British Journal of Pharmacology (1999) 127, 161–171
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AMP is a partial agonist at the sheep cardiac ryanodine receptor

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1 We have investigated the ability of AMP to modulate the native sheep cardiac ryanodine receptor (RyR) channel at various cytosolic [Ca^{2+}]. Channels were incorporated into planar phospholipid bilayers and current fluctuations through the bilayer were monitored under voltage clamp conditions.

2 We demonstrate that AMP only exhibits agonist activity if the cytosolic [Ca^{2+}] is sufficiently high. Even in the presence of a high cytosolic [Ca^{2+}] (65 μM), AMP cannot fully open the channel and the maximum open probability (Po) observed is approximately 0.3 at 2 mM AMP.

3 Concentrations of AMP above the maximally activating level cause inactivation of the channel.

4 Our experiments indicate that AMP is an agonist with such low efficacy at the ATP sites on the cardiac RyR that it is effectively an antagonist of ATP-induced increases in Po. Our study demonstrates that the number of phosphates attached to the 5′-carbon of the ribose ring of adenine-based compounds determines the efficacy of the ligand to increase the Po of the cardiac RyR. Substitution of groups at this position may lead to the identification of potent antagonists at ATP sites on RyR.

Keywords: Ryanodine receptor; AMP; ATP; sarcoplasmic reticulum; Ca^{2+}-release channel

Abbreviations: DAT, Digital Audio Tape; EGTA, ethylene glycol-bis(β-aminoethyl ether)-N,N,N,N′-tetraacetic acid; HEPES, N′-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid; pdf, probability density function; Po, open probability; RyR, ryanodine receptor; SR, sarcoplasmic reticulum; Tris, tris(hydroxymethyl)-methylamine

Introduction

ATP has been shown to stimulate the release of Ca^{2+} from sarcoplasmic reticulum (SR) membrane vesicles isolated from mammalian cardiac and skeletal muscle (Meissner, 1984; Meissner *et al*., 1986; Meissner & Henderson, 1987) and to activate SR Ca^{2+}-release/ryanodine receptor channels (RyR) incorporated into planar phospholipid bilayers (Smith *et al*., 1986; Kermode *et al*., 1998). We have previously demonstrated that ATP can almost fully activate single sheep cardiac RyR with an EC_{50} value of approximately 200 μM in the presence of 10 μM Ca^{2+} (Kermode *et al*., 1998). Maximal activation of the channel by ATP occurs at approximately 2 mM ATP and higher concentrations lead to inactivation of the channel. Cytosolic levels of ATP in cardiac cells are thought to be between 5 and 10 mM (Allue *et al*., 1996) and therefore it is likely that ATP plays a very important role in regulating the gating of the cardiac RyR and hence the release of sarcoplasmic reticulum (SR) Ca^{2+} during a normal contractile cycle.

The ATP sites on the cardiac RyR are very different to cell surface P_2 purinoceptors judged by the ability of other adenine-based compounds including adenosine, ADP and cyclic ADP-ribose (cADPR) to activate the cardiac RyR (McGarry & Williams, 1994b; Sitsapesan *et al*., 1994; Kermode *et al*., 1998). Agents often used as antagonists at P_1 (xanthines (Fredholm *et al*., 1994)) and P_2 (suramin (Dunn & Blakeley, 1988; Hoyle *et al*., 1990)) purinoceptors do not antagonize the effects of ATP on cardiac RyR but actually activate the channel by binding to different sites (Sitsapesan & Williams, 1996; McGarry & Williams, 1994b). We have recently discovered, however, that ADP is a partial agonist at the ATP sites on the cardiac RyR (Kermode *et al*., 1998). By removing one phosphate group the efficacy of the ligand is reduced by 30%. A reduction in affinity of the ligand for RyR was also observed (Kermode *et al*., 1998). Thus, by using single channel recording we can define how the potency of an agent in activating SR Ca^{2+}-release can depend not only on the affinity of the agent for the binding sites but also on the efficacy of the agent once bound. Since the removal of one phosphate group from ATP caused such marked effects on affinity and efficacy we investigated the possibility that removal of a second phosphate group could lead to an agent which may bind to the ATP sites on RyR but have very little agonist effect once bound. We have therefore examined the effects of AMP on the gating of the sheep cardiac RyR incorporated into planar lipid bilayers. As we have demonstrated that ATP can only fully activate the cardiac RyR in the presence of activating cytosolic [Ca^{2+}] we have examined if the ability of AMP to bind to the channel and its ability to open the channel once it is bound can be modulated by changing cytosolic [Ca^{2+}]. Our results demonstrate that AMP is not very effective at activating the sheep cardiac RyR and is completely Ca^{2+}-dependent. The results also indicate that AMP competes with ATP for the same binding sites on the sheep cardiac RyR and can effectively act as an antagonist at these sites.

Methods

Preparation of SR membrane vesicles and planar lipid bilayer methods

Heavy SR membrane vesicles were prepared from sheep cardiac muscle as previously described by Sitsapesan *et al*. (Sitsapesan *et al*., 1991b). Heavy SR membrane vesicles were frozen rapidly and stored in liquid nitrogen. Vesicles were fused with planar phosphatidylethanolamine lipid bilayers as previously described (Sitsapesan *et al*., 1991b). The vesicles

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incorporated into the bilayer 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 was held at potentials relative to ground. After fusion of vesicles, the *cis* chamber was perfused with a solution containing N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES) (250 mM), Tris(hydroxymethyl)methylamine (Tris) (125 mM), pH 7.2 buffered to maintain the free Ca^{2+} concentration required (65, 10, 0.1 μM or approximately 100 pM) with ethylene glycol - *bis*(β-aminoethyl ether)- N, N, N′, N′-tetraacetic acid (EGTA) and $CaCl_2$. The *trans* chamber was perfused with a solution of glutamic acid (250 mM), HEPES (10 mM), pH to 7.2 with $Ca(OH)_2$ (free [Ca^{2+}] was approximately 60 mM). All experiments were performed at $23 \pm 1^{\circ}$C. Additions of ATP and AMP were made to the *cis* chamber. The free [Ca^{2+}] and pH of the solutions in the absence and presence of AMP were measured at 23°C with a Ca^{2+} electrode (93-20, Orion Research, Boston, MA, U.S.A.) and Ross-type pH electrode (Orion 81-55) as previously described in detail (Sitsapesan *et al*., 1991b). Due to the low efficacy of AMP, EMD 41000, an agent which acts at the caffeine sites (McGarry & Williams, 1994a), was added at the end of each experiment to determine the number of channels present in the bilayer.

