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Adenosine Discriminates between the Caffeine and Adenine Nucleotide Sites on the Sheep Cardiac Sarcoplasmic Reticulum Calcium-Release Channel

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Abstract. Calcium-release channels of sheep cardiac sarcoplasmic reticulum were incorporated into phosphatidylethanolamine bilayers and single channel currents were recorded under voltage-clamp conditions. The effect of adenosine on single channel conductance and gating was investigated, as were the interactions between adenosine and caffeine and adenosine and α,β-methylene ATP.

Addition of adenosine (0.5–5 mM) to the cytosolic but not the luminal side of the membrane increased the open probability of single calcium-activated calcium-release channels by increasing the frequency and duration of open events, yielding an EC_{50} of 0.75 mM at 10 μM activating Ca^{2+}.

Addition of 1 mM caffeine potentiated the effects of adenosine at 10 or 100 μM-activating cytosolic calcium, but had no effect on the inability of adenosine to activate the channel at 80 pM calcium, suggesting discrete sites of action on the calcium-release channel for adenosine and caffeine. In contrast, addition of 100 μM α,β-methylene-ATP decreased single channel open probability in the presence of adenosine, suggesting that these compounds act on the same site on the channel.

Activation of single channel opening by adenosine, or by adenosine together with caffeine, had no effect on single channel conductance or the $Ca^{2+}/Tris^{+}$ permeability ratio. Channels activated by adenosine were characteristically modified by ryanodine and blocked by μM ruthenium red or mM magnesium.

These results show that adenosine activates the sheep cardiac sarcoplasmic reticulum Ca^{2+}-release channel by increasing the frequency and duration of open events in a Ca^{2+}-dependent manner. The receptor site on the channel for adenosine is distinct from that for caffeine but probably the same as that for adenine nucleotides.

Key words: Adenosine — Sarcoplasmic reticulum — Cardiac Ca^{2+}-release channel — Caffeine — Adenine nucleotides

Introduction

Calcium-induced calcium-release, where calcium influx during the action potential causes rapid efflux of calcium from the sarcoplasmic reticulum (SR), is thought to be the main mechanism for releasing stored calcium in cardiac muscle cells (Fabiato, 1985). When isolated cardiac SR vesicles are incorporated into planar phospholipid bilayers, a calcium-selective channel is seen which has a conductance of 80–120 pS with Ca^{2+} as the permeant ion (Rousseau et al., 1986; Ashley & Williams, 1990). Single channel, radioisotope flux and ^{3}H-ryanodine binding studies using isolated SR vesicles indicate that activation of the Ca^{2+}-release channel, rapid release of $^{45}Ca^{2+}$ from and stimulation of ^{3}H-ryanodine binding to populations of these vesicles is, in general, Ca^{2+} dependent and that other ligands may act in conjunction with Ca^{2+} to increase its effect (Rousseau et al., 1986; Meissner & Henderson, 1987; Holmberg & Williams, 1990; Pessah & Zimanyi, 1991). The activation of these processes by Ca^{2+} is enhanced by several positively inotropic compounds including xanthines, ATP and cardiac glycosides (Rousseau et al., 1986; Meissner, 1986; Meissner & Henderson, 1987; McGarry & Williams, 1993*a*) and inhibited by magnesium, ruthenium red and calmodulin (Meissner &

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Henderson, 1987). Single channel experiments, efflux studies and binding assays have also yielded important results about the skeletal Ca^{2+}-release channel and its modulation by drugs such as ATP and caffeine (Palade, 1987; Rousseau et al., 1988; Chu et al., 1990; Zimanyi & Pessah, 1991). However, it would be unwise to conclude that agents acting on the skeletal isoform of the Ca^{2+}-release channel act by a similar mechanism on the cardiac channel, as some drugs, for example, cardiac glycosides, are specific activators of only the cardiac channel (McGarry & Williams, 1993*a*); also, the different sensitivities of the isoforms of the channel to Ca^{2+} mean that drugs which act by a Ca^{2+}-dependent mechanism will have different potencies on cardiac and skeletal channels (Chu et al., 1991). Hence, this study will concentrate on cardiac SR Ca^{2+}-release channels.

