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RESEARCH PAPER

Diadenosine pentaphosphate is a potent activator of cardiac ryanodine receptors revealing a novel high-affinity binding site for adenine nucleotides

L Song, SM Carter, Y Chen and R Sitsapesan

Department of Physiology & Pharmacology, University of Bristol, School of Medical Sciences, University Walk, Bristol, UK

Background and purpose: Diadenosine polyphosphates are normally present in cells at low levels, but significant increases in concentrations can occur during cellular stress. The aim of this study was to investigate the effects of diadenosine pentaphosphate (Ap5A) and an oxidized analogue, oAp5A on the gating of sheep cardiac ryanodine receptors (RyR2).
Experimental approach: RyR2 channel function was monitored after incorporation into planar bilayers under voltage-clamp conditions.
Key results: With10 μmol·L^{-1} cytosolic Ca^{2+}, a significant 'hump' or plateau at the base of the dose–response relationship to Ap5A was revealed. Open probability (Po) was significantly increased to a plateau of approximately 0.2 in the concentration range 100 pmol·L^{-1}–10 μmol·L^{-1}. High Po values were observed at >10 μmol·L^{-1} Ap5A, and Po values close to 1 could be achieved. Nanomolar levels of ATP and adenosine also revealed a hump at the base of the dose–response relationships, although GTP did not activate at any concentration, indicating a common, high-affinity binding site on RyR2 for adenine-based compounds. The oxidized analogue, oAp5A, did not significantly activate RyR2 via the high-affinity binding site; however, it could fully open the channel with an EC_{50} of 16 μmol·L^{-1} (Ap5A EC_{50} = 140 μmol·L^{-1}). Perfusion experiments suggest that oAp5A and Ap5A dissociate slowly from their binding sites on RyR2.
Conclusions and implications: The ability of Ap5A compounds to increase Po even in the presence of ATP and their slow dissociation from the channel may enable these compounds to act as physiological regulators of RyR2, particularly under conditions of cellular stress.
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Keywords: ryanodine receptor; sarcoplasmic reticulum; ATP; diadenosine polyphosphates; Ca^{2+}-release; cardiac

Abbreviations: AMP-PCP, adenosine-5′-(β, γ-methylenetriphosphate); Ap5A, diadenosine pentaphosphate; RyR2, cardiac ryanodine receptor; SR, sarcoplasmic reticulum

Introduction

The cardiac ryanodine receptor (RyR2) plays an important role in coupling excitation to contraction by acting as the primary pathway for the release of Ca^{2+} from the sarcoplasmic reticulum (SR). ATP is thought to participate in the physiological regulation of RyR2 by potentiating the activation of the channel by cytosolic Ca^{2+} (Chan *et al.*, 2003). ATP itself, is minimally effective as an activator of RyR2, but in the presence of activating levels of cytosolic Ca^{2+}, and with Ca^{2+} as the permeant ion, it enables almost full activation of the channel (Meissner, 1994; Kermode *et al.*, 1998; Chan *et al.*, 2003). The significance of ATP as a physiological regulator of RyR2 in cardiac cells can be appreciated from experiments in permeabilized cardiac myocytes where intracellular ATP levels have been manipulated. These studies show that severe reductions in ATP concentrations lead to the complete abolition of Ca^{2+} sparks and Ca^{2+}-induced Ca^{2+}-release (Smith and O'Neill, 2001;Yang and Steele, 2001).

A number of ligands other than ATP also bind to the adenine nucleotide binding sites on RyR2 with varying degrees of affinity and efficacy. These include adenosine, AMP and the non-hydrolysable ATP analogue, adenosine-5′-(β, γ-methylenetriphosphate) (AMP-PCP) (Ching *et al.*, 1999; Chan *et al.*, 2000; 2003). Ligands without any phosphate groups (e.g. adenosine) can bind to RyR2 and increase open probability (Po) but have a minimal ability to increase the duration of channel openings. Adenosine therefore is a partial agonist with a limited capacity as an activator of RyR2 because it influences only the frequency of channel opening and not the duration of openings. It appears that the phosphate groups of ATP confer the ability to maximally

Correspondence: Dr R Sitsapesan, Department of Physiology & Pharmacology, University of Bristol, School of Medical Sciences, University Walk, Bristol BS8 1TD, UK. E-mail: r.sitsapesan@bris.ac.uk
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activate RyR2, enabling long open events to take place (Chan *et al.*, 2003).

A family of compounds characterized by having two adenosine moieties linked by multiple phosphate groups (2–6 phosphates), the diadenosine polyphosphates (ApnA where $n = 2$–6 phosphates), have been termed 'alarmones' because of their increased cellular production under conditions of metabolic stress (Lee *et al.*, 1983; Varshavsky, 1983; Bochner *et al.*, 1984; Baxi and Vishwanatha, 1995; Flores *et al.*, 1999). Certain ApnAs have been shown to stimulate [^{3}H]-ryanodine binding to brain homogenates and skeletal and cardiac microsomes (Holden *et al.*, 1996) and to stimulate SR Ca^{2+}-release from skeletal muscle (Morii and Makinose, 1992). Some ApnAs were found to be more effective at stimulating [^{3}H]-ryanodine binding than AMP-PCP, and it was suggested that they are high-affinity ligands for RyR channels. The effects of diadenosine compounds on the single-channel function of RyR channels have not been investigated. In view of the possible high-affinity interactions between ApnAs and RyR channels that might take place at the adenine nucleotide binding sites, we investigated how diadenosine pentaphosphate (Ap5A) and its oxidized analogue oAp5A could affect the gating of RyR2. Our results demonstrate that Ap5A and oAp5A (a periodate-oxidized Ap5A) exhibit high affinity for RyR2 and both are capable of inducing high Po values with long opening events. The binding of both ligands to RyR2 appears to be only slowly reversible. Our experiments also show that Ap5A, ATP and adenosine can activate RyR2 at concentrations less than 1 $\mu mol \cdot L^{-1}$ indicating that RyR2 possesses a novel high-affinity binding site for adenine nucleotides.

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.* (1991). SR 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.*, 1991). 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 $mmol \cdot L^{-1}$ HEPES, 125 $mmol \cdot L^{-1}$ Tris, 10 $\mu mol \cdot L^{-1}$ free Ca^{2+} buffered with EGTA and $CaCl_2$, pH 7.2. The *trans* chamber was perfused with a solution containing 250 $mmol \cdot L^{-1}$ glutamic acid, 10 $mmol \cdot L^{-1}$ HEPES, pH 7.2 with $Ca(OH)_2$ (free $[Ca^{2+}] \approx 50$ $mmol \cdot L^{-1}$). The free $[Ca^{2+}]$ and pH of the solutions in the presence and absence of ATP, adenosine, Ap5A and oAp5A were measured at 22°C by using a calcium electrode (Orion 93-20) and Ross-type pH electrode (Orion 81-55) and were maintained constant as described previously in detail (Sitsapesan *et al.*, 1991).

Data acquisition and analysis

Single-channel recordings were displayed on an oscilloscope and recorded on digital audio tape. Current recordings were filtered at 1 kHz (– 3 dB) and digitized at 20 kHz by using Pulse (HEKA Elektronik Lambrecht/Pfalz, Germany). Po was determined over 3 min of continuous recording, unless otherwise stated, by using the method of 50% threshold analysis (Colquhoun and Sigworth, 1983). The average Po for multiple channels was calculated as previously described (Sitsapesan and Williams, 1995). The total number of channels in the bilayer was determined by maximally activating the channels with caffeine at the end of each experiment. 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. Individual lifetimes were fitted to a probability density function (pdf) by the method of maximum likelihood (Colquhoun and Sigworth, 1983) according to the equation:

$$g(x) = \sum_{i=1}^{N} a_i g_o(x - \ln \tau_i)$$

Where $\ln \tau_i$ is the logarithm of the ith time constant, and a_i is the fraction of the total events represented by that component (Sigworth and Sine, 1987). A likelihood ratio test (Blatz and Magleby, 1986) was used to compare fits of up to four exponentials by testing the chi-squared distribution at the 1% level. Measurements of current amplitude were made by using the WinEDR programme (John Dempster, Strathclyde University) by manually assessing the closed and open current levels by using cursors.

Chemicals and solutions

Tris, HEPES, glutamate, $Ca(OH)_2$, PIPES were purchased from BDH (BDH Biochemical Laboratories, Poole, UK). EGTA, Ap5A, ATP, oAp5A and adenosine were from Sigma (Sigma-Aldrich Company Ltd. Gillingham, Dorset, UK).