Data acquisition and analysis

Single-channel recordings were displayed on an oscilloscope and the analogue voltage signal was stored in digital form on Digital Audio Tape (DAT) (Biologic, Intracel, Cambridge). All steady state recordings were carried out at 0 mV. Under the above ionic conditions at 0 mV, the permeant ion, Ca^{2+} flows through the RyR from the luminal to the cytosolic side of the channel. Current recordings were filtered at 600 Hz and digitized at 2 kHz. Channel open probability (Po) and the lifetimes of the open and closed events were determined over 3 min of steady state recording using the method of 50% threshold analysis (Colquhoun & Sigworth, 1983). When more than one channel was incorporated into the bilayer, *NPo* was calculated from the formula:

$$NPo = [T_{open1} + 2(T_{open2}) + 3(T_{open3}) \cdots + n(T_{open\ n})]/T_{total}$$

where T_{open1}, T_{open2} and T_{open3} are the times in the first, second and third open channel levels respectively and T_{total} is the total recording time. 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 therefore excluded from lifetime analysis. Open and closed lifetimes accumulated from approximately 3 min of recording were stored in sequential files and displayed in non-cumulative histograms. Individual lifetimes were fitted to a probability density function (pdf) using the method of maximum likelihood (Colquhoun & Sigworth, 1983) according to the equation:

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

where τ is the time constant in milliseconds and a is the relative area. A missed events correction was applied as described by Colquhoun and Sigworth (Colquhoun & Sigworth, 1983). A likelihood ratio test (Blatz & Magleby, 1986) was used to compare fits of 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. All Po and conductance values are quoted as mean $\pm$ s.e.mean where $n \geqslant 4$. For $n=3$, standard deviation (s.d.) is given. The AMP dependence of Po was characterized using the equation:

$$Po = P_{max}(1/(1 + (EC_{50}/[AMP]H_1)) \ast (1 - (1/(1 + (IC_{50}/[AMP])H_2)))$$

where P_{max} equals the maximum Po attained, EC_{50} and IC_{50} equal the half maximum concentrations required for activation and inhibition respectively and H_1 and H_2 are the Hill coefficients of activation and inhibition respectively.

AMP (>99% purity) and ATP (99% purity) were both obtained from Sigma (Poole, U.K.) and EMD 41000 was a gift from Merck (64271 Darmstadt, Germany). All solutions were prepared using MilliQ deionized water (Millipore, Harrow, U.K.) and filtered through a Millipore membrane filter (pore size 0.45 μm) before use.

Results

Effects of AMP on the gating of RyR in the presence of 10 μM cytosolic Ca^{2+}

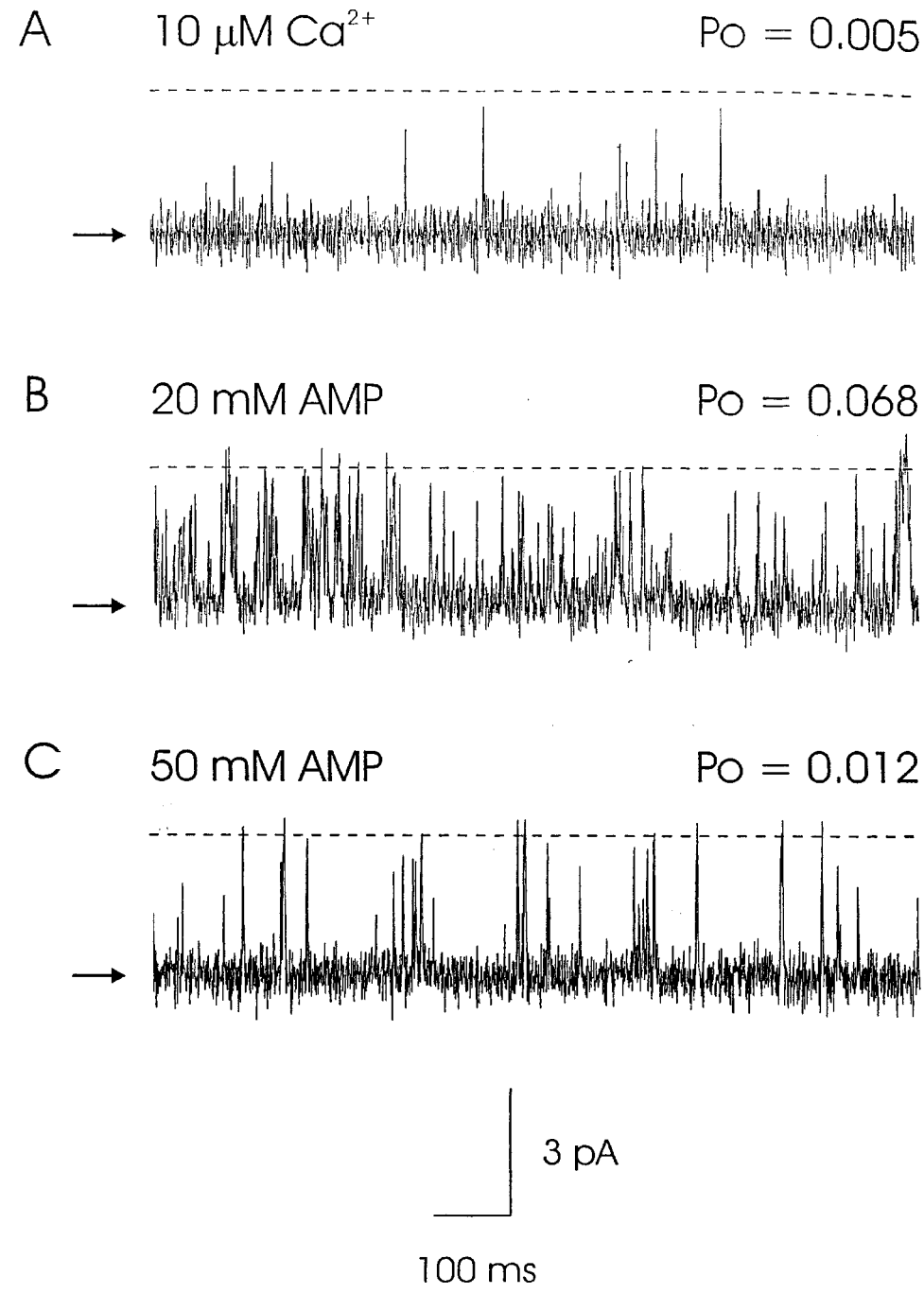
The ability of AMP to activate the sheep cardiac RyR in the presence of 10 μM cytosolic free Ca^{2+} is shown in Figure 1. High levels of AMP were required to produce significant activation of the channel and maximal activation occurred at 20 mM AMP with a Po of 0.071 ± 0.045 (s.e.mean; $n=10$). Further increases in [AMP] tended to inactivate the channel (Po $= 0.036 \pm 0.012$ at 50 mM AMP; s.e.mean; $n=6$). Half-maximal activation was obtained at 3 mM AMP and the Hill coefficient for channel activation was 0.78.

Mean open and closed times were 0.73 ± 0.19 and 237.17 ± 57.34 ms (s.e.mean; $n=4$) respectively in the presence of 10 μM Ca^{2+} alone and were 1.24 ± 0.20 and 36.02 ± 16.16 ms (s.e.mean; $n=4$) respectively after the addition of 20 mM AMP, demonstrating that AMP predominantly increases Po by increasing the frequency of channel opening. Lifetime analysis confirms this mechanism of activation. Figure 2 shows the effect of AMP on the open and closed lifetime distributions from a representative channel. In the presence of 10 μM Ca^{2+}, addition of 5 mM AMP reduced the duration of all three closed states indicating an increase in the frequency of opening.