Caffeine has been used extensively to induce positive inotropy and to cause calcium release in whole cells from SR vesicles and to activate SR Ca^{2+}-release channels incorporated into lipid bilayers (Chapman & Miller, 1974; Rousseau & Meissner, 1989; Sitsapesan & Williams, 1991). The mechanism of this activation was shown by Sitsapesan and Williams (1991) to involve both a Ca^{2+}-dependent increase in the frequency and duration of open events and a Ca^{2+}-independent action, usually requiring higher concentrations of caffeine.

Although caffeine and adenosine are very similar in structure, and are antagonistic at cell surface adenosine receptors (Olsson & Pearson, 1991), the effect of adenosine on cardiac SR Ca^{2+}-release channel gating has not been studied. The site of action of adenosine with respect to other adenine-containing drugs, or the structurally related drug caffeine, on the cardiac channel has also not been distinguished, although Rousseau and Meissner (1989) suggested, from a limited amount of data, that ATP and caffeine may act at different sites on the cardiac Ca^{2+}-release channel.

The binding of adenosine to high affinity cell surface receptors and the subsequent effects elicited are well characterized (Olsson & Pearson, 1991). However, the intracellular effects of adenosine, in contrast to caffeine, are less well known. Adenosine is a weak inhibitor of type III phosphodiesterase and inhibits adenylate cyclase by binding to the inhibitory, "P," site of the enzyme (Rodbell, 1983). Here, evidence is presented that adenosine, like caffeine, also acts on the SR Ca^{2+}-release channel.

The investigation of the activation mechanism of the Ca^{2+}-release channel by adenosine will firmly establish whether or not adenosine and caffeine act at the same or distinct sites on the cardiac Ca^{2+}-release channel protein. Clarifying this will allow the full characterization of the Ca^{2+}-release channel pharmacology using both adenosine and caffeine analogues. Additionally, this work may indicate whether or not adenosine (an adenine nucleoside) and α,β-methylene ATP (a nucleotide) act at the same or distinct sites on the channel protein, aiding the structure-activity study of the adenosine and/or caffeine site(s).

Materials and Methods

Preparation of SR Membrane Vesicles

The methods for the isolation of SR membrane vesicles were as previously described (Sitsapesan & Williams, 1990). Fresh sheep hearts were obtained from the local abattoir and transported to the laboratory in cold modified cardioplegic solution (Tomlins et al., 1986). Left ventricle and septum (approximately 100 g) were stripped of fat and connective tissue prior to homogenization in a solution containing 300 mM sucrose, 20 mM potassium piperazine-*N'N'*-bis-ethanesulfonic acid (PIPES) and 1 mM phenylmethanesulphonyl chloride (PMSF), pH 7.4. The homogenate was centrifuged at 8,000 × g_{av} in a Sorvall GSA rotor for 20 min and the pellet was discarded. The supernatant was then centrifuged at 100,000 × g_{av} in a Sorvall T647.5 rotor for 40 min. The mixed membrane population sedimented by this step was resuspended in a solution containing (mM): 400 KCl, 0.5 $MgCl_2$, 0.5 $CaCl_2$, 0.5 1,2-di(2-aminoethoxy)ethane-*N,N,N',N'*-tetraacetic acid (EGTA), 25 PIPES, pH 7.0 and 10% sucrose w/v, and subfractionated on discontinuous sucrose-density gradients. The membrane suspension was layered onto identical salt solutions containing 20, 30 and 40% w/v sucrose and sedimented at 100,000 × g_{av} for 120 min in a Sorvall AH629 rotor. Heavy SR (HSR) membrane vesicles collecting at the 30–40% interface were collected and diluted into 400 mM KCl and pelleted by centrifugation at 100,000 × g_{av} for 40 min in a Sorvall T647.5 rotor before resuspension in a solution containing 400 mM sucrose, 5 mM N'-2-hydroxyethylpiperazine-*N'*-2-sulfonic acid (HEPES) titrated to pH 7.4 with tris(hydroxymethyl)-methylamine (Tris). Membrane vesicles were snap-frozen in liquid nitrogen and stored at −80°C.