Statistics

Means ± s.e.mean are shown where the number of experiments ≥4. For $n = 3$, standard deviation is given. Where appropriate, Student's *t*-test was used to assess the difference between mean values. A *P*-value <0.05 was taken as significant. Where comparison of three or more groups was required, we used ANOVA followed by Dunnett's test.

Results

Figure 1 illustrates the effects of a wide range of doses of Ap5A on the current fluctuations through a representative sheep cardiac RyR2 channel incorporated into a planar phospholipid bilayer. In the presence of an activating level of cytosolic Ca^{2+} (10 $\mu mol \cdot L^{-1}$), even a very low concentration of 0.1 $nmol \cdot L^{-1}$ Ap5A, when added to the cytosolic side of the bilayer, caused an increase in Po (Figure 1A). The traces demonstrate that the channel openings remain very brief and the gating is characterized by short opening and closing events giving rise to an appearance of 'flickery' gating. Subsequent increases in Ap5A concentration up to 10 $\mu mol \cdot L^{-1}$ did not cause any further significant increases in Po (see Figure 1A iii and iv) and the flickery appearance of gating remained. Po

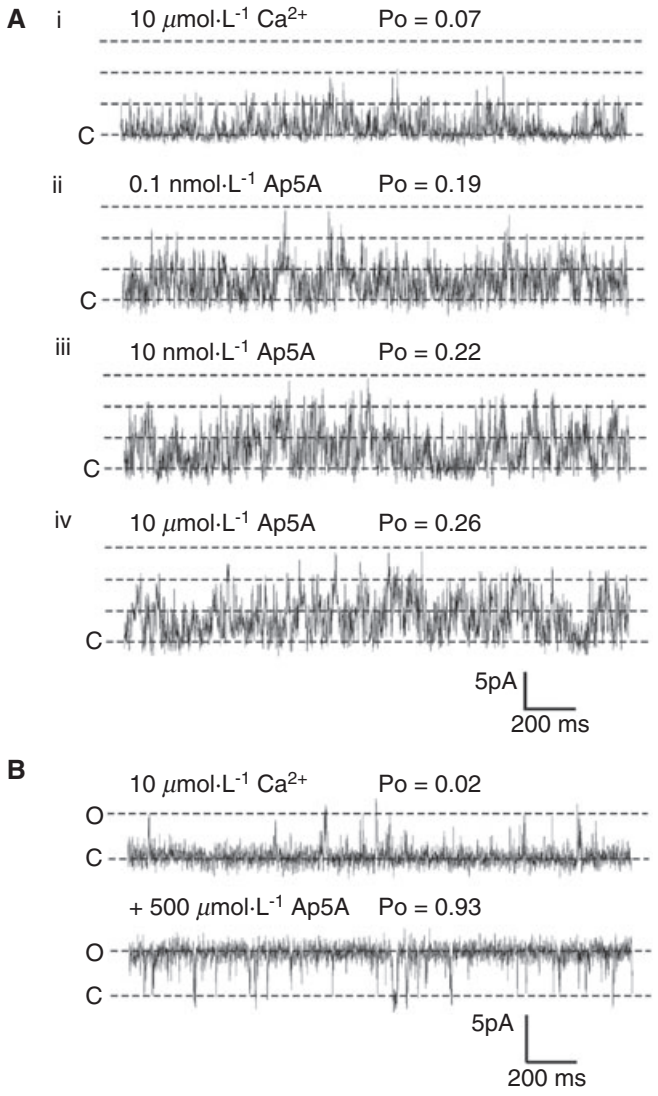

Figure 1 Effects of nanomolar and micromolar concentrations of Ap5A on RyR2 channels. In (A), a typical experiment where three channels incorporated into the bilayer are shown. In (A) the channel was activated solely by 10 μmol·L^{-1} cytosolic Ca^{2+}. Subsequent additions of 0.1 nmol·L^{-1} (ii), 10 nmol·L^{-1} (iii) and 10 μmol·L^{-1} Ap5A (iv) were made to the cytosolic channel side. The holding potential was 0 mV. The dotted lines indicate the open channel levels, and the 'C' indicates the zero current level. Channel Po is indicated above each trace. In (B), a single channel has incorporated into the bilayer. In the top trace the channel was activated solely by 10 μmol·L^{-1} cytosolic Ca^{2+}. Subsequent addition of 500 μmol·L^{-1} Ap5A to the cytosolic channel side almost fully opened the channel. Ap5A, diadenosine pentaphosphate; Po, open probability; RyR2, cardiac ryanodine receptor.

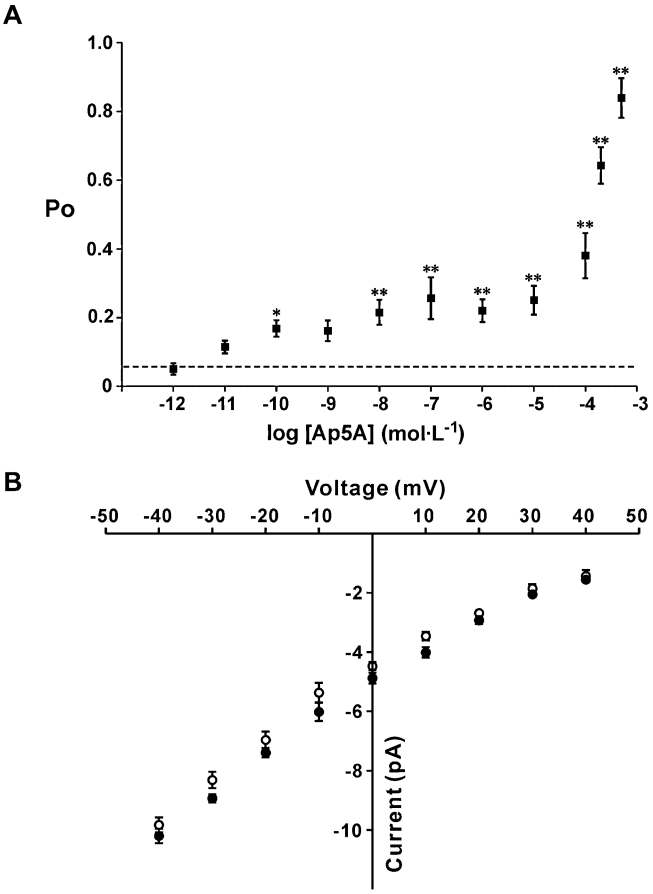

Figure 2 Effects of Ap5A on Po and conductance. (A) The dose–response relationship of cytosolic Ap5A in the presence of 10 μmol·L^{-1} cytosolic free Ca^{2+}. The dashed line indicates the mean control Po (0.060 ± 0.007, $n = 37$) in the presence of 10 μmol·L^{-1} Ca^{2+} alone. The numbers of experiments for each dose on the plateau portion of the dose–response curve were (starting at 10 pmol·L^{-1}): 10, 10, 11, 9, 8, 8 and 9. Elsewhere, mean values ± s.e.mean are shown for $n \geq 4$. The asterisks show where values are significantly different from the controls $^*P < 0.05$, $^{**}P < 0.01$. (B) The current–voltage relationship of RyR2 in the presence (filled circles) and absence (open circles) of 100 μmol·L^{-1} Ap5A. Single-channel conductance was calculated from the slope of the regression lines between − 40 mV and + 20 mV and was 120.0 ± 4.9 pS for controls and 122.1 ± 4.0 pS after addition of 100 μmol·L^{-1} cytosolic Ap5A. The mean values ± s.e.mean are shown ($n = 4$). Ap5A, diadenosine pentaphosphate; Po, open probability; RyR2, cardiac ryanodine receptor.

appeared to plateau at approximately 0.2. Only when Ap5A was increased above 10 μmol·L^{-1} were further increases in Po observed. As shown in Figure 1B, Ap5A could almost fully activate the channel at high micromolar concentrations, and these high Po values were characterized by long open events. Figure 2A shows the dose–response curve for Ap5A and clearly illustrates the long Po plateau at the base of the relationship. The dashed line shows the control Po in the presence of 10 μmol·L^{-1} Ca^{2+}. Figure 2B shows the current–voltage relationship in the presence and absence of Ap5A and demonstrates that Ap5A had no effect on single-channel conductance.