The ineffectiveness of AMP as an activator of the cardiac RyR can be observed more clearly when compared to ATP under identical experimental conditions (Figure 3). In the presence of 10 μM cytosolic free Ca^{2+}, 1 mM ATP increased Po from 0.004 ± 0.003 (s.e.mean; $n=4$) to 0.747 ± 0.080 (s.e.mean; $n=4$) while an equivalent concentration of AMP only increased Po to 0.031 ± 0.016 (s.d.; $n=3$). Mean open and closed times with 1 mM ATP were 8.47 ± 1.47 and 2.06 ± 0.63 (s.d.; $n=3$) respectively. Clearly the effectiveness of ATP as an activator of RyR stems from its ability to increase both the duration and the frequency of channel openings whereas AMP only increases the frequency of opening. AMP had no effect on the single channel conductance of RyR. Slope conductance between +20 and −40 mV was 96 ± 0.8 pS (s.d.; $n=3$) before and 97 ± 4 pS (s.d.; $n=3$) after activation of the channel by AMP.

Ca^{2+}-dependence of AMP action

We have investigated if changes in cytosolic [Ca^{2+}] can alter the ability of AMP to activate the channel and if so, whether the affinity or the efficacy of AMP is affected. Figure 4 illustrates the effect of AMP on a typical cardiac RyR in the



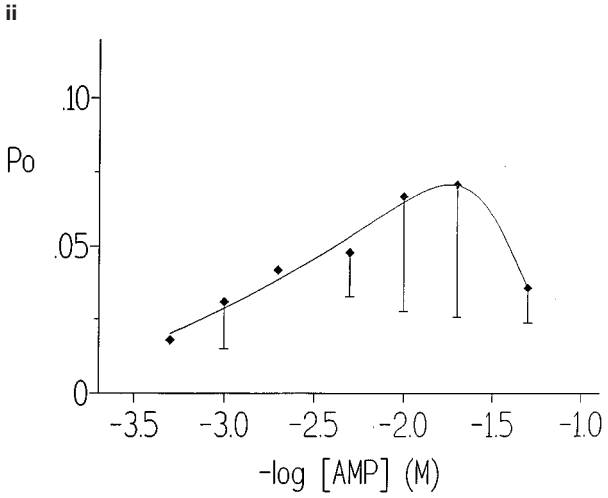


Figure 1 (i) Current fluctuations through a typical single native sheep cardiac RyR incorporated into a planar phospholipid bilayer held at 0 mV. At this holding potential current flows from the luminal to the cytosolic side of the channel. The arrows indicate the closed channel level and the dotted lines indicate the open channel level. Channel Po is shown above each trace. In the first trace (A), the channel is activated solely by 10 μM cytosolic free Ca^{2+}. Subsequent addition of 20 mM AMP to the cytosolic side of the channel further activated the channel (B). The lower trace (C) shows the same channel inactivated after increasing [AMP] to 50 mM. (ii) The relationship between Po and [AMP] (filled triangles) in the presence of 10 μM Ca^{2+}. The data points are the mean of 3–10 observations. Standard error bars are shown for $n \geqslant 4$.

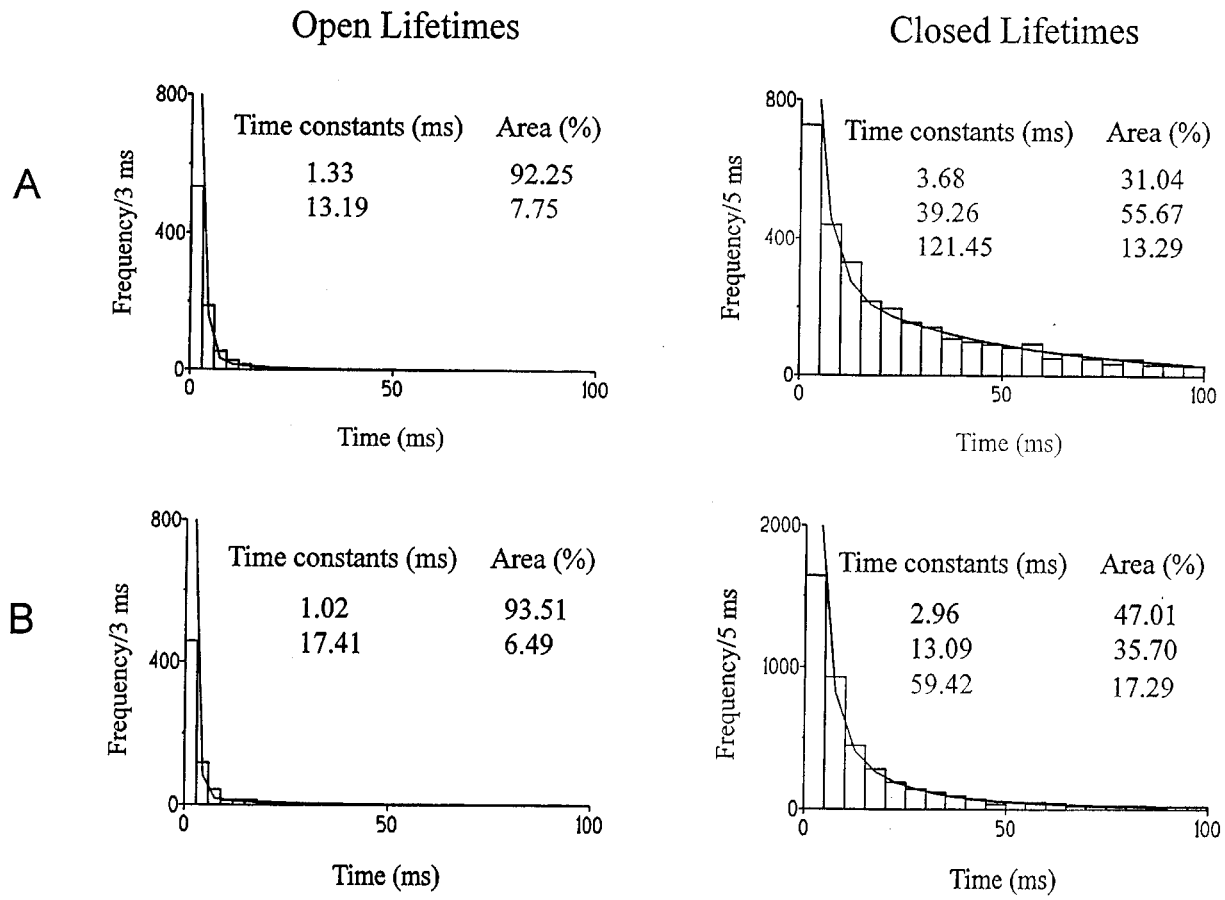


Figure 2 Open and closed lifetime distributions and pdfs for a representative single sheep cardiac RyR activated by (A) 10 μM Ca^{2+} alone and (B) 10 μM Ca^{2+} + 5 mM AMP. Similar results were obtained in two further experiments. The best fits to the data were obtained by the method of maximum likelihood and the resulting time constants and corresponding percentage areas are shown.

