactivate the cardiac RyR (Sitsapesan et al., 1991a). The inactivation of the channel by [ATP] >5 mM is clearly demonstrated in Fig. 1. As the [ATP] is increased above the optimal level, increased numbers of channel closing events lead to the fall in P_o.

ATP can activate the sheep skeletal RyR at subactivating levels of cytosolic Ca^{2+} (Sitsapesan and Williams, 1995) but it has been reported that at nanomolar levels of Ca^{2+}, while ATP can still effectively stimulate Ca^{2+} efflux in skeletal SR vesicles, in cardiac vesicles ATP has very little effect (Meissner and Henderson, 1987). We therefore tested the hypothesis that ATP could only activate the cardiac channel in the presence of activating levels of cytosolic Ca^{2+}. Fig. 3 shows the effects of ATP on a representative cardiac RyR in the absence of activating cytosolic Ca^{2+}. In

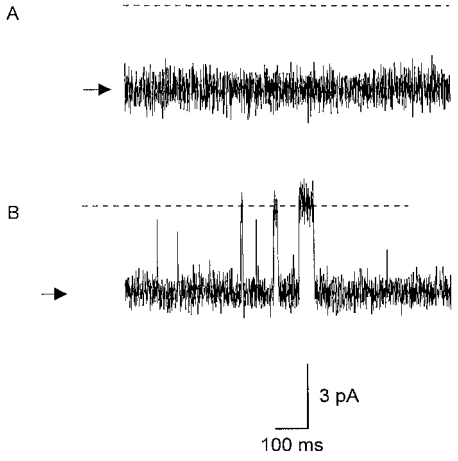

FIGURE 3 ATP can activate RyR by a Ca^{2+}-independent mechanism. (*A*) A single cardiac channel has incorporated into the bilayer and the cytosolic free [Ca^{2+}] has been lowered to picomolar levels by the addition of 12 mM EGTA. Under these conditions there are no channel openings. The dotted lines indicate the open channel level and the arrows indicate the zero current level. (*B*) 2 mM ATP has been added to the *cis* chamber and long but infrequent openings occur.

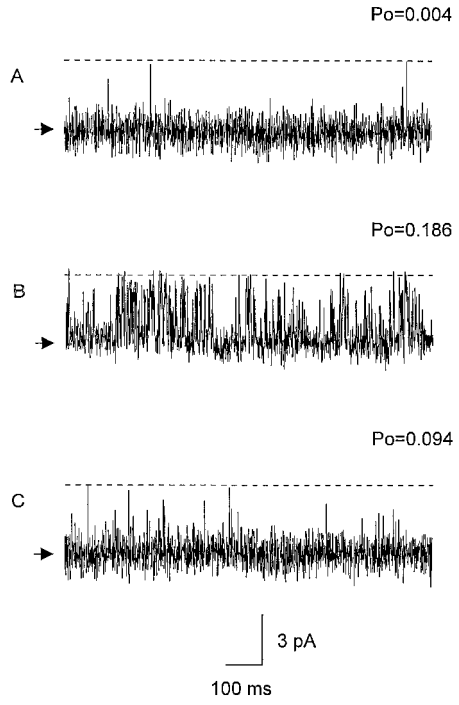

FIGURE 4 Effects of ADP on a single cardiac RyR. The channel was activated by 10 μM cytosolic Ca^{2+} alone (*A*) and by 10 μM cytosolic Ca^{2+} in the presence of 10 mM (*B*) and 50 mM ADP (*C*). Dotted lines indicate the open channel level and arrows indicate the zero current level. Channel open probability is indicated above each trace.

trace *A* the cytosolic [Ca^{2+}] was lowered to picomolar levels by adding 12 mM EGTA to the cytosolic channel face, and the channel remained completely closed. After the addition of 2 mM ATP to the *cis* chamber (*trace B*), channel openings were observed and P_o was increased from 0 ± 0 to 0.016 ± 0.014 (SD; $n = 3$). The mean open time was 2.55 ± 1.83 ms, which is longer than that observed when the channel is activated solely by cytosolic Ca^{2+} or by ATP in the presence of Ca^{2+} at comparable P_o values. In the absence of cytosolic Ca^{2+}, ATP was not able to fully activate the channel (at least in concentrations ≤10 mM). The ATP-activated channel therefore exhibits different gating kinetics to the channel activated by ATP plus cytosolic Ca^{2+}.