Planar Lipid Bilayer Methods

Lipid bilayers containing phosphatidylethanolamine in decane (30 mg ml^{-1}) were formed across a 200 μm diameter hole in the partition between two fluid-filled styrene copolymer chambers, referred to as *cis* (volume 0.5 ml) and *trans* (1.0 ml). The *trans* chamber was held at ground and the *cis* chamber clamped at holding potentials relative to this. Current flow through the bilayer was measured using an operational amplifier as a current-voltage converter (Miller, 1982). Initially both chambers contained (mM): 50 choline chloride, 10 HEPES and 5 $CaCl_2$, with the pH adjusted to 7.4 with Tris. HSR vesicles were added to the *cis* chamber and the choline chloride concentration increased to given a 7:1 gradient *cis-trans* to promote vesicle fusion with the bilayer. Fusion was marked by the appearance of Cl^--selective channels (Smith, Coronado & Meissner, 1985). SR vesicles incorporated into the bilayer in a fixed orientation such that the cytosolic face of the Ca^{2+}-release channel was directed towards the *cis* chamber and the luminal face to the *trans* chamber. Following vesicle fusion, the *cis* and *trans* chambers were perfused with solutions allowing resolution of only Ca^{2+}-release channel currents. The *cis* chamber was perfused with (mM) 250 HEPES, 125 Tris, pH 7.4; and the *trans* with 250 glutamic acid, 10 HEPES, with the pH adjusted to 7.4 with $Ca(OH)_2$, giving a $[Ca^{2+}]$ on the *trans* side of 67 mM. The concentration of Ca^{2+} in the *cis* chamber was buffered to the desired level by the addition of $CaCl_2$ and EGTA. The concentrations of Ca-

Cl_2 and EGTA were calculated using EQCAL (Biosoft, Cambridge). Experiments were performed at room temperature (22 ± 1°C).

Data Acquisition and Analysis

Single channel data were displayed on an oscilloscope and recorded on video tape. Current recordings were replayed, low-pass filtered using an 8-pole Bessel filter (Frequency Devices 902) at 1 kHz and digitized at 2 kHz using an AT-based system (Intracel, Cambridge). Channel P_o and the lifetimes of open and closed events were determined by 50% threshold analysis at a holding potential of 0 mV. Single channel P_o values are displayed as mean ± standard error of the mean (SEM). Lifetimes, accumulated from 3 min steady-state recordings, were stored in sequential files and displayed in noncumulative histograms. Individual lifetimes from each single channel were fitted to a probability density function using the method of maximum likelihood (Colquhoun & Sigworth, 1983). Lifetimes lasting less than 1 msec were incompletely resolved under these data acquisition conditions and were therefore excluded from the fitting procedure. A missed events correction was applied (Colquhoun & Sigworth, 1983) and a likelihood ratio test was used to compare fits to double and triple exponentials (Blatz & Magleby, 1986).

Single channel conductance was obtained from the slope of the linear regression lines drawn through single channel current amplitude data monitored at holding potentials within the range (+40 to −40 mV). Reversal potentials (E_{rev}) were obtained from extrapolations of these lines. The $Ca^{2+}/Tris^+$ permeability ratio was calculated from the equation given by Fatt & Ginsborg (1958):

$$pX^{2+}/pY^{+} = [Y^{+}]/4[X^{2+}]\exp(E_{rev}F/RT)\,[\exp(E_{rev}F/RT) + 1] \quad (1)$$

It should be noted, however, that using linear regression to calculate the E_{rev} will result in an underestimation of the $Ca^{2+}/Tris^+$ permeability ratio, since the current-voltage relationship for the Ca^{2+}-release channel in the presence of $Tris^+$ is not linear (*see* Tinker, Lindsay & Williams, 1992, for discussion).