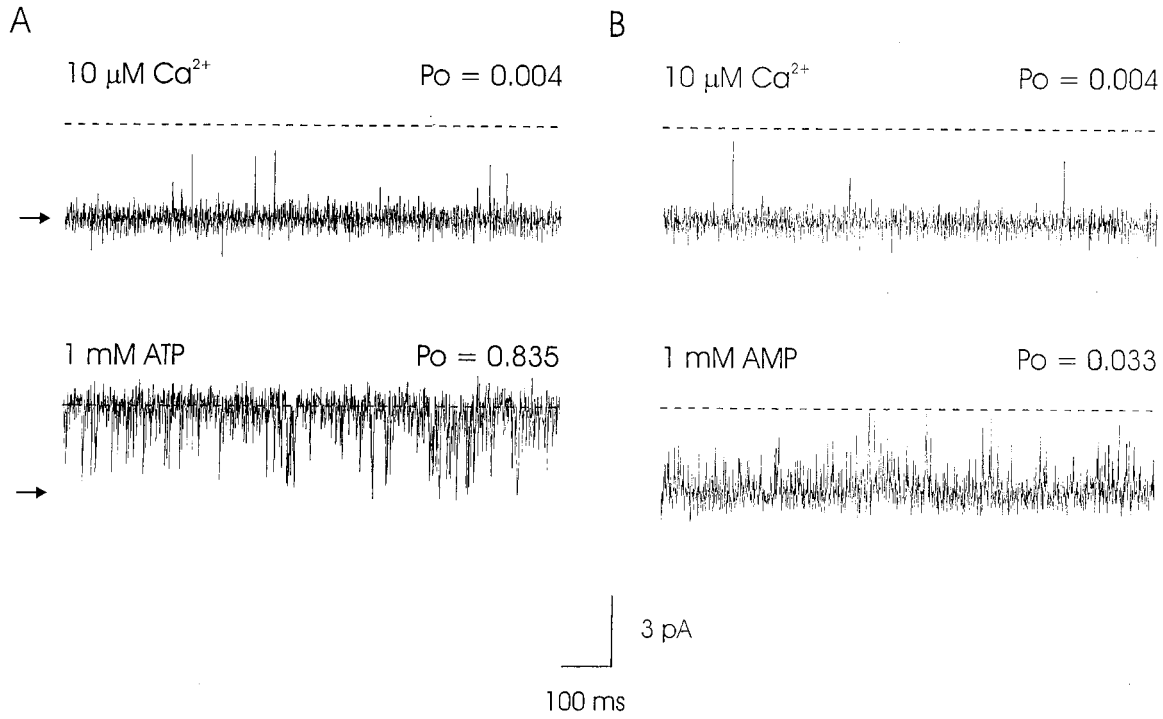


Figure 3 A comparison of the effects of an equivalent concentration of ATP and AMP on the gating of the sheep cardiac RyR. The arrows indicate the closed channel level and the dotted lines indicate the open channel level. On the left (A), is shown a channel activated by 10 μM cytosolic Ca^{2+} alone (top) and 1 mM ATP in the presence of 10 μM Ca^{2+} (bottom). On the right (B), is shown another channel activated solely by 10 μM cytosolic Ca^{2+} (top) and by 1 mM AMP in the presence of 10 μM Ca^{2+} (bottom). Channel open probability is given above each trace.