Effects of ADP on the Ca^{2+}-release channel

As for ATP, we found that ADP caused no significant changes to the current-voltage relationship of the cardiac RyR (data not shown). In the presence of 10 μM cytosolic Ca^{2+}, ADP increased P_o in a concentration-dependent manner but was far less effective than ATP. The effects of two concentrations of ADP on a single RyR are shown in Fig. 4. 10 mM ADP was required to increase P_o from 0.020 ± 0.009 to 0.490 ± 0.197 (mean ± SE; $n = 4$). In comparison, ~0.2 mM ATP activates the channel to the same degree. The relationship between [ADP] and P_o is illustrated in Fig. 5. The EC_{50} value for channel activation by ADP was 1.2 mM and the Hill coefficient was 0.966. An important observation was that ADP was unable to fully activate the channel even in the presence of activating levels of Ca^{2+} (10 μM). The highest P_o value observed with ADP was 0.578 ± 0.214 (mean ± SE; $n = 4$) at 20 mM ADP. Further increases in [ADP] led to reductions in P_o. At 50 mM ADP, P_o was 0.275 ± 0.240 (mean ± SE; $n = 4$).

There are obvious similarities in the mechanisms by which ATP and ADP activate the channel. A comparison of Figs. 1 and 4 demonstrates that both agonists are effective at increasing the frequency of channel opening. However, ADP does not cause very large increases in mean open time. Even at optimum ADP concentrations the increase in mean open time is not so marked as that occurring in the presence

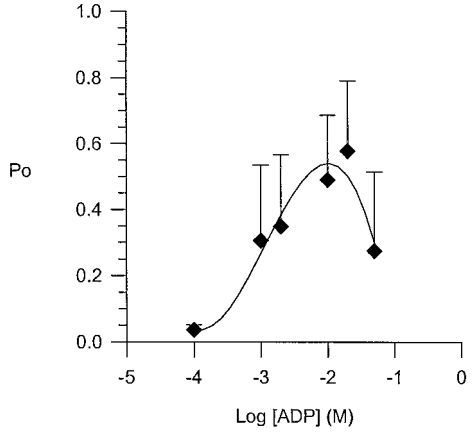

FIGURE 5 The relationship between [ADP] and P_o in the presence of 10 μM cytosolic free Ca^{2+}. The mean and standard error of four observations are shown.

of ATP. For example, in a typical channel activated by the optimal concentration of 20 mM ADP, the P_o increases from 0.004 to 0.407 but the mean open lifetime only increases from 0.55 ms to 1.37 ms. As for ATP, ADP was able to induce Ca^{2+}-independent openings, although even at the concentration of 20 mM the openings were infrequent (<10 openings per minute).

Site of action of ADP

ADP possesses structural similarities to ATP, and therefore may compete with ATP for the adenine nucleotide binding site/s on the RyR. If the two agents share common binding sites, then it would be expected that one would not potentiate the other. In fact, we find that ADP decreases the P_o of channels already activated by a submaximal concentration of ATP. A typical example of such an experiment is shown in Fig. 6. P_o decreased from 0.839 ± 0.133 (SD; $n = 3$) in the presence of 10 μM cytosolic Ca^{2+} and 500 μM ATP to 0.689 ± 0.208 (SD; $n = 3$) after the addition of 1 mM ADP. A further decrease in P_o to 0.401 ± 0.353 (SD; $n = 3$) was seen when [ADP] was increased to 10 mM. Thus the actions of ADP support the idea that ADP is acting at the same sites as ATP. Moreover, since ADP is unable to fully activate the channel (Fig. 5) and is apparently antagonizing the effects of ATP to some degree, ADP is behaving as a partial agonist at the adenine nucleotide binding sites.