It is likely that Ap5A and ATP bind to overlapping sites on RyR2, but we have not previously observed a hump at the base of the dose–response relationship for ATP or any other adenine nucleotide. Because only small increases in Po were observed at the 10 μmol·L^{-1} dose, we did not previously investigate the effects of lower concentrations. We therefore examined whether ATP and adenosine could also activate RyR2 at nanomolar concentrations. Figure 3 demonstrates that low concentrations of both ATP and adenosine give rise to activation of RyR2 to a maximum Po of approximately 0.2, although the shape of the hump is different for Ap5A, ATP and adenosine. The low-dose activation hump for adenosine (Figure 3B) peaks at around 10 nmol·L^{-1} and then appears to decline back

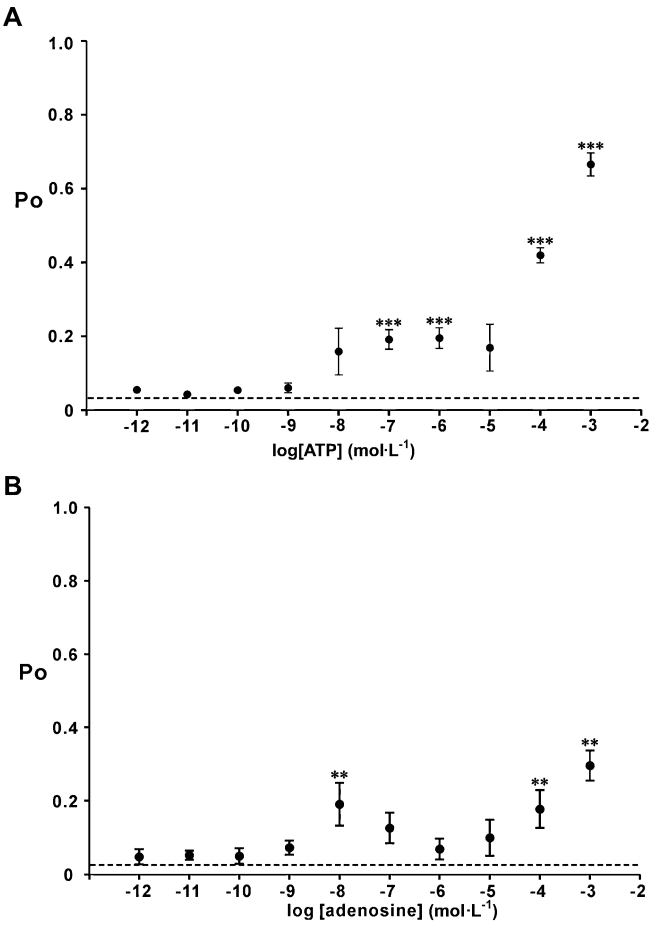

Figure 3 RyR2 Po dose–response relationships for ATP and adenosine. Panels (A) and (B) show the RyR2 Po dose–response relationship for ATP and adenosine respectively. In each case the dashed line indicates the control Po in the presence of 10 μmol·L^{-1} cytosolic free Ca^{2+} alone, which was 0.049 ± 0.008 ($n = 45$) for ATP and 0.025 ± 0.008 ($n = 16$) for adenosine. The numbers of experiments for each dose on the plateau portion of the ATP dose–response curve were (starting at 1 nmol·L^{-1}): 10, 10, 13, 13 and 7. Elsewhere, mean values ± s.e.mean are shown for $n \geq 10$. The numbers of experiments for each dose on the plateau portion of the adenosine dose–response curve were (starting at 1 nmol·L^{-1}): 8, 6, 7, 7 and 8. Elsewhere, mean values ± s.e.mean are shown for $n \geq 5$. The asterisks show where values are significantly different from the controls $^{**}P < 0.01$, $^{***}P < 0.001$. Po, open probability; RyR2, cardiac ryanodine receptor.

towards the baseline Po with higher concentrations of adenosine. This could indicate that adenosine binds to a high-affinity activation site and a slightly lower-affinity inactivation site. The binding of adenosine to an even lower-affinity site then gives rise to the increase in Po observed at micromolar and millimolar concentrations. We have previously shown that adenosine has a low efficacy for activation of RyR2 and this has also been shown for RyR1 (Chan *et al.*, 2000; Laver *et al.*, 2001), and the figure confirms this showing that even micromolar and millimolar adenosine do not activate RyR2 above a Po of approximately 0.3. To investigate the specificity of the high-affinity site we examined the effects of low doses of GTP on RyR2 channel function. [We have previously shown that GTP at concentrations 100 μmol·L^{-1}–10 mmol·L^{-1} has no effect on RyR2 Po (Chan *et al.*, 2000)]. We observed no effect on Po at any concentration of GTP [control Po was 0.069 ± 0.008, and the Po values after the addition of 1 pmol·L^{-1}, 1 nmol·L^{-1}, 1 μmol·L^{-1} and 100 μmol·L^{-1} GTP were 0.069 ± 0.006, 0.073 ± 0.013, 0.081 ± 0.009 and 0.059 ± 0.011 ($n = 4$–6) respectively], suggesting a requirement for the adenine nucleotide part of the molecule.

Figure 4 shows a comparison of the dose–response curves of Ap5A, ATP and adenosine. Although all cause a hump at the base of the dose–response curve, these humps appear different. The Ap5A hump is evident at 10 pmol·L^{-1} whereas adenosine and ATP do not increase Po until approximately 10 nmol·L^{-1}. In addition, the shape of the adenosine hump suggests that doses of adenosine above 10 nmol·L^{-1} may have an inhibitory component. Thus, the data suggest that there may be high-affinity and low-affinity activation sites for adenine nucleotides but there may also be an intermediate-affinity site that is inhibitory. Previous work has shown that there must also be an additional very low-affinity inhibitory site that operates at millimolar concentrations (Kermode *et al.*, 1998).

To address the mechanisms underlying the changes in Po caused by the low concentrations of these ligands, we compared the changes in mean open and closed times for Ap5A, ATP and adenosine, as shown in Figure 4B,C. These measurements cannot be made if multiple channels incorporate into a bilayer, only if a single channel is present. The figures demonstrate that the parameter that changes most at low doses of the adenine nucleotide is the mean closed time. The initial rises in Po are associated with decreases in mean closed time, which tends to plateau during the plateau in Po. As Po increases again at the micromolar concentrations of the ligands, mean closed time is further reduced. In contrast, mean open times change less at low doses of nucleotide. There appears to be a slight tendency for an increase in mean open time during the Po plateau phase, but this is only significant for Ap5A at 10 nmol·L^{-1} [mean open time increases from 0.80 ± 0.04 ms ($n = 17$) to 2.70 ± 0.87 ms ($n = 5$, $P < 0.05$)]. At the micromolar concentrations of Ap5A and ATP, large increments in Po are associated with large changes in mean open time. Adenosine, on the other hand, does not increase mean open time even at micromolar and millimolar concentrations and is therefore not capable of large increments in Po, as demonstrated previously (Chan *et al.*, 2000).

Lifetime analysis, again carried out when only a single channel was present in the bilayer, confirms the data shown in Figure 4. Figure 5 illustrates the changes that occur to open and closed lifetime distributions for a typical channel activated by Ap5A. Under the experimental conditions of this study, in the control situation with 10 μmol·L^{-1} cytosolic Ca^{2+} as the sole activating ligand, RyR2 channel gating is characterized by brief channel openings. Two open and three closed states can be identified, and most of the opening events occur to the shortest time constant (Figure 5A). It can be seen that the main change in the lifetime distributions at 0.1 and 1 nmol·L^{-1} Ap5A is the reduction in closed lifetimes. Little further change in closed lifetimes is observed until micromolar Ap5A, when further reductions in closed lifetimes are evident. Open lifetimes, in contrast, change little until 10 nmol·L^{-1}, when a third, longer open state can be detected and fewer openings occur to the briefest time constant. At micromolar Ap5A, when the huge increases in Po are evident, there are significant shifts