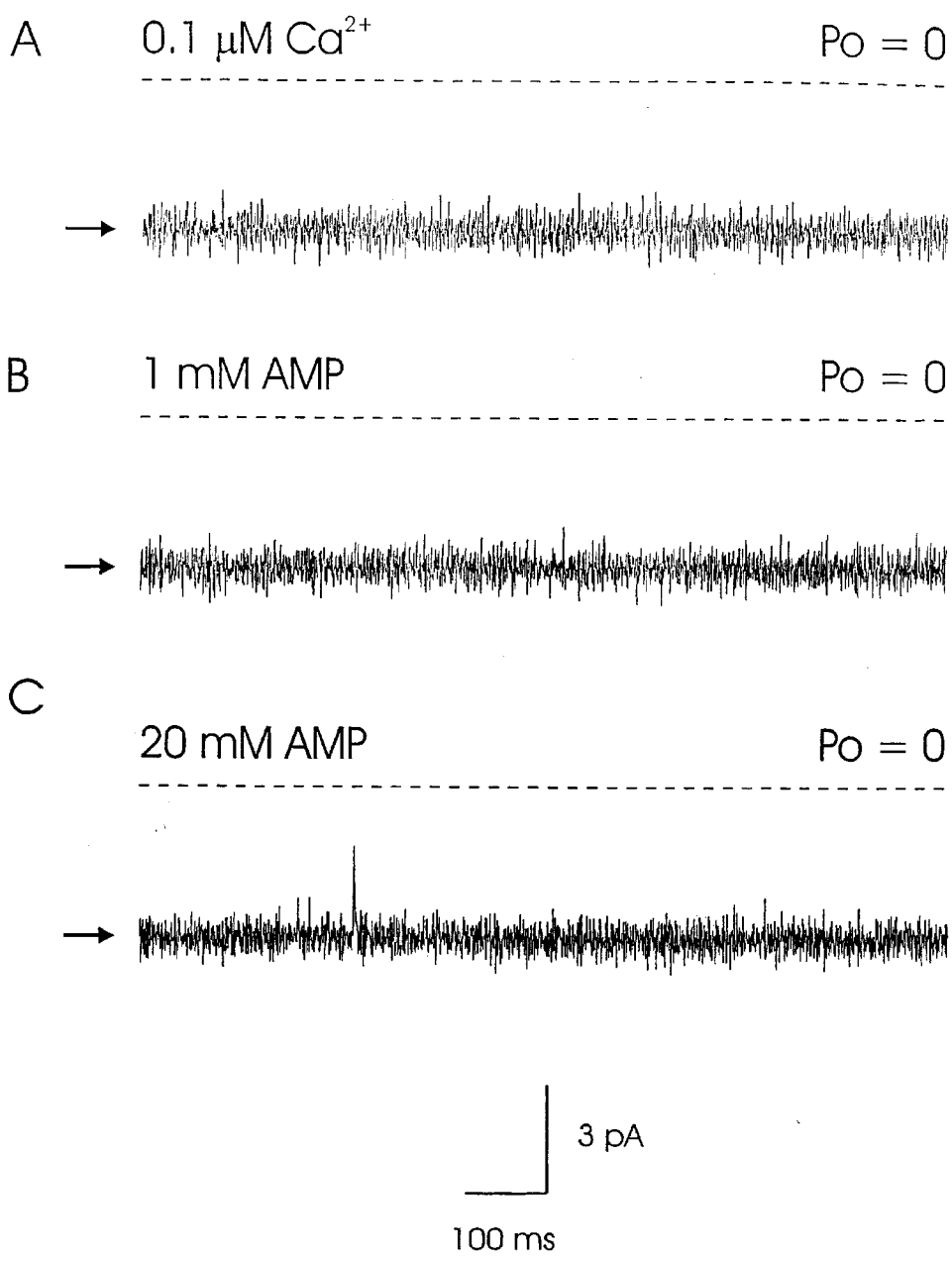


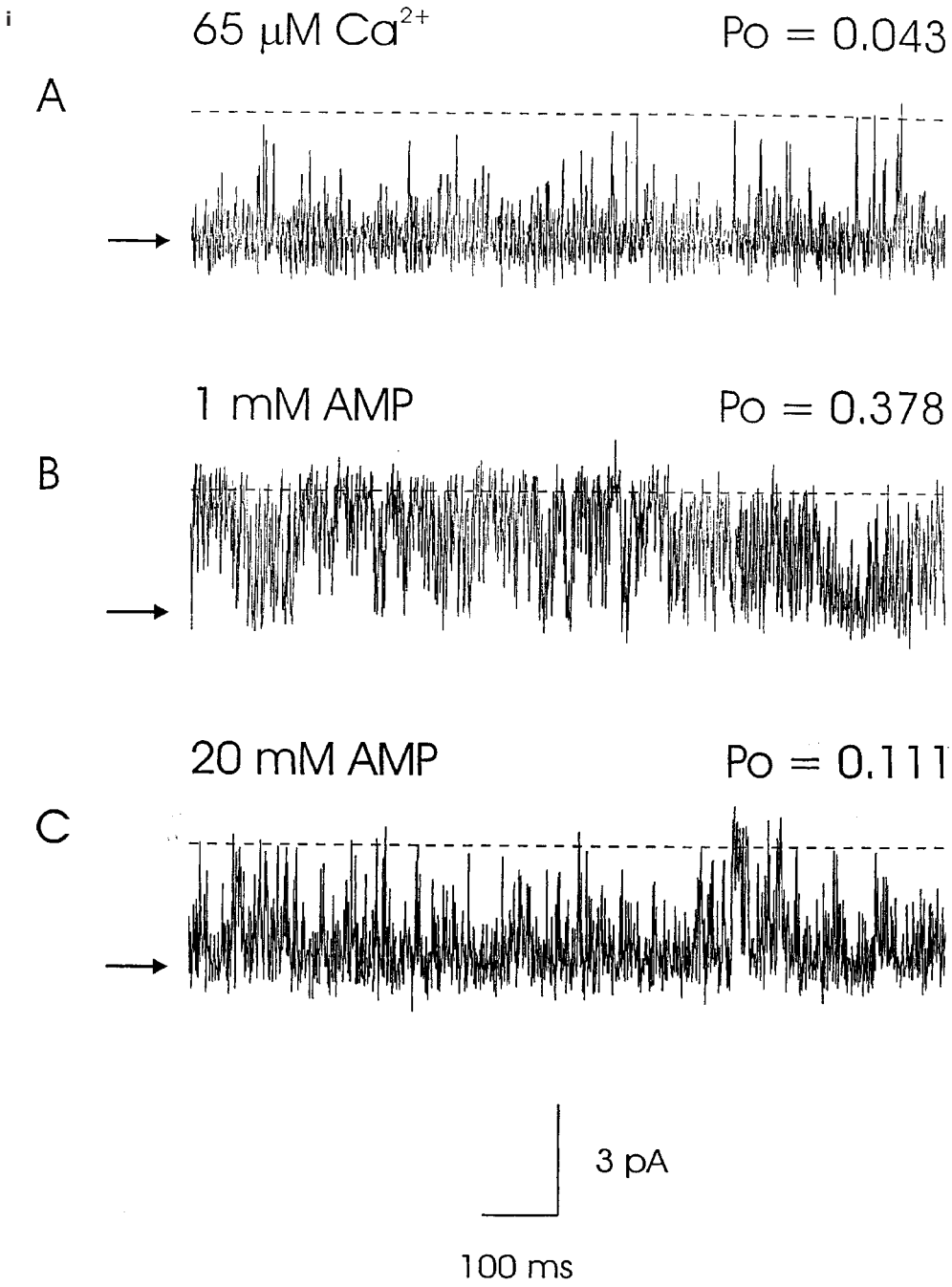
Figure 4 Effects of AMP on the gating of a representative cardiac RyR in the presence of 0.1 μM [Ca^{2+}]. The arrows indicate the closed channel level and the dotted lines indicate the open channel level. Trace (A) shows the channel in the presence of 0.1 μM Ca^{2+} alone. Po was 0. Subsequent additions of (B) 1 mM AMP and (C) 20 mM AMP did not activate the channel and no increase in Po was observed.

presence of 0.1 μM Ca^{2+}. At this [Ca^{2+}], addition of 1 and 20 mM AMP did not activate the channel demonstrating that the agonist action of AMP is totally Ca^{2+}-dependent. Similarly, AMP was unable to activate the channel when cytosolic Ca^{2+} was lowered to picomolar levels by addition of EGTA (12 mM) (results not shown).

Figure 5 demonstrates the effects of AMP in the presence of 65 μM Ca^{2+}. At this [Ca^{2+}], AMP was a much more effective activator of RyR than in the presence of 10 μM Ca^{2+} and was able to activate the RyR to a maximal Po value of 0.280 ± 0.147 (s.e.mean; $n=4$). Half maximum activation occurred at 0.54 mM AMP and the Hill coefficient was 1.5. In the presence of 65 μM Ca^{2+}, AMP can still only partially activate the channel and concentrations above 2 mM cause inactivation (Half maximal inactivation occurred at approximately 7 mM). Analysis of single channel open and closed lifetimes after activation of the channel by AMP in the presence of 65 μM cytosolic Ca^{2+} indicates that they are now best described by three open and two closed states. This is demonstrated in Figure 6. In comparison to AMP induced activation at 10 μM Ca^{2+} (see Figure 2B), more openings now occur to the second longer open state but the most dramatic changes are the shortening of the closed lifetime durations and the loss of the third, longest closed state. These results suggest that although AMP can slightly increase the duration of channel openings at higher [Ca^{2+}], the main mechanism by which AMP increases Po is still predominantly by increasing the frequency of channel opening.

Competition with ATP

If AMP and ATP activate the sheep cardiac RyR by binding to different sites on the channel then the two agents would be expected to potentiate each other. If, however, AMP is acting



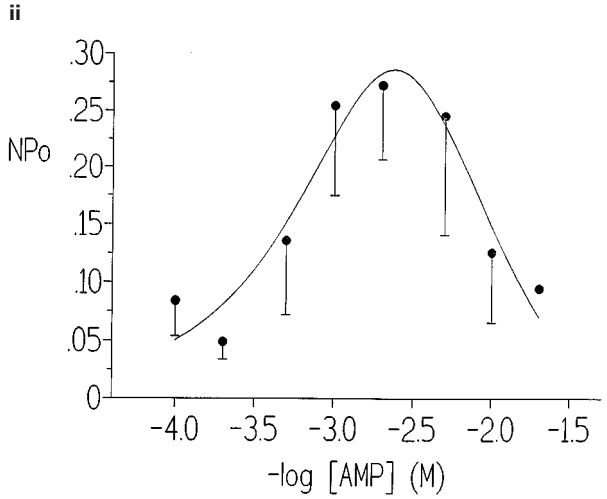


Figure 5 (i) Effects of AMP on the gating of a typical cardiac RyR in the presence of 65 μM cytosolic free Ca^{2+}. The arrows indicate the closed channel level and the dotted lines indicate the open channel level. In (A) a single channel is activated by 65 μM cytosolic free Ca^{2+} alone. The same channel was maximally activated by 1 mM AMP, still in the presence of 65 μM Ca^{2+} (B) and in (C) the channel was inactivated when [AMP] was increased to 20 mM. Po is shown above each trace. (ii) The relationship between NPo and [AMP] in the presence of 65 μM cytosolic free Ca^{2+}. The data points are the mean of 3–10 observations. Standard error bars are shown for $n \geqslant 4$.