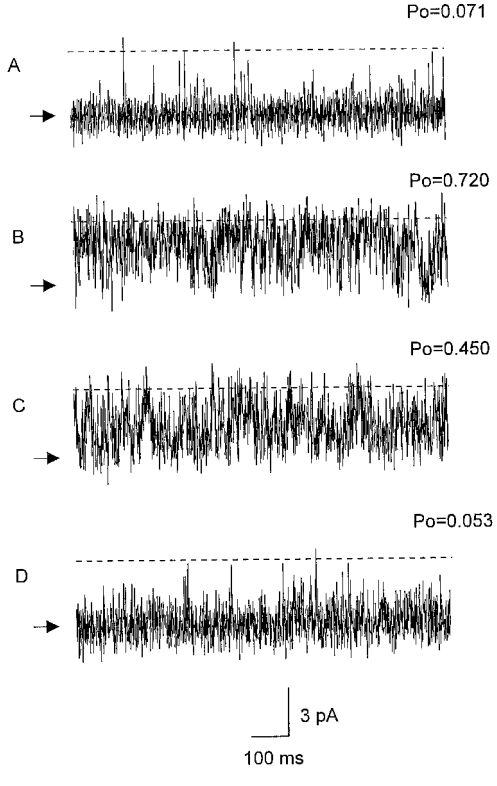

FIGURE 6 Effects of ADP on cardiac RyR already activated by Ca^{2+} plus ATP. The dotted lines indicate the open channel level and the arrows indicate the zero current level. In (*A*) the channel is activated by 10 μM cytosolic Ca^{2+} alone. In (*B*) 500 μM ATP has been added and P_o has increased to 0.720. In (*C*) the subsequent addition of 1 mM ADP decreases P_o to 0.450. In (*D*) the [ADP] has been increased to 10 mM and P_o is further reduced to 0.053.

Channel activation by P_i

Fruen et al. (1994a, b) reported that millimolar levels of P_i increased the P_o of purified skeletal RyR incorporated into bilayers and increased ryanodine binding to skeletal SR vesicles, but did not affect ryanodine binding to cardiac SR. However, Xiang and Kentish (1995) suggested that P_i may slightly activate SR Ca^{2+} release by directly activating the cardiac RyR. In order to resolve this issue we investigated the effects of P_i on the cardiac RyR. Fig. 7 demonstrates the typical effects of inorganic phosphate (P_i) on the gating of a single cardiac RyR. In the presence of 10 μM cytosolic free Ca^{2+}, millimolar concentrations of P_i caused a concentration-dependent increase in P_o. For example, 20 mM P_i increased P_o from 0.009 ± 0.003 (mean ± SE; $n = 5$) to 0.077 ± 0.025 (mean ± SE; $n = 5$). Further increasing [P_i] to 100 mM increased P_o to 0.244 ± 0.206 (SD; $n = 3$). The [P_i]-P_o relationship is shown in Fig. 8. The effect of P_i was not due to an increase in ionic strength as adding KCl (≤100 mM) to the cytosolic channel side under the same ionic conditions had no effect on P_o (P_o was 0.079 ± 0.016 and 0.063 ± 0.061 (SD; $n = 3$), respectively, in the absence and presence of 100 mM KCl). Relatively high concentrations of P_i are required to produce significant increases in