Drugs

Stock solutions were prepared using deionized water produced by a Milli-Q water purification system (Millipore, UK) and diluted into *cis* or *trans* solutions. Adenosine (9-β-D-ribofuranosyladenine), α,β-methylene-ATP and caffeine were obtained from Sigma (Poole, UK), ryanodine from Cambridge Bioscience (Cambridge, MA) and ruthenium red from BDH (Dagenham, UK). Phosphatidylethanolamine was from Avanti Polar Lipids (Birmingham, AL).

Results

Effect of Increasing Concentrations of Adenosine

The effect of adding increasing concentrations of adenosine to the cytosolic face of the channel is shown in Fig. 1. At an activating $[Ca^{2+}]$ of 10 μM on the cytosolic face of the channel the P_o increases from 0.040 ± 0.013 (n = 12) to 0.311 ± 0.062 (n = 5), 0.670 ± 0.131 (n = 8), 0.980 ± 0.030 (n = 3) and 0.99 ± 0.045 (n = 4) on addition of 0.5, 1, 3 and 5 mM adenosine to the cytosolic side with 50% maximal activation occurring at approximately 0.75 mM adenosine. The Hill slope of the fitted binding curve was approximately two, suggesting that at least two adenosine molecules may bind to and activate the SR Ca^{2+}-release channel at 10 μM activating Ca^{2+} (*see* Fig. 6). The activation occurred almost immediately on addition of the drug and was readily reversed on washout of the *cis* chamber. There was no effect of adenosine when added to the luminal face of the Ca^{2+}-release channel.

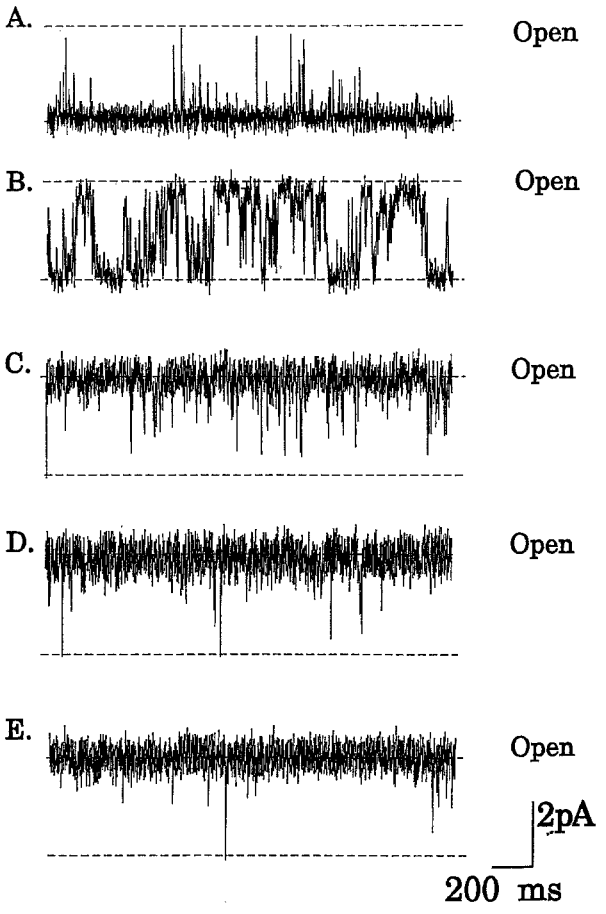

Fig. 1. Representative portions of data from a single SR Ca^{2+}-release channel held at 0 mV. Single channel P_o, determined from 3 min of continuous recordings, was 0.049 at 10 μM activating cytosolic Ca^{2+} (*A*). Addition of 0.5, 1, 3 and 5 mM adenosine to the cytosolic face of the channel (*B–E*) increased the P_o to 0.323, 0.710, 0.969 and 0.983, respectively. Adenosine did not affect single channel conductance. Open channel level is indicated above each respective portion of data.