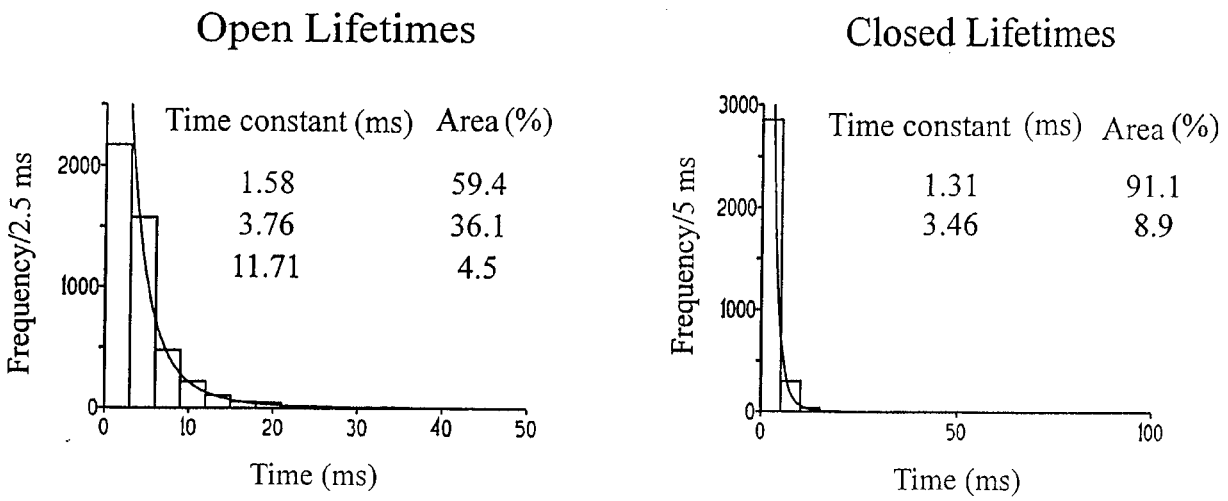


Figure 6 Open and closed lifetime distributions and pdfs for a typical single cardiac RyR activated by 1 mM AMP in the presence of 65 μM cytosolic Ca^{2+}. Similar results were obtained in four further experiments. The best fits to the data were obtained by the method of maximum likelihood and the resulting time constants and percentage areas are shown.

as a partial agonist at the ATP sites then the simultaneous effects of AMP and ATP would be expected to be no greater than the sum of their individual effects. We found that a range of [AMP] did not potentiate activation of the channel by ATP (Figure 7). In fact, AMP was found to antagonize the effects of ATP in a concentration-dependent manner as would be expected if AMP was a partial agonist at the ATP site. Addition of 1 and 2 mM AMP to channels already activated by 1 mM ATP in the presence of 10 μM cytosolic free Ca^{2+}, lowered Po from 0.746 ± 0.080 to 0.554 ± 0.236 and 0.309 ± 0.122 (s.e.mean; $n=4$) respectively. Two mM AMP, therefore, can produce more than 50% inhibition of the activation conferred by 1 mM ATP. This is likely to be antagonism of the effects of ATP by AMP rather than inactivation of the channels caused by high levels of adenine nucleotides since there is no evidence of inactivation by AMP (at 10 μM Ca^{2+}) until concentrations exceed 20 mM.

The effects of EMD 41000 on channels inactivated by high [AMP]

Following inactivation of the channels by high [AMP] in the presence of 10 μM Ca^{2+}, we found that EMD 41000, a caffeine-analogue which has been reported to fully activate the sheep cardiac RyR in the presence of 10 μM Ca^{2+} (McGarry & Williams, 1994a), was relatively ineffective at increasing Po. The effects of EMD 41000 on activation of the cardiac RyR in the presence and absence of 50 mM AMP is illustrated in Figure 8. Addition of 100 μM EMD 41000 to channels inactivated by 50 mM AMP only increased Po to 0.181 ± 0.082 (s.e.mean; $n=4$). However, after removal of AMP from the cytosolic side of the channel by perfusion with the *cis* solution containing 10 μM free Ca^{2+}, 100 μM EMD 41000 activated the channel to a Po of 0.787 ± 0.095 (s.e.mean, $n=4$).

Discussion

Effectiveness of AMP as an activator of the cardiac RyR: comparison with other adenine nucleotides

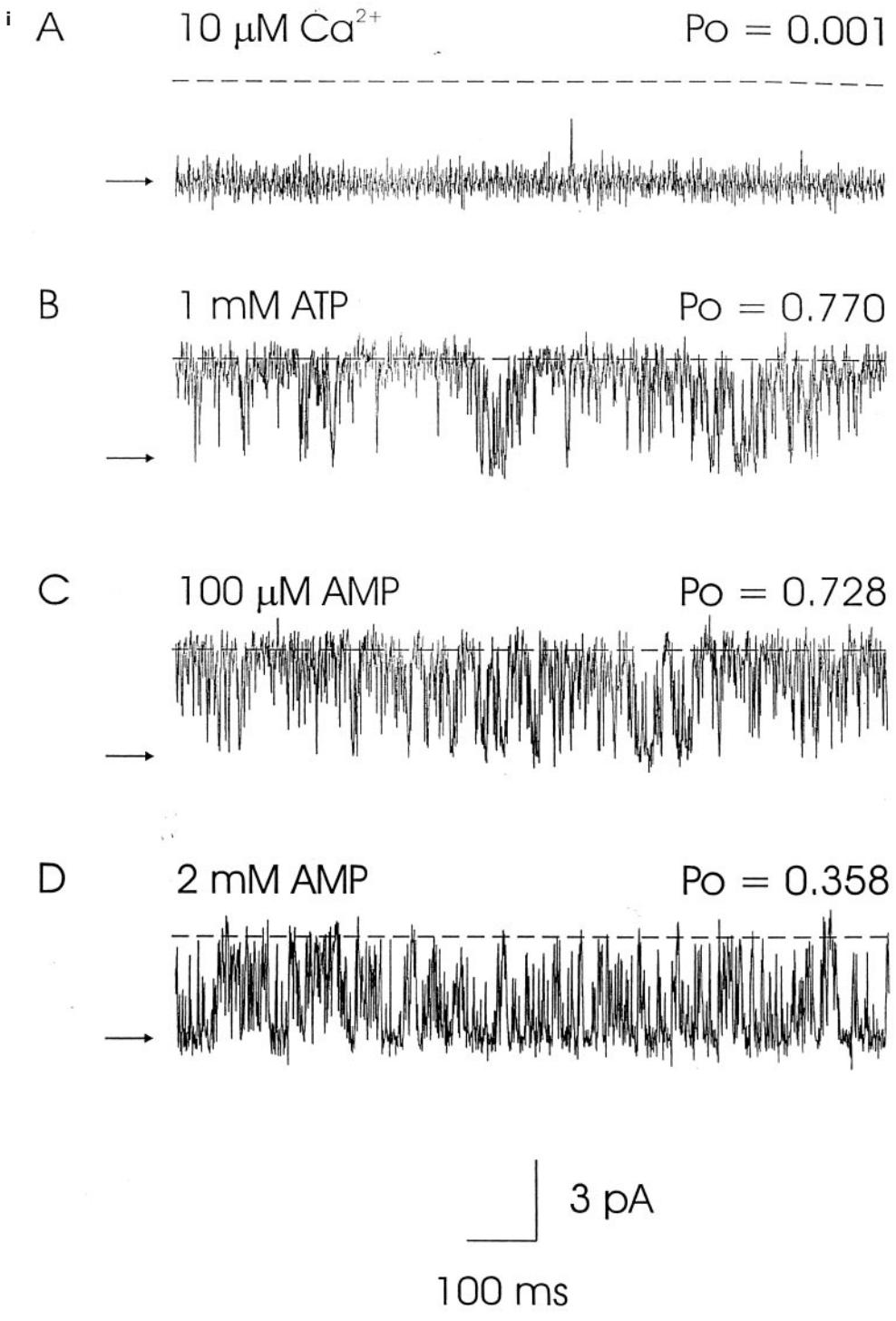
The structural similarities of ATP, ADP and AMP and the fact that ADP (Kermode *et al*., 1998) and AMP (Figure 7) both appear to act as partial agonists at the ATP sites suggests that all three ligands could bind to the same sites on RyR. In fact, the low efficacy of AMP at 10 μM Ca^{2+} allows it to effectively antagonize the effects of ATP. In the presence of 10 μM Ca^{2+}, maximum Po values achieved with ATP, ADP and AMP were approximately 0.9, 0.6 and 0.07 respectively, demonstrating that reducing the number of phosphate groups markedly reduces the efficacy of the ligand. ADP and AMP, therefore, both act as partial agonists although AMP is virtually without effect at this free [Ca^{2+}]. Reducing the number of phosphate groups also reduces the apparent affinity of the ligand for the binding sites. The EC_{50} values for ATP, ADP and AMP were 0.22 mM (Kermode *et al*., 1998), 1.2 mM (Kermode *et al*., 1998) and 3 mM. The reduction in affinity and efficacy of the ligands as the number of phosphates is reduced is also mirrored by a reduction in the Hill coefficient for channel activation in the presence of 10 μM Ca^{2+}. This was 1.5 for ATP (Kermode *et al*., 1998), 0.97 for ADP (Kermode *et al*., 1998) and 0.78 for AMP indicating that positive or negative cooperativity of binding of the adenine nucleotides to RyR may play an important role in determining the affinity and efficacy of the ligands. The reduction in the Hill coefficient may reflect the ability of the ligand to bind to the open channel state and increase the duration of open lifetimes as this appears to be crucial in governing adenine nucleotide efficacy and occurs in the following descending order of ability: ATP > ADP > AMP.