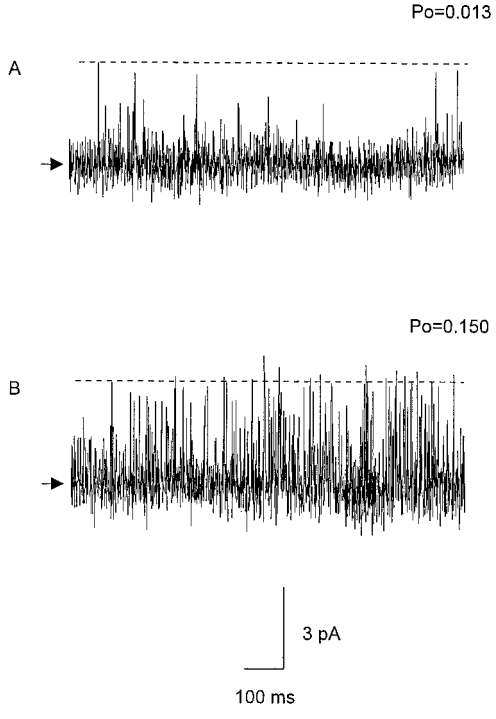

FIGURE 7 Activation of a representative single cardiac RyR by P_i. The dotted lines indicate the open channel level and the arrows indicate the zero current level. The channel is activated by 10 μM cytosolic free Ca^{2+} alone (*A*) and by 10 μM cytosolic free Ca^{2+} in the presence of 50 mM P_i (*B*).

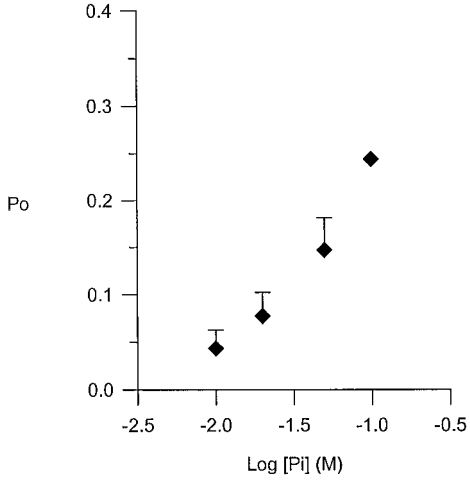

FIGURE 8 The effect of P_i on the P_o of sheep cardiac RyR. The experiments were performed in the presence of 10 μM cytosolic free Ca^{2+}. The data points are the mean of three to five observations. Standard error bars are shown for $n > 3$.

P_o. With 10 μM cytosolic free Ca^{2+} as the only other activating ligand, P_i is far less effective at elevating P_o than ATP or ADP. However, the concentration of P_i required to increase P_o and the degree of elevation in P_o observed in the cardiac channel in the present study is similar to that reported by Fruen et al. (1994b) in the skeletal channel.

Fig. 7 demonstrates that P_i increases the frequency of channel openings without any evidence that the duration of the open events are increased. This is confirmed by lifetime analysis (Fig. 9). The open lifetime distribution is virtually unchanged by P_i but the closed lifetime constants are reduced, demonstrating that P_i increases P_o solely by increasing the frequency of channel opening.

The effects of P_i were investigated in the absence of activating cytosolic [Ca^{2+}] (picomolar Ca^{2+}). Even at concentrations up to 100 mM, P_i was unable to induce any channel openings (data not shown).

It has been suggested that P_i may exert an effect on the cardiac RyR via the adenine nucleotide binding sites on the channel (Xiang and Kentish, 1995). To examine this hypothesis we investigated the effects of the simultaneous presence of ATP and P_i on the P_o of the channels. Fig. 10 illustrates a representative experiment where more than one channel has incorporated into the bilayer. The figure illustrates the gating of the channels in the presence of cytosolic Ca^{2+} alone (*trace A*), after the addition of a supramaximal concentration of ATP (10 mM; *trace B*) and after the addition of 20 mM P_i (*trace C*). Channel P_o increased from 0.597 $\pm$ 0.381 (mean $\pm$ SE; $n = 4$) in the presence of 10 mM ATP to 0.702 $\pm$ 0.291 (mean $\pm$ SE; $n = 4$) upon the addition of 20 mM P_i. Fig. 1 (*bottom*) demonstrates that concentrations of ATP above the optimum (2 mM) tend to reduce P_o. Therefore, 10 mM ATP is on the inactivating portion of the [ATP]-P_o relationship. The subsequent addition of an agent binding to the same sites as ATP would be expected to produce further inactivation of channel gating. P_i actually causes an increase in P_o to a level higher than