Adenosine did not affect single Ca^{2+}-release channel conductance or the $Ca^{2+}/Tris^+$ permeability ratio. In the presence of 10 μM Ca^{2+}, slope conductance was 88 ± 3 pS (±SEM, n = 5) and the $Ca^{2+}/Tris^+$ permeability ratio was 13.1 ± 0.2 (n = 5). Addition of 1 mM adenosine changed these values to 89 ± 3 pS (n = 5) and 13.3 ± 0.2 (n = 5), respectively. That the adeno-

sine-activated channels displayed an almost identical conductance and relative permeability to channels activated by Ca^{2+} alone suggests that the adenosine-activated channels were Ca^{2+}-release channels.

Ca^{2+}-Dependence of Adenosine Action

The activation by adenosine was dependent on the channel first being activated by *cis* Ca^{2+}. At sub-activating [Ca^{2+}] (80 pM), the P_o was zero and was not increased on addition of 1 mM adenosine. In fact, addition of up to 12.5 mM adenosine failed to increase P_o at pM Ca^{2+} (*not shown*), demonstrating the lack of a Ca^{2+}-independent action of adenosine. Figure 2 demonstrates the Ca^{2+} dependence of the adenosine activation of the Ca^{2+}-release channel. At 10 μM Ca^{2+}, the P_o increased from 0.040 ± 0.013 (n = 12) to 0.670 ± 0.131 (n = 8); at 100 μM Ca^{2+}, the P_o increased from 0.217 ± 0.050 (n = 4) to 0.837 ± 0.100 (n = 4) on addition of 1 mM adenosine. That the increase in P_o was Ca^{2+} dependent was supported by the experiments conducted with Mg^{2+}, which is thought to compete with Ca^{2+} for the activation site on the Ca^{2+}-release channel (Ashley & Williams, 1990). The P_o of single channels activated by adenosine in the presence of 10 μM Ca^{2+} was reduced to zero or near zero on addition of 5 mM Mg^{2+}. Similarly, channels activated by Ca^{2+} where the P_o was reduced to zero by 5 mM Mg^{2+} could not be activated by adenosine (*not shown*).

Experiments where the single channel P_o was increased independently of Ca^{2+} by 1 mM (+)-sulmazole showed that adenosine failed to augment this increase (*not shown*). Addition of 1 mM (+)-sulmazole to the channel at pM Ca^{2+} increased P_o from zero to 0.362 ± 0.156 (n = 3). Further addition of up to 3 mM adenosine to the cytosolic face of the channel failed to affect single channel P_o.

Single Ca^{2+} release channels activated by Ca^{2+} or by Ca^{2+} and adenosine were closed on addition of 10 μM *cis* ruthenium red and were also modified to a characteristic long-lasting but reduced conductance state on addition of 100 nM ryanodine to the *cis* chamber (*not shown*).

The mechanism of the increase in P_o induced by adenosine involved an increase in the frequency and duration of open events. The gating of the channel in the presence of 10 μM cytosolic Ca^{2+}, with Ca^{2+} as the permeant ion, can be described by at least two open and three closed states. Addition of 0.5 mM adenosine to the cytosolic face of the channel changed the gating such that the fit required at least three open and three closed states. Higher concentrations of adenosine (1–5 mM) caused a further decrease of closed lifetime durations and an increase in open lifetime durations such that gating was best described by three open and two closed states (Fig. 3).