The adenine base appears to be required for agonist activity at the ATP sites on RyR as GTP cannot increase the Po of RyR channels incorporated into bilayers (Sitsapesan, unpublished observations) and other nucleotides (GTP, CTP and UTP) cannot release Ca^{2+} from isolated SR (Meissner, 1984; Morii & Tonomura, 1983). It is also now clear that substitutions at the 5′ position of the ribose moiety lead to large changes in apparent affinity and efficacy of the ligands. As such large differences in the activity of the adenine nucleotides can be achieved by the removal or addition of a single phosphate group, electrostatic interactions between the phosphates and the amino acid residues in the binding sites may play an important role in determining the ability of a ligand to bind to and activate the channel. This is supported by results from Ca^{2+}-flux and [^{3}H]-ryanodine binding experiments using various diadenosine polyphosphate (Ap_nA) compounds (Holden *et al*., 1996; Morii & Makinose, 1992)

where the more phosphates separating the two adenosine moieties, the more effective and potent the compound.

Ca^{2+}-dependence of AMP activation

Raising the free cytosolic [Ca^{2+}] has two very obvious effects on AMP modulation of the cardiac RyR channel gating. Both the affinity of AMP for RyR and the ability of AMP to open the channel are increased as the [Ca^{2+}] is increased. At 10 μM Ca^{2+}, AMP increases Po by increasing the frequency of channel opening without affecting the open lifetimes and therefore AMP predominantly binds to the closed state of the channel. At 65 μM Ca^{2+}, AMP increases both the frequency and the duration of the open times indicating that AMP must interact with at least one open and one closed state of the channel. Clearly, the Ca^{2+}-induced increased efficacy is due predominantly to the Ca^{2+}-dependent ability of AMP to bind to at least one open channel state and cause an increase in the duration of the open events. Presumably this is reflected by the apparent increase in the Hill coefficient from approximately 0.8 to 1.5 by raising cytosolic Ca^{2+} from 10 to 65 μM. It is known from previous work that maximal activation of the sheep cardiac RyR with Ca^{2+} as the only activating ligand requires the binding of least three to four Ca^{2+} ions (Sitsapesan &



Williams, 1994). An explanation which might account for the shift in cooperativity we observed with higher cytosolic [Ca^{2+}] is that the binding of more Ca^{2+} ions may select for conformational state(s) which have a higher affinity for AMP and will give more response when AMP is bound. This is supported by the fact that AMP was unable to activate the channel at all at picomolar or 0.1 μM free Ca^{2+}.

Channel inactivation by high [AMP]

An important observation was that addition of AMP above the optimal level (>20 mM at 10 μM Ca^{2+} and >2 mM at 65 μM Ca^{2+}) caused the channel to inactivate. Similar occurrences of channel inactivation were also seen with ATP and ADP (Sitsapesan *et al*., 1991a; Kermode *et al*., 1998) which suggest that inactivation may occur through the binding of adenine nucleotides to low-affinity inactivation sites. By comparing inactivation caused by AMP at the two different [Ca^{2+}], our results demonstrate that inactivation occurs at a lower concentration of AMP in the presence of higher [Ca^{2+}]. If, indeed, the binding of more Ca^{2+} ions shifts the equilibrium to the conformational state of the channel with increased affinity for AMP (both the activation and inactivation sites), this would explain why inactivation at 65 μM Ca^{2+} was observed with a lower [AMP]. Evidence suggests that inactivation of the cardiac RyR by other adenine nucleotides is similarly altered by the free cytosolic [Ca^{2+}] (Kermode *et al*., 1998). This is likely to be particularly important for agents with a higher affinity for the channel such as ATP which are also present in relatively high levels in cardiac cells. During excitation-contraction coupling, entry of Ca^{2+} into the cell through L-type Ca^{2+} channels results in a small increase in [Ca^{2+}] at the cytosolic face of RyR channels. This small increase in [Ca^{2+}] would be expected to produce a large increase in RyR Po due to the potentiating effects of millimolar levels of ATP. Following the release of SR Ca^{2+}, however, the much larger rise in intracellular [Ca^{2+}] may be enough to cause adenine nucleotide-induced inactivation of the cardiac RyR thus terminating SR Ca^{2+}-release.

Another significant observation was that EMD 41000 was relatively ineffective at increasing the Po of channels already inactivated by 50 mM AMP. The same channels however, were highly activated by EMD 41000 after removal of AMP by perfusion. There are two possible explanations for this observation: (1) Inactivation by an adenine nucleotide plus Ca^{2+} may lead to a channel state which cannot be reversed without removing or lowering the level of either Ca^{2+} or the adenine nucleotide. The addition of channel activators which bind to different sites would therefore be expected to have little effect. As most activators of RyR channels are Ca^{2+}-dependent, it is not entirely surprising that a channel inactivated by high Ca^{2+} and adenine nucleotide concentrations becomes refractory to other ligands. (2) Although there is strong evidence that caffeine analogues and adenine-based compounds activate the cardiac RyR by binding to different sites on the channel (McGarry & Williams, 1994a,b), the structural similarities between caffeine and ATP analogues suggest that it is feasible for caffeine-related agents to bind with low affinity (and perhaps low efficacy) to the ATP binding sites and vice versa. It is possible therefore, that high concentrations of AMP (50 mM) may also bind with low efficacy to the caffeine sites thereby preventing EMD 41000 from binding to the same sites. Further work is required for a clearer understanding of this effect.

Does AMP have a physiological role as a modulator of cardiac RyR gating?