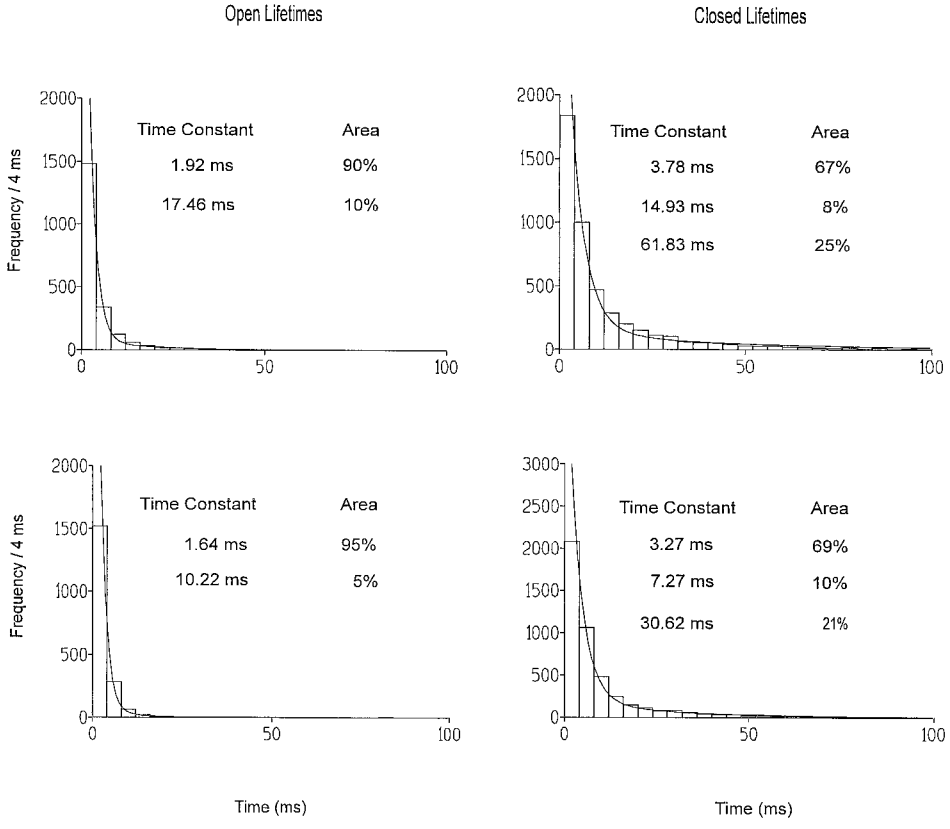

FIGURE 9 Effects of P_i on open (*left*) and closed (*right*) lifetime distributions and probability density functions (pdf). The time constants and percentage areas obtained from the best fit to the data are shown. Lifetimes were determined as described in Methods. The channel was activated by 10 μM cytosolic Ca^{2+} alone (*top*) and by 10 μM cytosolic Ca^{2+} plus 50 mM P_i (*bottom*).