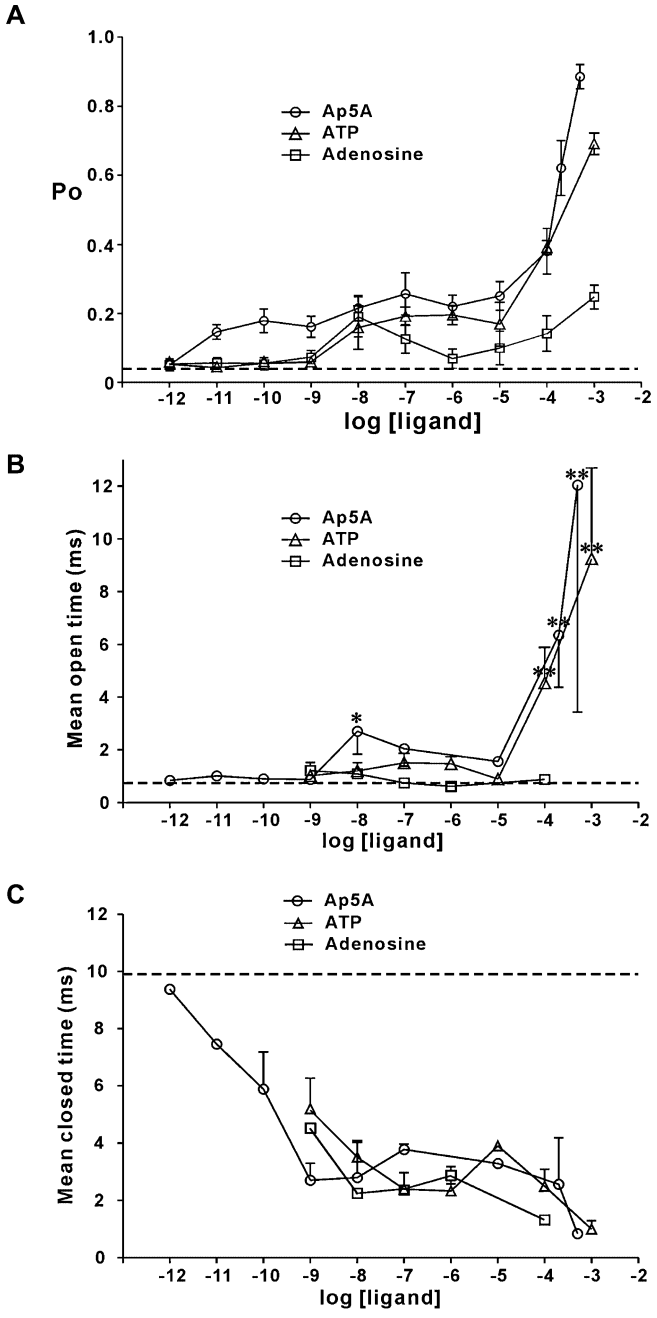

Figure 4 Comparison of the Ap5A, ATP and adenosine dose–response relationships, and their effects on the mean open and closed times. The relationships between ligand concentration and Po (A), mean open time (B) and mean closed time (C) are shown. In (A), (B) and (C), the dashed lines indicate the average control Po level (0.048 ± 0.005, $n = 112$), the average control mean open and the average control mean closed time respectively. In (B) and (C) only experiments where single-channels incorporated into the bilayer were used. The asterisks in (B) show where values are significantly different from the controls $^*P < 0.05$, $^{**}P < 0.01$. In (C), the asterisks denoting values significantly different from controls are omitted for clarity; however, all values ≥1 nmol·L^{-1} for Ap5A and all values ≥10 nmol·L^{-1} for ATP and adenosine are significant ($P < 0.05$ or $P < 0.01$). Mean values ± s.e.mean are shown for $n \geq 4$. Ap5A, diadenosine pentaphosphate; Po, open probability.

in the open lifetime distributions such that only 34% of openings occur to the shortest open time.

To address the question of whether the effects of low and high doses of Ap5A are reversible, we perfused the Ap5A away from the cytosolic chamber. The histogram shown in Figure 6A demonstrates that after 3 min in the presence of a low dose of Ap5A (100 pmol·L^{-1}), its effects were not reversible. Inspection of the individual traces showed that although, on average, Po was not lowered after washout, reversal of Po towards control values did occur in two out of eight experiments. The reversibility of a high dose of Ap5A (200 μmol·L^{-1}) is illustrated in Figure 6B. Again, the figure highlights that Ap5A is not fully reversible. Figure 6C shows the Po before and after washout for the individual experiments used in the histogram in Figure 6B. It can be seen that there was a high level of variability in the level to which 200 μmol·L^{-1} Ap5A increased Po. In two of seven channels, Ap5A only activated to a Po of approximately 0.2, and in these channels the effects were fully reversible. The Po of the remaining channels was much higher and was either not changed at all (two channels) or was partially reversible (three channels). Thus, the effects of both high and low doses of Ap5A are not completely reversible on the time scale of a single-channel experiment. Figure 6D demonstrates that, in contrast, the effects of ATP are completely reversible; 2 mmol·L^{-1} ATP was present in the cytosolic chamber for 10 min before it was perfused away. Po was reversed back to control levels in all experiments.

We then wanted to investigate if Ap5A and ATP were binding to the same binding sites on RyR2. If this were the case, the two ligands would not be expected to potentiate each other; the simultaneous effect of the two ligands would be no greater than the maximum effect of ATP alone. Therefore, adding a low dose of ATP to a low dose of Ap5A (or vice versa) should not increase Po above the plateau level of approximately 0.2 (the maximum Po for either compound at doses up to 10 μmol·L^{-1}). Figure 7A shows a comparison of the effects of a low dose of Ap5A (100 pmol·L^{-1}) and a low dose of ATP (100 nmol·L^{-1}), both of which increase Po to the plateau level of approximately 0.2. The dose–response curves of these ligands shown in Figures 2A and 3A demonstrate that at least 1000-fold these doses are required to increase Po significantly. If both 100 nmol·L^{-1} ATP and 100 pmol·L^{-1} Ap5A are present simultaneously; however, Po is significantly raised (Figure 7A). This suggests either that the two ligands are binding to independent domains on RyR2 or that the binding of one ligand somehow interferes with the binding of the other ligand.

The irreversible or slowly reversible nature of Ap5A binding to RyR2 would be expected to contribute to its ability to activate RyR2. Ap5A appears to have a higher affinity for RyR2 at both the high-affinity binding site (as evidenced by the appearance of the 'hump' in the dose–response relationship at lower concentrations than ATP) and the low-affinity activation site [EC_{50} for Ap5A is 140 μmol·L^{-1} and for ATP is 220 μmol·L^{-1} (Kermode *et al.*, 1998) at the low-affinity activation site]. A ligand that has a higher affinity than ATP for ATP binding sites and also dissociates very slowly from the sites may be capable of sustaining RyR2 Po even when cardiac cells become metabolically compromised and ATP levels are low. We therefore investigated how 100 μmol·L^{-1} Ap5A [a level