Increasing the cytosolic [Ca^{2+}] increases P_o by mainly decreasing single channel closed lifetime durations, with little or no effect on open lifetimes (Ashley & Williams, 1990). Ca^{2+} acting as the sole ligand is unable to fully activate the SR Ca^{2+}-release channel. Addition of cytosolic Ca^{2+} in the μM range increases single channel P_o. P_o reaches a maximum at between 100 μM–1 mM activating Ca^{2+} and decreases at higher concentrations (Sitsapesan & Williams,1993). Addition of adenosine at 100 μM cytosolic Ca^{2+} causes a further increase in P_o, reflected in a further increase in the frequency and duration of open events.

This results in kinetics best described by three open and two closed states (Fig. 4). As adenosine increased both the frequency and duration of open events at maximally activating cytosolic Ca^{2+}, it would appear unlikely that adenosine increased P_o by sensitizing the channel protein to the effects of Ca^{2+}.

Potentiation of Adenosine Activation by Caffeine

That there was no Ca^{2+}-independent action of adenosine could be interpreted as suggesting that the adenosine activation site on the channel is distinct from the caffeine site, since caffeine is able to activate the channel in a Ca^{2+}-independent manner (Sitsapesan & Williams, 1991). Support for this is provided by the potentiation of adenosine activation by caffeine (Figs. 5 and 6). At an activating [Ca^{2+}] of 10 μM, 1 mM caffeine increased P_o in channels activated by 0.5, 1 and 3 mM adenosine from 0.311 ± 0.062 (n = 5), 0.670 ± 0.131 (n = 8) and 0.980 ± 0.030 (n = 3) to 0.791 ± 0.093 (n = 3), 0.936 ± 0.071 (n = 5) and 0.992 ± 0.039 (n = 4), respectively. The presence of 1 mM caffeine decreased the EC_{50} for activation of the channel by adenosine to 0.316 mM and the Hill slope was slightly, but not significantly, reduced to 1.80, at 10 μM activating Ca^{2+}. The potentiation of P_o by caffeine was reflected in a further increase in the frequency and duration of open events caused by adenosine (in the presence of 10 μM Ca^{2+}). The open and closed lifetime durations were best described by three open and two closed states (*not shown*).

At 100 μM activating Ca^{2+}, caffeine potentiated the adenosine-induced increase in P_o to a slightly higher P_o than at 10 μM Ca^{2+}, although at 100 μM Ca^{2+}, 1 mM adenosine increased P_o to near maximal levels, so the possible potentiation by caffeine was limited. At 10 μM Ca^{2+}, 1 mM caffeine increased P_o in channels activated by 1 mM adenosine from 0.670 ± 0.131 to 0.936 ± 0.071 (n = 5); at 100 μM activating Ca^{2+}, 1 mM caffeine increased P_o from 0.837 ± 0.100 (n = 5) to 0.960 ± 0.052 (n = 4).

Competition with Adenine Nucleotides

Since activation of the SR Ca^{2+}-release channel by adenosine occurs at a site distinct from that of caffeine,