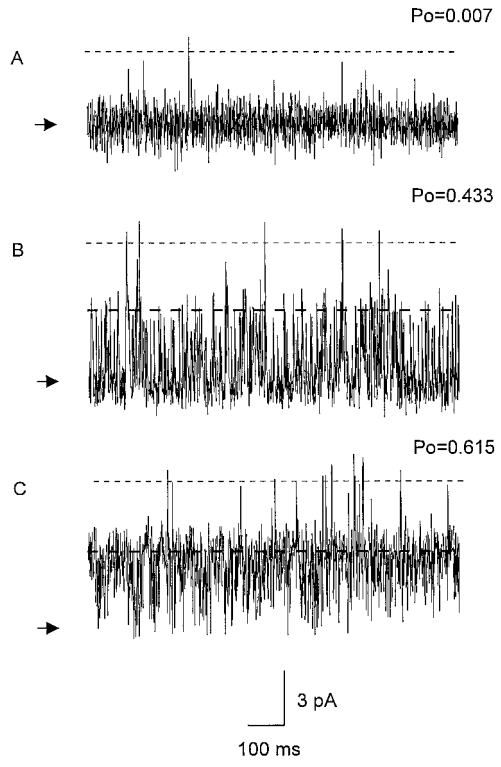

FIGURE 10 Effects of P_i on channels already activated by 10 μM cytosolic Ca^{2+} plus 10 mM ATP. Dotted lines indicate the open channel level and arrows indicate the zero current level. In (*A*) the channel is activated by 10 μM cytosolic Ca^{2+} alone. After the addition of 10 mM ATP in (*B*), P_o is increased to 0.433. This concentration of ATP is on the inactivating portion of the [ATP]-P_o relationship. Subsequent addition of 20 mM P_i (*C*) results in an increase in P_o to 0.61

that expected if the individual effects of ATP and P_i are added together. This is good evidence that P_i does not exert effects on channel gating by binding to the same sites as ATP.

DISCUSSION

Mechanisms of ATP activation

This is the first study to demonstrate the full relationship between cytosolic [ATP] and the P_o of a native RyR. In the presence of activating cytosolic Ca^{2+}, activation of the channel by ATP appears to be a positively cooperative process, as the Hill slope is 1.5. Lifetime analysis of events >1 ms in duration indicate that in the presence of cytosolic Ca^{2+} ATP increases both the frequency and the duration of channel openings. Therefore, ATP must bind to both a closed and an open state of the channel. At low concentrations of ATP ($\leq EC_{50}$ value) the primary mechanism for the increase in P_o is the increase in frequency of opening. As the [ATP] increases above this level, large increases in the duration of the open states cause the further elevations in P_o. At P_o values close to unity, the longest closed state and the shortest open state cannot be detected. The channel may predominantly enter the long open states via the short closed states and enter the short open state via long closings. As yet we are uncertain whether free ATP or Ca^{2+}-bound ATP (or both) are binding to the channel to cause the observed changes in P_o. While the fact that adenosine (McGarry and Williams, 1994) can fully activate the channel indicates that, in general, adenine nucleotides do not need to be bound to divalent cations to be effective activators of RyR, it is possible that the different forms of ATP may exert different effects on the channel.

A large amount of information on adenine nucleotide-stimulated Ca^{2+} efflux from SR vesicles isolated from both cardiac and skeletal muscle has been accumulated by Meissner and co-workers (Meissner and Henderson, 1987; Meissner et al., 1986; Meissner, 1984). However, much of this work was performed with the nonhydrolyzable analog of ATP, AMP-PCP, which appears to be slightly less potent than ATP (Meissner et al., 1986). In skeletal SR vesicles, the EC_{50} for ATP-stimulated Ca^{2+} efflux was 1 mM compared with 2 mM for AMP-PCP. ADP was reported to be much less effective at stimulating Ca^{2+} efflux and our single-channel data indicate that this is because ADP has both a lower affinity and efficacy at the adenine nucleotide sites than ATP. Meissner and Henderson (1987) reported that adenine nucleotides were more effective stimulators of Ca^{2+}-release from skeletal than from cardiac SR vesicles. However, in their study this only appears to be the case when the cytosolic [Ca^{2+}] is very low (2 nM). Our single channel study demonstrates that ATP is a more potent activator of cardiac RyR ($EC_{50} = 0.22$ mM) than would be predicted from the flux studies by Meissner and co-workers. Although not precisely determined, data from our own laboratory suggest that ATP is approximately equipotent at activating the sheep cardiac and skeletal isoforms of the channel (Sitsapesan and Williams, 1995). Herrmann-Frank and Varsányi (1993) reported that ATP increased the P_o of the purified skeletal RyR with an EC_{50} value of 2.2 mM, which decreased to 0.5 mM when the channel was phosphorylated by protein kinase A. A direct comparison of our results with those of Herrmann-Frank and Varsányi would indicate that the sheep skeletal and cardiac channels are far more sensitive to ATP than the rabbit skeletal channel. Herrmann-Frank and Varsányi, however, carried out their experiments on purified channels, and since ATP appears to be more effective in the vesicle flux studies of Meissner et al. (1986) from the same species, it is possible that purification of the RyR may affect ATP binding sites or Ca^{2+}-binding sites, thereby affecting the synergistic effects of ATP and Ca^{2+} on channel gating.