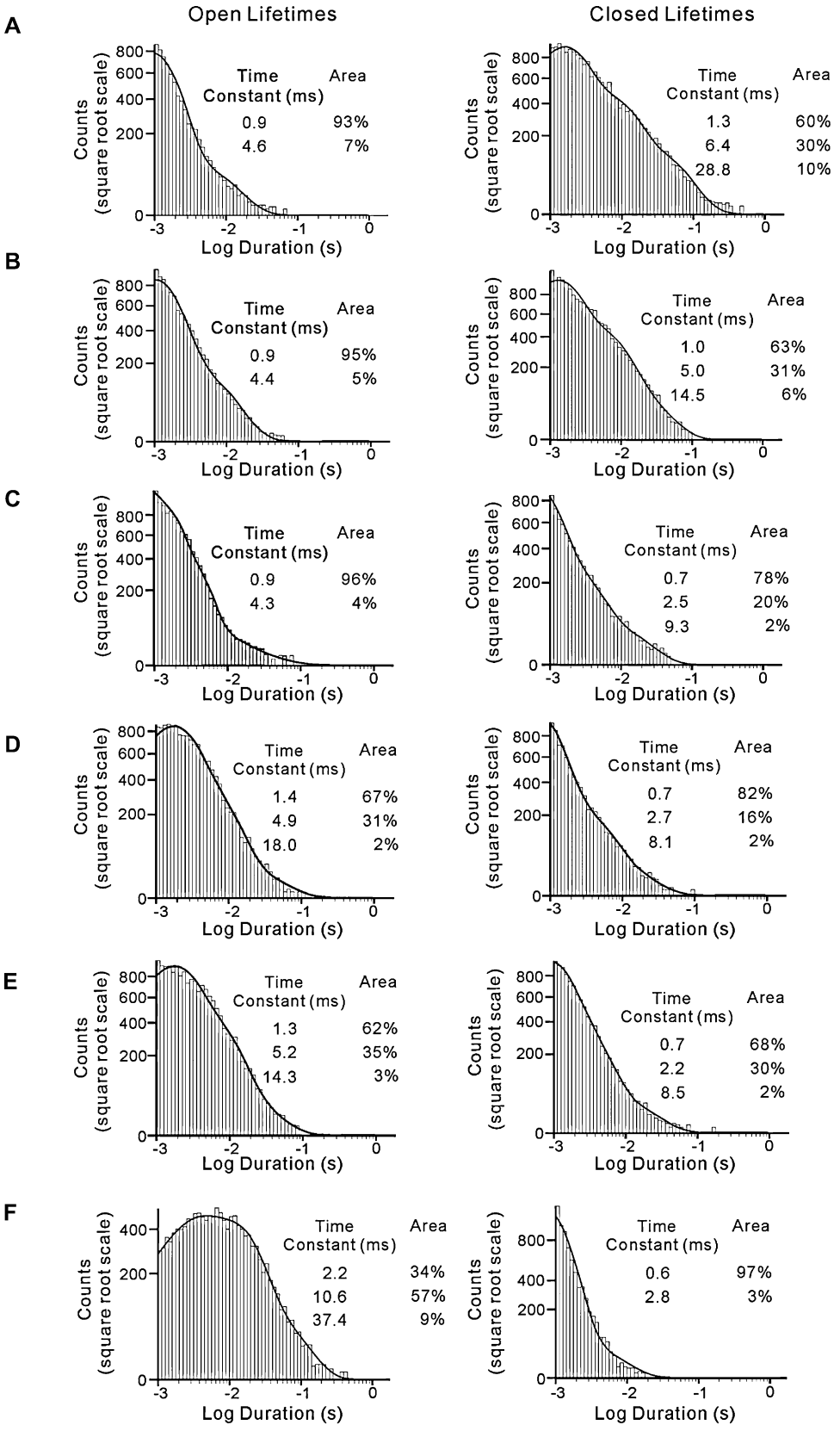

Figure 5 The effects of Ap5A on open (left) and closed (right) lifetime distributions and probability density functions (pdf). The best fits to the data were obtained by the method of maximum likelihood fitting (Colquhoun and Sigworth, 1983). The resulting time constants and corresponding percentage areas are shown. In (A), the channel was activated by 10 μmol·L^{-1} cytosolic Ca^{2+} alone. In (B), (C), (D), (E) and (F) the effects of 0.1 nmol·L^{-1}, 1 nmol·L^{-1}, 10 nmol·L^{-1}, 100 nmol·L^{-1} and 500 μmol·L^{-1} Ap5A, respectively, are shown. Ap5A, diadenosine pentaphosphate.

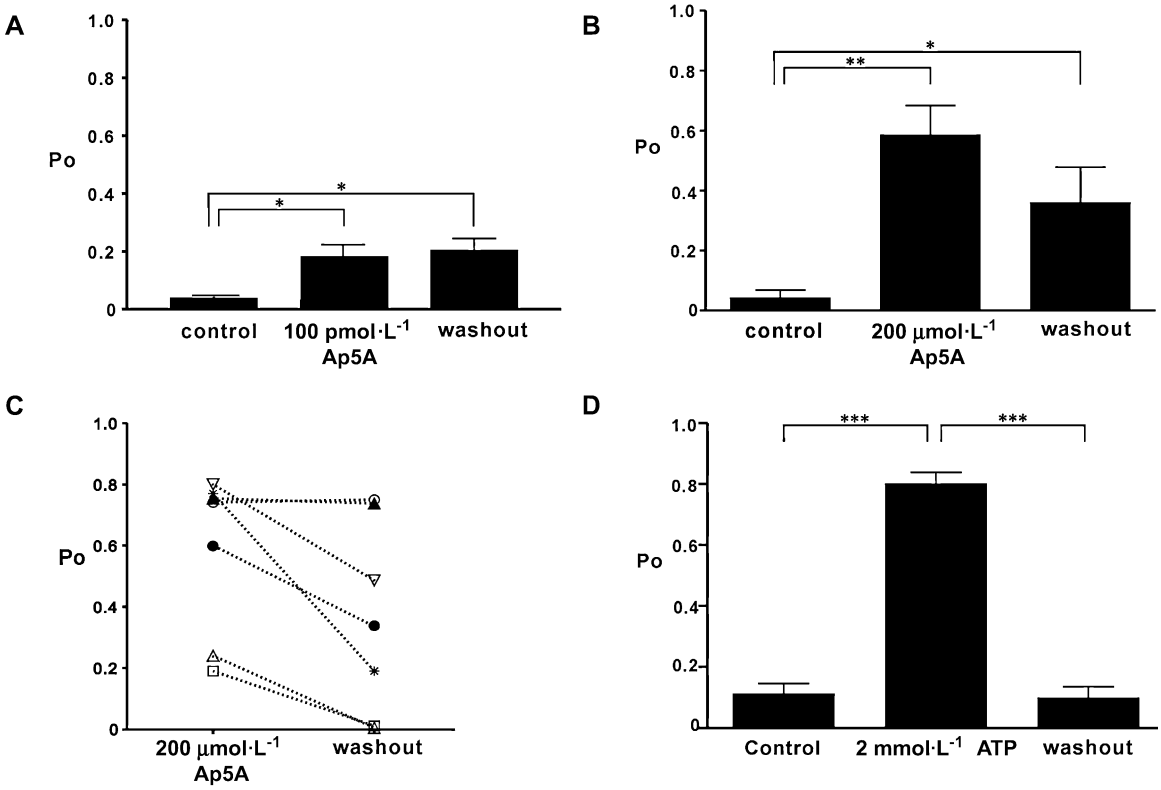

Figure 6 The reversibility of low doses (100 pmol·L^{-1}) and high doses (200 µmol·L^{-1}) of Ap5A. In (A), the histogram shows the mean Po before addition of Ap5A (control), in the presence of 100 pmol·L^{-1} Ap5A and after washout of Ap5A from the cytosolic chamber. The Po values were determined over a 3 min recording period at the holding potential of 0 mV. Mean values ± s.e.mean ($n = 8$) are shown. In (B) the histogram shows the mean Po before addition of Ap5A (control), in the presence of 200 µmol·L^{-1} Ap5A and after washout of Ap5A from the cytosolic chamber. The Po values before and after washout are not significantly different. Mean values ± s.e.mean ($n = 7$) are shown. (C) The Po of the individual channels in the presence of 200 µmol·L^{-1} Ap5A and after washout of Ap5A from the cytosolic chamber. In (D) the histogram shows the mean Po in the presence of 10 µmol·L^{-1} cytosolic Ca^{2+} (control), in the presence of 2 mmol·L^{-1} ATP and after washout of ATP from the cytosolic chamber. The Po values were determined over a 3 min recording period for controls and washout values. The Po in the presence of ATP was measured for 10 min. Mean values ± s.e.mean are shown ($n = 4$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. Ap5A, diadenosine pentaphosphate; Po, open probability.

that may be reached during stress (Holden *et al.*, 1996;Luo *et al.*, 2004)] affects the gating of RyR2 in the presence of a low ATP concentration (100 µmol·L^{-1}) and whether the Po of the channels can be maintained after perfusing away the ATP. Figure 7B shows the average Po (recorded over 3 min) for six individual channels incubated with 100 µmol·L^{-1} Ap5A + 100 µmol·L^{-1} ATP and the subsequent time course of the Po changes after the ATP and Ap5A had been perfused away. It can be seen that Po remains fairly high for several minutes in all channels after washout but gradually falls away. This is interesting given that the effects of ATP alone are immediately reversible (Figure 6D). Figure 7C shows a comparison of the Po in response to 100 µmol·L^{-1} ATP alone and 100 µmol·L^{-1} Ap5A alone with the simultaneous presence of the two ligands and the average Po in the first 2 min after washout and the second 2 min after washout. As can be seen the effects of Ap5A and ATP are approximately additive and, significantly, their effects are only very slowly reversible. In a cellular context, this could have important consequences during metabolic stress when ATP levels are reduced; the maintained binding of Ap5A to RyR2 would be predicted to enable the continued activation of RyR2.