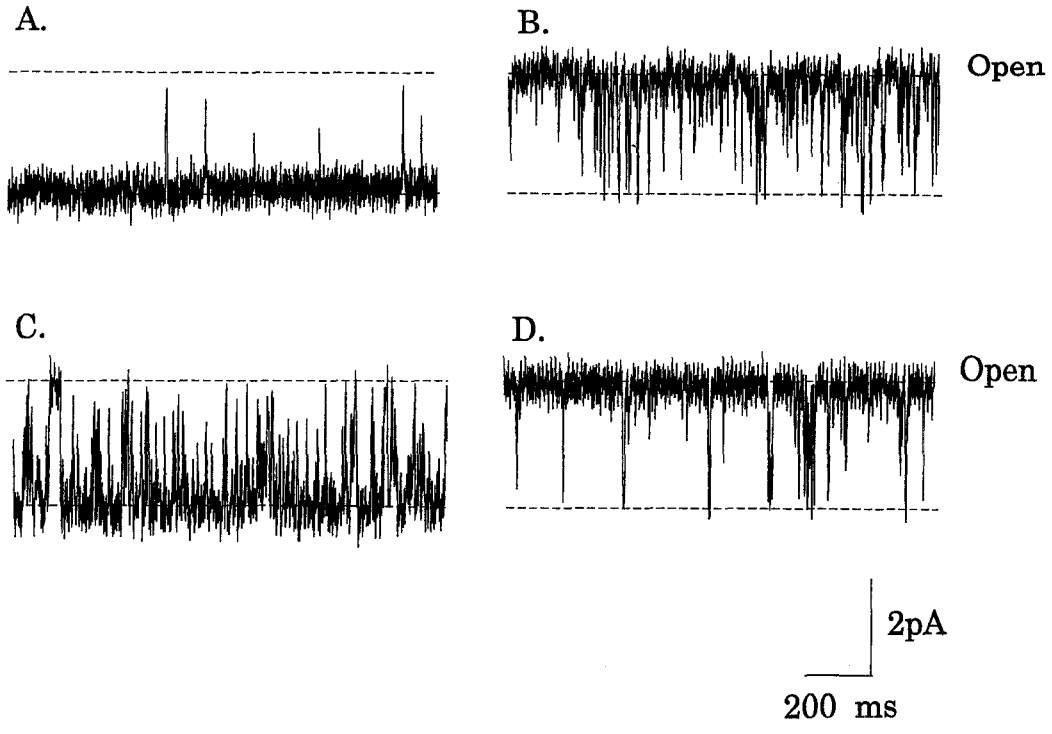

Fig. 2. Data from single Ca^{2+}-release channels held at 0 mV. The channels were first activated by 10 μM Ca^{2+} (*A*) or 100 μM Ca^{2+} (*C*) and then 1 mM adenosine was added to the *cis* chamber (*B* and *D*). Single channel P_o increased as cytosolic Ca^{2+} was raised. Addition of 1 mM adenosine at 10 μM Ca^{2+} increased P_o from 0.032 to 0.650 (*A* and *B*). At 100 μM activating Ca^{2+} (*C*), P_o was 0.221 and this was elevated to 0.877 on addition of 1 mM adenosine (*D*). Single channel P_o values were calculated from 3 min of continuous recordings. Single channel open level is indicated above each portion of data.