An important observation of this study is that concentrations of ATP (and ADP) above the optimum level cause a decrease in P_o. The inactivation results from an increase in the frequency of channel closing and therefore the duration of the open events is reduced. The cause of the increase in the frequency of channel closing is not yet clear. This may result from ATP and ADP binding to low-affinity inactivation sites. The experiments performed at subactivating levels of cytosolic Ca^{2+} indicate that the shape of the dose-

response curve for ATP (and ADP) may be dependent on the cytosolic [Ca^{2+}]. At subactivating [Ca^{2+}], ATP could induce channel openings but was very ineffective even at a concentration of 10 mM. No peak in P_o was achieved and therefore no inactivation was observed at the higher [ATP]. At high levels of ATP, the concentration of cytosolic Ca^{2+} will determine whether the channel is on the activating or the inactivating portion of the ATP dose-response curve. In fact, what we have termed "high" ATP concentrations are close to the normal physiological levels of ATP (10 mM) thought to be present intracellularly in cardiac cells (Hohl et al., 1992). Inactivation of the cardiac RyR by high ATP concentrations may therefore play a role in terminating Ca^{2+} release from the SR during EC coupling.

Mechanisms of ADP activation

In the presence of cytosolic Ca^{2+}, ADP increases P_o predominantly by increasing the frequency of channel openings. At optimal [ADP] the duration of the open lifetimes is slightly increased, but not to the same extent as occurs in the presence of ATP. Whereas the Hill coefficient for ATP activation indicates that the interaction of ATP with the RyR is a positively cooperative process, the Hill coefficient for ADP activation suggests that this is not the case for ADP. These values may reflect the ability of ATP to bind to both the open and closed channel states, whereas ADP may bind predominantly to the closed state. Alternatively, ADP may bind to the open state without producing a measurable response. Unlike ATP, ADP cannot fully activate the cardiac RyR. Presumably the inability of ADP to cause marked increases in open lifetime duration explains why ADP can only partially activate the channel.

Similarities in the mechanisms of channel activation suggest that ADP and ATP share a common binding region. Both agents increase the frequency of channel opening at low concentrations. Both agents act synergistically with Ca^{2+} to increase P_o, but can also activate the channel in a Ca^{2+}-independent manner by invoking an apparently different kinetic gating scheme with longer open events. In addition, ATP does not potentiate the effects of ADP. Fig. 7 demonstrates that ADP acts as if it were competing with ATP for the same binding site/s. ADP cannot fully activate the channel and therefore, when present in high enough concentrations, effectively antagonizes the effects of ATP. Our results therefore suggest that ADP is a partial agonist at adenine nucleotide binding sites.