ATP is present in millimolar levels in normal cardiac cells (Hohl *et al*., 1992; Allue *et al*., 1996) while measurement of phosphorus-containing metabolites in ferret-hearts using [^{31}P]-nuclear magnetic resonance (Allen *et al*., 1985) have indicated

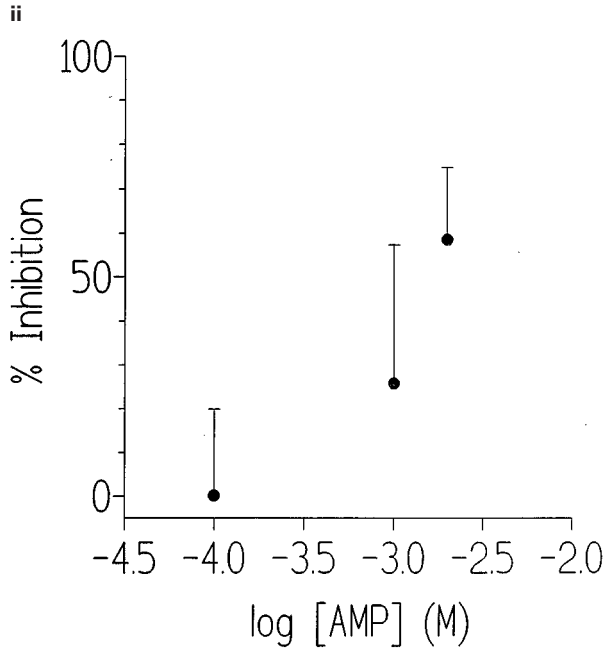


Figure 7 (i) The effects of AMP on a channel already activated by 10 μM Ca^{2+} and ATP. The arrows indicate the closed channel level and the dotted lines indicate the open channel level. Po is given above each trace. In (A) the channel is activated solely by 10 μM cytosolic Ca^{2+}. In (B) the channel is further activated by addition of 1 mM ATP to the *cis* chamber. Subsequent additions of (C) 100 μM AMP and (D) 2 mM AMP to the *cis* chamber reduces Po. (ii) The relationship between the percentage reduction in Po of channels activated by 10 μM Ca^{2+} and 1 mM ATP (percentage inhibition) and [AMP]. The Po of the channels activated by 10 μM Ca^{2+} and 1 mM ATP was taken to be 100%. The data points shown are the mean$\pm$s.e.mean of four observations.

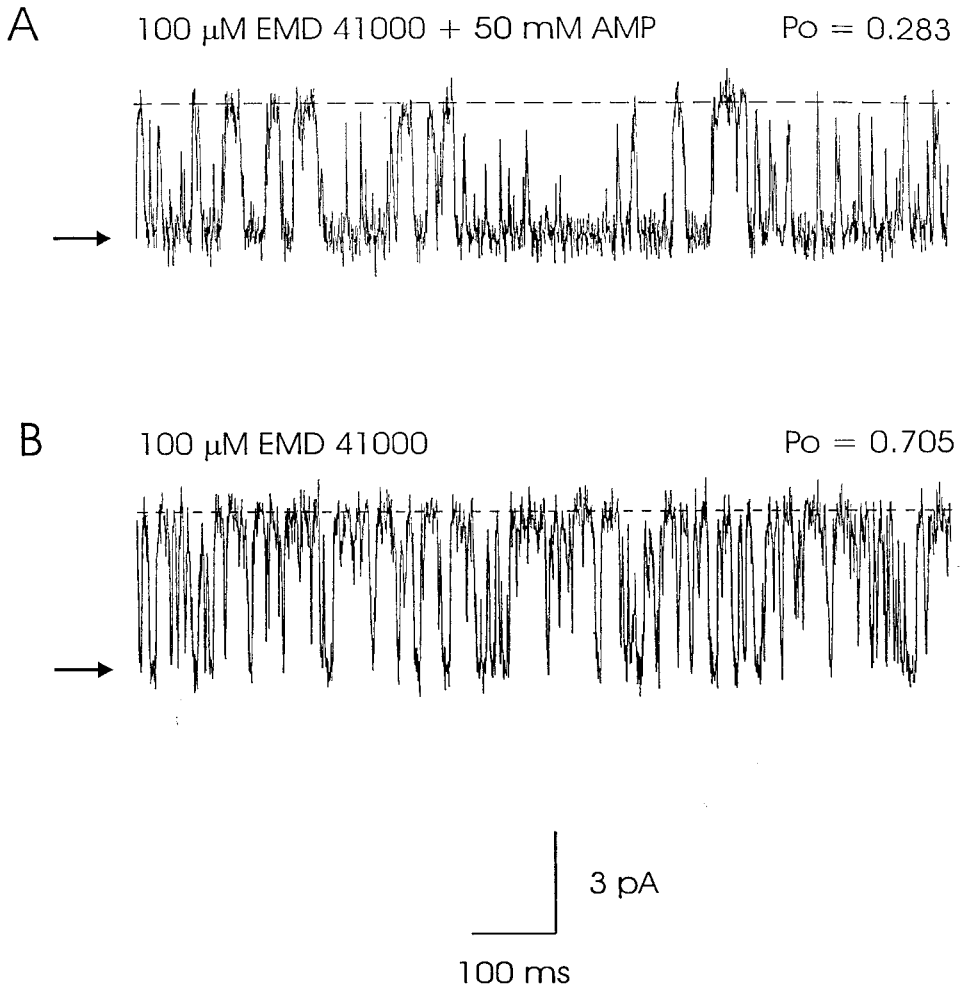


Figure 8 The effects of EMD 41000 on cardiac RyR already inactivated by high [AMP] (50 mM) in the presence of 10 μM cytosolic Ca^{2+}. The arrows represent the closed channel level and the dotted lines represent the open channel level. Po is given above each trace. In (A) 100 μM EMD 41000 was added to channels inactivated by 50 mM AMP and was relatively ineffective at increasing Po. In (B) the *cis* chamber was perfused to remove the AMP and EMD 41000. A large increase in Po was observed after re-addition of 100 μM EMD 4100.

that, in normal hearts, levels of AMP are hardly detectable. In addition, the affinity of AMP for the channel is lower than that of ATP which suggests that it is unlikely that AMP would have any effect on channel gating given that it would have to compete with high concentrations of ATP for the binding sites. Under conditions of ischaemia, however, when ATP levels fall and the levels of other adenine based compounds including AMP increase, the binding of AMP to the channel would be more likely to modulate gating. Given the low efficacy of AMP it would be expected to reduce Po by antagonizing the effects of ATP. Unfortunately, it is difficult to be precise about the possible effects of AMP without knowing the intracellular free [Ca^{2+}] and the concentration, affinity and efficacy of other adenine nucleotides and nucleosides present in the cell.

In conclusion, our results indicate that AMP binds to the same sites on the sheep cardiac RyR as ATP but because it has a very low efficacy it can effectively antagonize the effects of ATP on channel gating. Both the ability of AMP to increase Po and the affinity of the channel for AMP are highly dependent on the cytosolic free [Ca^{2+}]. While the adenine moiety appears to be crucial for binding to the ATP sites on the channel, this study demonstrates that changes to the group attached to the 5′-carbon of the ribose ring play an important role in determining the affinity and efficacy of the ligand and provides the basis for an understanding of the structural requirements of ligands for the ATP sites on the cardiac RyR.

We are grateful to the British Heart Foundation for support.

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(Received October 22, 1998
Revised January 5, 1999
Accepted January 26, 1999)