We then examined whether the oxidized analogue of Ap5A (oAp5A) is even more potent. oAp5A might be expected to form a Shiff base on binding to RyR2 leading to a more permanent ligand-receptor complex. In fact, as shown in Figure 8, we found that picomolar and nanomolar oAp5A did not significantly activate RyR2. Po was increased only when oAp5A concentrations were raised above 1 µmol·L^{-1}, and then it was very effective; 10 µmol·L^{-1} oAp5A caused significant activation of all three channels in the bilayer, and this was characterized by rapid flickery gating. The dose of 100 µmol·L^{-1} oAp5A caused almost maximal activation of the channels. In Figure 8B, the dose–response relationship is shown. The EC_{50} and Hill slope for the relationship were 16 µmol·L^{-1} and 1.74 respectively. The EC_{50} value demonstrates that oAp5A has a higher affinity for RyR2 than any other compound thought to interact with the adenine nucleotide binding site. In Figure 9A, the effects of washing out the oAp5A are shown for a single channel. For this particular channel it can be seen that the effects of oAp5A are, at least within the recording period of the experiment (for 3 min after washout), completely irreversible. Because the effects of Ap5A are not fully reversible and because the oxidation of

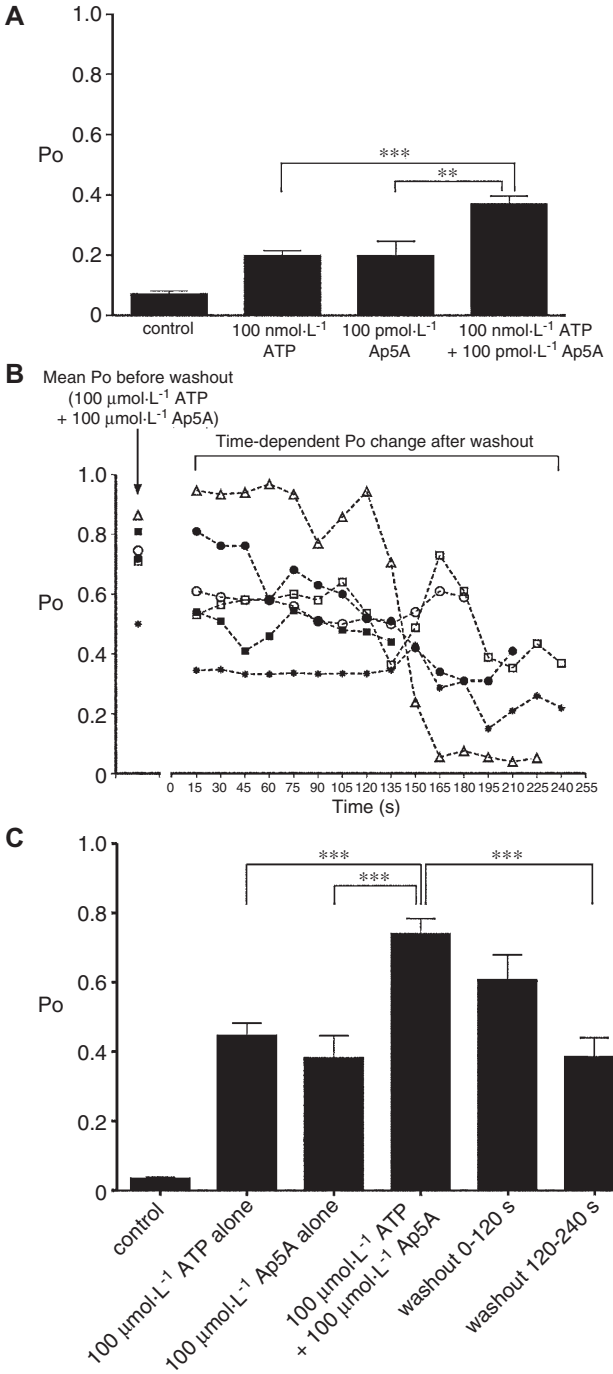

Figure 7 The effects of simultaneously incubating ATP and Ap5A on the Po of RyR2. In (A), low doses of both ligands were used. The histogram shows the Po of RyR2 channels in the presence of 10 µmol·L^{-1} cytosolic Ca^{2+} only (control), and after addition of either 100 nmol·L^{-1} ATP, 100 pmol·L^{-1} Ap5A or the simultaneous addition of 100 nmol·L^{-1} ATP plus 100 pmol·L^{-1} Ap5A. Channel activity in the presence of both ligands together was significantly greater than that in the presence of only ATP or Ap5A. Mean values ± s.e.mean are shown ($n \geq 5$). In (B), RyR2 was simultaneously incubated with 100 µmol·L^{-1} ATP and 100 µmol·L^{-1} Ap5A for 3 min. Both compounds were perfused away, and the time course of the decline in Po was then monitored for each individual channel. The period of perfusion was 2 min in all experiments. In (C) the mean data for $n = 6$ are presented in the form of a histogram. $^{**}P < 0.01$, $^{***}P < 0.001$. Ap5A, diadenosine pentaphosphate; Po, open probability; RyR2, cardiac ryanodine receptor.

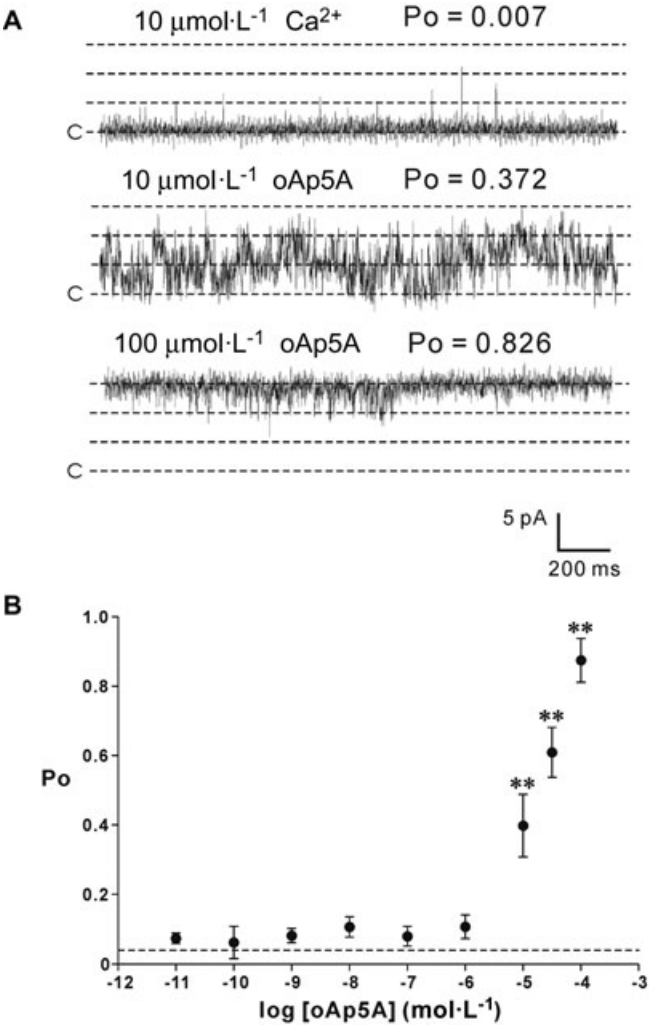

Figure 8 The effects of cytosolic oAp5A on RyR2 channel gating. In (A), a typical experiment is shown where three channels have incorporated into the bilayer. In the top trace the channel was activated by 10 µmol·L^{-1} cytosolic Ca^{2+} only. Subsequent additions of 10 µmol·L^{-1} and 100 µmol·L^{-1} oAp5A were then made to the cytosolic chamber. The holding potential was 0 mV. The dotted lines indicate the open channel levels and the 'C' indicates the zero current level. The oAp5A Po dose–response relationship is shown in (B). The dashed line indicates the control Po level (0.035 ± 0.009, $n = 14$) in the presence of 10 µmol·L^{-1} cytosolic free Ca^{2+}. Mean values ± s.e.mean are shown ($n \geq 4$). The asterisks show where values are significantly different from the controls. $^{**}P < 0.01$. oAp5A, oxidized analogue of diadenosine pentaphosphate; Po, open probability; RyR2, cardiac ryanodine receptor.