How do these results relate to the control of channel gating within the cell? The EC_{50} value for ADP is more than 5 times greater than that for ATP. Therefore, coupled with the fact that ATP may be present in concentrations >10 mM compared with submillimolar levels of ADP, it is unlikely that ADP will have any effect during normal physiological conditions. During prolonged conditions of ischemia, ATP levels will fall and ADP levels will rise (Allen and Orchard, 1987). If ADP levels rise high enough and ATP levels fall low enough, then it is possible that ADP may play a role in regulating the gating of the cardiac RyR. However, ADP would not be expected to cause an increased efflux of Ca^{2+} from the SR. Once the levels of ADP became high enough to compete effectively with ATP so that ADP was the predominant agonist, this would be more likely to reduce the P_o of the channels or at least prevent them from being fully activated. When considering the possible consequences of ischemia it should be remembered that levels of other adenine nucleotides and nucleosides also change during ischemia. For example, levels of adenosine, cAMP, and AMP increase during ischemia and the relative concentrations, affinity, and efficacy of these agents for the adenine nucleotide binding site would determine how ADP affected the gating of the RyR in situ. Our results do indicate, however, that it is unlikely that the Ca^{2+} efflux from the SR observed by Smith and Steele (1992) or by Xiang and Kentish (1995) is caused by stimulation of the RyR by ADP since millimolar levels of ATP were present in their experiments. If anything, under the experimental conditions of their experiments, ADP would be expected to reduce the P_o of the channels. More difficult to explain is the potentiation of CICR by ADP and the further increase in Ca^{2+}-release in the presence of P_i plus ADP observed by Xiang and Kentish (1995). One possible explanation for this effect could be the inclusion of di-adenosine pentaphosphate (AP_5A) in all their solutions containing ADP. AP_5A is a myokinase inhibitor and was included to prevent ATP production from ADP. We have performed preliminary experiments with this compound indicating that it is a potent activator of the cardiac RyR (data not shown). Therefore, in the experiments of Xiang and Kentish (1995) it may be AP_5A, rather than ADP, which is stimulating CICR. P_i, if acting via a separate binding site on the channel, may then potentiate the effects of AP_5A to cause further activation of the RyR.

Mechanisms of P_i activation

By isolating cardiac SR and monitoring current flow through the RyR we have established that increases in cytoplasmic [P_i] can activate sheep cardiac RyR. The activation requires millimolar concentrations and is purely a Ca^{2+}-dependent process. P_i has no effect in the absence of activating cytosolic [Ca^{2+}] at concentrations up to 100 mM. In the presence of Ca^{2+}, P_i increases P_o solely by increasing the frequency of channel opening. No lengthening of the open events occurs. Thus the mechanism for P_i activation of the channel is identical to that for Ca^{2+} activation of the channel. These results suggest two possible mechanisms for P_i activity: either P_i can sensitize the channel to Ca^{2+} or P_i can only bind to and affect the gating of the Ca^{2+}-bound channel. It was suggested by Fruen et al. (1996) that P_i interacts with specific sites on the skeletal RyR, whereas other anions such as perchlorate, nitrate, and chloride may affect channel gating because of their chaotropic properties. An interesting finding of their experiments was that P_i and

other inorganic anions could increase ryanodine binding to and Ca^{2+} efflux from skeletal, but not cardiac, SR. We are uncertain why no apparent activation of the cardiac channels was observed by Fruen et al. (1994a, b) whereas we clearly observed P_i-induced increases in P_o. Possible reasons include differences between species or differences in the optimal conditions required for ryanodine binding or Ca^{2+} efflux from cardiac and skeletal SR.

An investigation of the effects of P_i in the presence of ATP indicates that P_i does not bind to adenine nucleotide sites on the RyR (Fig. 10). Although in skinned cardiac cells a recent report suggested that P_i increased SR Ca^{2+} efflux by a non-RyR mechanism (Steele et al., 1996) our results demonstrate that it would be expected that increases in P_i to the levels occurring during ischemia would be able to potentiate the effects of agents acting at adenine nucleotide binding sites on the RyR channels and cause an increase in P_o.

In conclusion, we have examined and described the mechanisms involved in ATP activation of the sheep cardiac RyR. We have shown that ADP binds to the same sites on the channel as ATP but has a much lower affinity. ADP also acts as a partial agonist at these sites. These findings should help in the elucidation of the structure-activity relationship of the ATP site. We have also demonstrated that P_i can activate the cardiac channel and that this action results from P_i interacting with sites distinct from the ATP sites. With Ca^{2+} as the only other ligand, ADP is more effective than P_i at increasing the P_o of cardiac RyR. In contrast, P_i would be predicted to exert more effect on the channel in situ since it would not have to compete with ATP for the same binding domain, but rather would potentiate the effects of ATP.

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