Ap5A is likely to produce a compound that could form a Schiff base with RyR channels (Hohenegger *et al.*, 1995) and therefore a more permanent interaction, this result is not surprising. The average data for all the channels are shown as a histogram in Figure 9B, demonstrating that oAp5A is not readily reversible. It was not possible to monitor the Po after washout of Ap5A in all the experiments because the bilayers frequently broke during perfusion; however, the time course of the Po changes in the successful perfusion experiments are shown in Figure 10. We know that multiple oAp5A molecules must bind to each RyR2 molecule for maximum Po levels to be attained. Perhaps Po drops an increment each time one

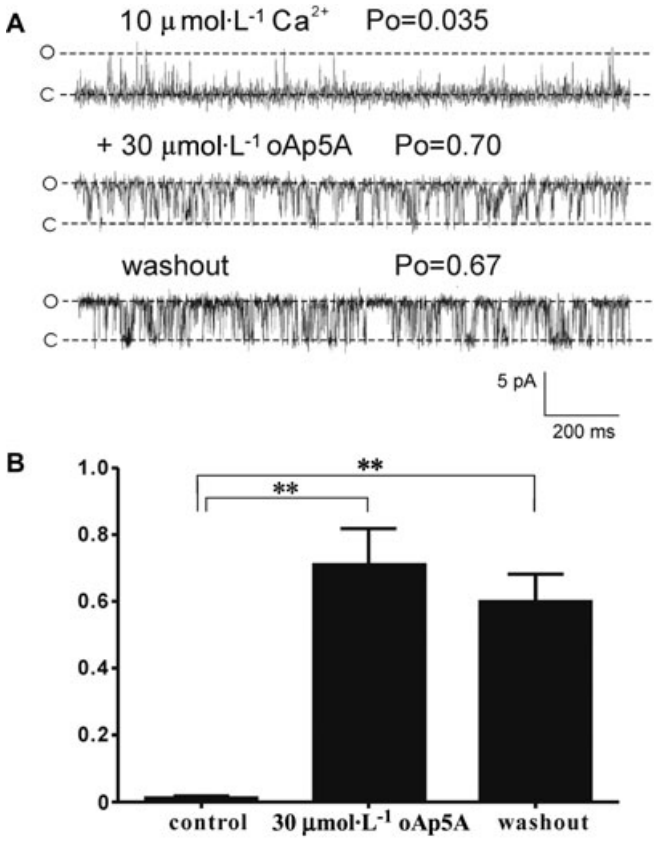

Figure 9 The persistent effects of oAp5A. In (A), an example of the effects of washing out the oAp5A from the cytosolic chamber are shown. The top trace shows the control, and the subsequent traces show channel gating in the presence of 30 μmol·L^{-1} oAp5A and after it had been perfused away. The histogram in (B) shows the average Po in the presence of 10 μmol·L^{-1} cytosolic Ca^{2+} (control), after addition of 30 μmol·L^{-1} oAp5A and after washout. The Po values were determined over a 3 min recording period at the holding potential of 0 mV. The Po values in presence of 30 μmol·L^{-1} Ap5A and after washout are not significantly different. Mean values ± s.e.mean are shown ($n \geq 4$). $^{**}P < 0.01$. Ap5A, diadenosine pentaphosphate; oAp5A, oxidized analogue of Ap5A; Po, open probability.

tightly bound oAp5A molecule dissociates from RyR2 thus giving rise to a very slow reversal of Po.

Discussion

We have demonstrated that Ap5A and oAp5A are potent activators of the sheep RyR2. Moreover, we have identified a novel high-affinity binding site for adenine nucleotides. Based on a bell-shaped dose–response curve, we previously suggested that there was a high-affinity activation and low-affinity inactivation site for adenine nucleotides on RyR2 (Kermode *et al.*, 1998). The results of this study now add to this data and indicate that there may be two activation sites, high-affinity and low-affinity. We did not previously investigate the ability of adenine nucleotides to activate RyR2 at concentrations lower than 1 μmol·L^{-1}, and therefore what we previously labelled as a high-affinity activation site, in fact now corresponds to the lower-affinity activation site.

Although no evidence for high- and low-affinity adenine nucleotide activation sites has previously been shown for RyR2, there is evidence for two adenine nucleotide activation sites on the skeletal ryanodine receptor (RyR1). This has been suggested by single-channel work using adenosine as an antagonist of ATP (Laver *et al.*, 2001) but also in a biphasic dose–response curve to ATP (Jona *et al.*, 2001). Additionally, use of spin-labelled ATP provides evidence that eight molecules of ATP bind per RyR1 tetramer with a K_d of approximately 170 μmol·L^{-1} (Dias *et al.*, 2006). As RyR channels are tetramers, this would fit with the idea that there would be four high-affinity and four low-affinity activation sites per tetramer. If a similar number of ATP molecules bound to RyR2 this would not account for the low-affinity inactivation site reported previously (Kermode *et al.*, 1998) or for the possibility that a high-affinity inactivation site could exist that would explain the adenosine dose–response relationship. We might therefore expect more ATP molecules to bind to RyR2.

Binding of Ap5A to the high-affinity activation binding site can increase Po to a maximum of approximately 0.2. The same is true for ATP and adenosine. Thus, the phosphate

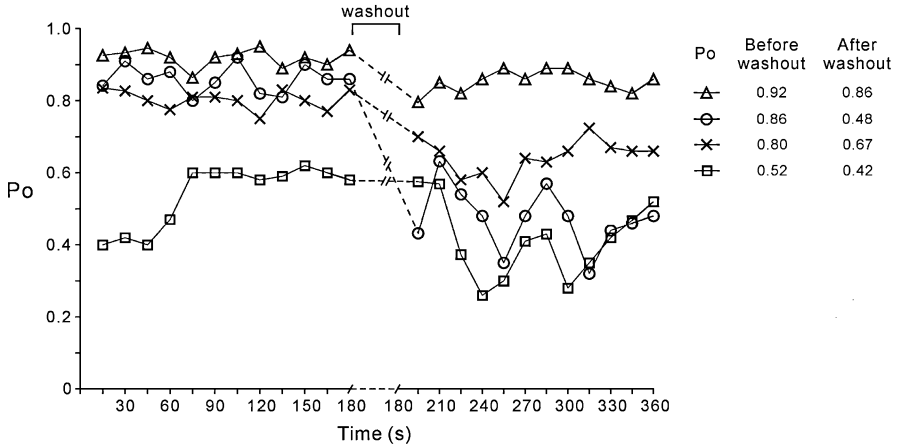

Figure 10 Single-channel reversibility profiles. The time course of the decline in Po following perfusion is shown for those experiments (from Figure 9) where the bilayer did not break during perfusion. Po was plotted every 15 s starting after 30 μmol·L^{-1} oAp5A was added to the cytosolic chamber. The time when oAp5A was perfused away is indicated by 'washout'. The period of perfusion was 2 min in all experiments. oAp5A, oxidized analogue of diadenosine pentaphosphate; Po, open probability.

groups are not essential for binding to this site nor is a diadenosine compound. Because GTP has no effect at any concentration (range 1 pmol·L^{-1}–10 mmol·L^{-1}) it appears that the adenine moiety is necessary, although there may be other ligands with higher affinity for this site and/or a greater ability to increase Po once bound (greater efficacy). Interestingly, the oxidized analogue, oAp5A, did not significantly increase Po at concentrations below 1 μmol·L^{-1}, suggesting either that structural change to the ribose groups does not allow it access to the high-affinity binding site or that it can bind but has no intrinsic ability to open the channel.

Diadenosine compounds have previously been shown to increase [^{3}H]-ryanodine binding to microsomes from rat brain and skeletal and cardiac muscle (Holden *et al.*, 1996) and to increase CICR from skeletal SR (Morii and Makinose, 1992), but this is the first report of the single-channel actions of these compounds. It has been suggested that a single ApnA compound could bridge two adenine nucleotide binding sites on different subunits of the RyR tetramer (Morii and Makinose, 1992). Our single-channel work, however, does not provide any clear evidence for such a model for Ap5A or oAp5A. In theory, Ap5A could bridge two low-affinity sites, two high-affinity sites or one low- and one high-affinity site. If we consider first the bridging of two low-affinity binding sites, the sites responsible for most of the increment in Po, the Hill slopes for the ATP [1.5, (Kermode *et al.*, 1998)], oAp5A (1.74) and Ap5A (1.7) dose–response curves are similar, suggesting that the nature of the positive co-operativity is also similar. If an Ap5A molecule was bridging two low-affinity adenine nucleotide binding sites, then it might be expected that the Hill slope would increase. The order of affinity (for the low-affinity activation site) is oAp5A > Ap5A > ATP, and this could be explained by the irreversible or slowly reversible nature of oAp5A and Ap5A (in comparison with ATP that is completely and rapidly reversible) rather than suggesting that a change in affinity is caused by a single Ap5A molecule accessing two ATP binding sites. Indeed, oxidation of ATP to give oATP (Hohenegger *et al.*, 1995) also increases the affinity (EC_{50}) of the ATP molecule for purified skeletal channels and gives a Hill coefficient of approximately 2, similar to that of ATP, Ap5A, oAp5A for RyR2. Thus, both the present study and previous work (Hohenegger *et al.*, 1995) suggest that oxidation of the ATP molecule can anchor it more firmly in the low-affinity activation site enabling the molecule to maintain long open states and high Po values. Alternatively, if the Ap5A molecules can bridge the low-affinity and high-affinity binding sites, it is possible that the apparent increase in affinity of oAp5A and Ap5A above ATP for the low-affinity sites is a direct result of the diadenosine molecules being bound to the high-affinity sites. oAp5A does not significantly increase Po at low doses, but that does not necessarily signify that it cannot bind to the high-affinity site; it may bind but have low efficacy. Ap5A and oAp5A are more potent than ATP or adenosine at the high-affinity sites. It is possible that this does result from the bridging of two high-affinity adenine nucleotide sites by the diadenosine compounds, but further work is required to determine the answer to these questions.

Does single-channel lifetime analysis provide any insight into the mechanisms underlying channel activation via the high- and low-affinity binding sites? The overall message that we can take from the results depicted in Figures 4 and 5 is that activation via the high-affinity binding sites predominantly occurs by reducing the duration of closed lifetimes. In other words there is an increase in the frequency of channel opening, suggesting that ligand binding to the closed channel state is responsible for mediating the effects of ligand binding to the high-affinity site. The plateaus in the dose–response curves coincide with plateaus in the reduction of the closed lifetime durations. In contrast, activation via the low-affinity site primarily occurs by increases in the duration of open lifetimes, although some reduction in the closed lifetime durations is also apparent. Hence binding to the open channel states may be responsible for the increases in Po via the low-affinity binding sites. This approximate demarcation in gating kinetics suggests that ligand binding to the high-affinity sites causes an increase in frequency of channel opening, whereas binding to the low-affinity site predominantly increases the duration of opening with some further reduction in the duration of closings. Unlike ATP and adenosine, Ap5A does cause a small increase in open lifetime durations within the plateau portion of the dose–response curve, so perhaps assigning these two types of gating to the high- and low-affinity sites is too inflexible. Alternatively, perhaps the Ap5A molecule does bridge low- and high-affinity sites, and its binding to high-affinity sites brings it into a more prolonged contact with low-affinity sites at low doses.

It is difficult to understand why low doses of ATP and Ap5A together give a greater increase in Po than the maximum effect of either compound alone (as shown in Figure 7). Doses of Ap5A or ATP up to 10 μmol·L^{-1} do not raise Po above approximately 0.2, yet very low doses of Ap5A and ATP together increase Po to approximately 0.4. The same result is achieved whether ATP or Ap5A is added first (results not shown). At this stage we cannot fully explain these data, but it is likely that the results hinge on the fact that Ap5A is irreversible and that it binds to a larger region of RyR2 than ATP. Possibly the extended binding domain of Ap5A allows both ATP and Ap5A to bind simultaneously to the high-affinity sites leading to a higher Po than that can be achieved with either ligand alone.

In summary, Ap5A and its periodate-oxidized version, oAp5A, can fully activate RyR2, both showing a higher affinity for RyR2 than ATP. The higher affinity of these compounds is likely to relate to their slow dissociation from the channel. We have also shown the presence of a novel high-affinity adenine nucleotide binding site on RyR2, which can be accessed by adenosine, ATP and Ap5A. The significance of this high-affinity binding site maybe that it enables other adenine nucleotides to potentiate the effects of ATP and therefore be physiologically active in the cell in the presence of ATP. Diadenosine compounds may be physiological ligands with this capability, allowing them to modulate and maintain SR Ca^{2+}-release when their levels are raised in response to cellular oxidative stress.

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Conflict of interest

None.

References

Baxi MD, Vishwanatha JK (1995). Diadenosine polyphosphates: their biological and pharmacological significance. *J Pharmacol Toxicol Methods* **33**: 121–128.

Blatz AL, Magleby KL (1986). A quantitative description of 3 modes of activity of fast chloride channels from rat skeletal muscle. *J Physiol* **378**: 141–174.

Bochner BR, Lee PC, Wilson SW, Cutler CW, Ames BN (1984). AppppA and related adenylylated nucleotides are synthesized as a consequence of oxidation stress. *Cell* **37**: 225–232.

Chan WM, Welch W, Sitsapesan R (2000). Structural factors that determine the ability of adenosine and related compounds to activate the cardiac ryanodine receptor. *Br J Pharmacol* **130**: 1618–1626.

Chan WM, Welch W, Sitsapesan R (2003). Structural characteristics that govern binding to, and modulation through, the cardiac ryanodine receptor nucleotide binding site. *Mol Pharmacol* **63**: 174–182.

Ching LL, Williams AJ, Sitsapesan R (1999). AMP is a partial agonist at the sheep cardiac ryanodine receptor. *Br J Pharmacol* **127**: 161–171.

Colquhoun D, Sigworth FJ (1983). Fitting and statistical analysis of single-channel recording. In: Sakmann B, Neher E (eds). *Single-channel Recording*. Plenum: New York & London, pp. 191–263.

Dias JM, Szegedi C, Jona I, Vogel PD (2006). Insights into the regulation of the ryanodine receptor: differential effects of Mg^{2+} and Ca^{2+} on ATP binding. *Biochemistry* **45**: 9408–9415.

Flores NA, Stavrou BM, Sheridan DJ (1999). The effects of diadenosine polyphosphates on the cardiovascular system. *Cardiovasc Res* **42**: 15–26.

Hohenegger M, Herrmann-Frank A, Richter M, Lehmann-Horn F (1995). Activation and labelling of the purified skeletal muscle ryanodine receptor by an oxidized ATP analogue. *Biochem J* **308**: 119–125.

Holden CP, Padua RA, Geiger JD (1996). Regulation of ryanodine receptor calcium release channels by diadenosine polyphosphates. *J Neurochem* **67**: 574–580.

Jona I, Szegedi C, Sarkozi S, Szentesi P, Csernoch L, Kovacs L (2001). Altered inhibition of the rat skeletal ryanodine receptor/calcium release channel by magnesium in the presence of ATP. *Pflugers Arch* **441**: 729–738.

Kermode H, Williams AJ, Sitsapesan R (1998). The interactions of ATP, ADP and inorganic phosphate with the sheep cardiac ryanodine receptor. *Biophys J* **74**: 1296–1304.

Laver DR, Lenz GK, Lamb GD (2001). Regulation of the calcium release channel from rabbit skeletal muscle by the nucleotides ATP, AMP, IMP and adenosine. *J Physiol* **537**: 763–778.

Lee PC, Bochner BR, Ames BN (1983). AppppA, heat-shock stress, and cell oxidation. *Proc Natl Acad Sci USA* **80**: 7496–7500.

Luo J, Jankowski V, Gungar N, Neumann J, Schmitz W, Zidek W *et al*. (2004). Endogenous diadenosine tetraphosphate, diadenosine pentaphosphate, and diadenosine hexaphosphate in human myocardial tissue. *Hypertension* **43**: 1055–1059.

Meissner G (1994). Ryanodine receptor/Ca^{2+} release channels and their regulation by endogenous effectors. *Annu Rev Physiol* **56**: 485–508.

Morii H, Makinose M (1992). Adenosine(5′)hexaphospho(5′) adenosine stimulation of a Ca(2+)-induced Ca(2+)-release channel from skeletal muscle sarcoplasmic reticulum. *Eur J Biochem* **205**: 979–984.

Sigworth FJ, Sine SM (1987). Data transformations for improved display and fitting of single-channel dwell time histograms. *Biophys J* **52**: 1047–1054.

Sitsapesan R, Williams AJ (1995). The gating of the sheep skeletal sarcoplasmic reticulum Ca^{2+}-release channel is regulated by luminal Ca^{2+}. *J Membr Biol* **146**: 133–144.

Sitsapesan R, Montgomery RAP, MacLeod KT, Williams AJ (1991). Sheep cardiac sarcoplasmic reticulum calcium release channels: modification of conductance and gating by temperature. *J Physiol* **434**: 469–488.

Smith GL, O'Neill SC (2001). A comparison of the effects of ATP and tetracaine on spontaneous Ca(2+) release from rat permeabilised cardiac myocytes. *J Physiol* **534**: 37–47.

Varshavsky A (1983). Diadenosine 5′, 5‴-P1, P4-tetraphosphate: a pleiotropically acting alarmone? *Cell* **34**: 711–712.

Yang Z, Steele DS (2001). Effects of cytosolic ATP on Ca(2+) sparks and SR Ca(2+) content in permeabilized cardiac myocytes. *Circ Res* **89**: 526–533.