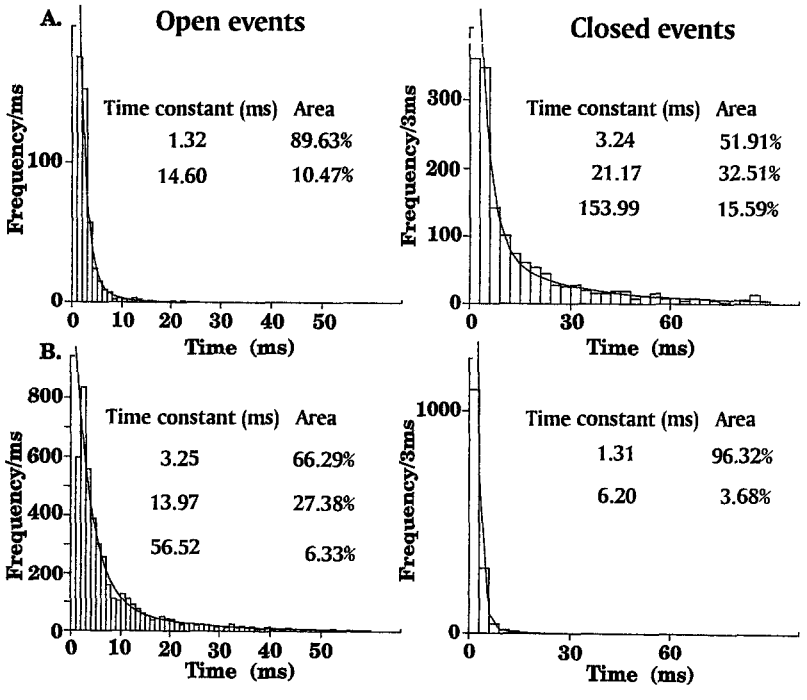

Fig. 3. Single sheep cardiac SR Ca^{2+}-release channel open and closed lifetime histograms, with probability density functions obtained by maximum likelihood fitting to individual lifetimes. Here, (*A*) corresponds to the channel activated by 10 μM Ca^{2+} and (*B*) to the channel activated by 10 μM Ca^{2+} + 1 mM adenosine. Channels were held at 0 mV. Lifetimes were obtained from 3 min of continuous recordings and are displayed in noncumulative histograms. Probability density functions are drawn according to:

$$f(t) = a_1(1/\tau_1)\exp(-t/\tau_1)+\ldots+a_n(1/\tau_n). \quad (2)$$

For open lifetimes at 10 μM Ca^{2+}, most likely fits were obtained to a double exponential. Open lifetimes for 10 μM Ca^{2+} + 1 mM adenosine were best fitted to a triple exponential. For closed lifetimes, most likely fits for 10 μM Ca^{2+} and 10 μM Ca^{2+} + 1 mM adenosine were obtained to triple and double exponentials, respectively.

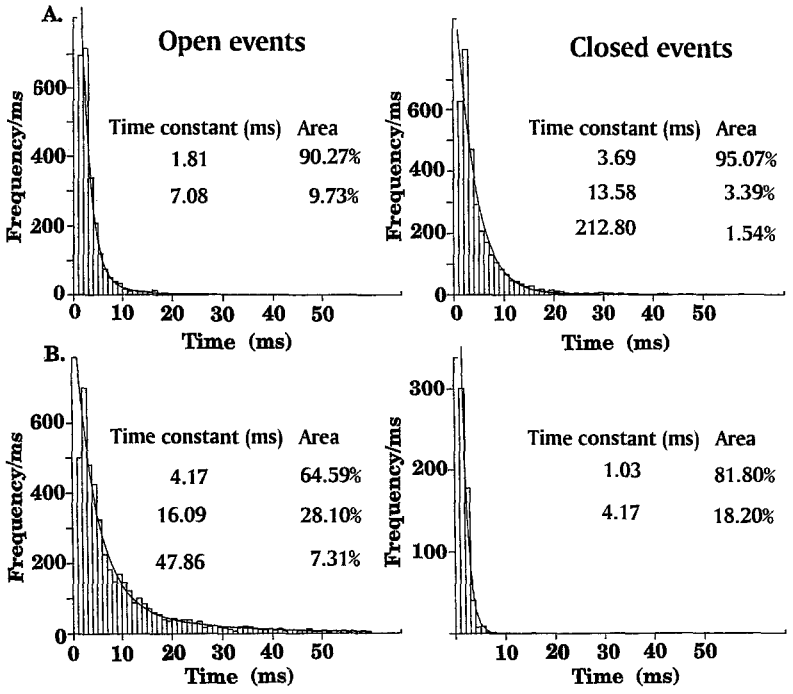

Fig. 4. Open and closed lifetime histograms, with probability density functions obtained by maximum likelihood fitting to individual lifetimes for a single sheep cardiac SR Ca^{2+}-release channel activated by (*A*) 100 μM Ca^{2+} and (*B*) 100 μM Ca^{2+} + 1 mM adenosine. Lifetimes are displayed in noncumulative histograms, with probability density functions drawn according to the equation given in Fig. 3. For open lifetimes, most likely fits were obtained to double and triple exponentials for 100 μM Ca^{2+} and 100 μM Ca^{2+} + 1 mM adenosine, respectively. For closed lifetimes, most likely fits were obtained to triple and double exponentials for 100 μM Ca^{2+} and 100 μM Ca^{2+} + 1 mM adenosine, respectively.

the question then arises as to whether adenosine acts at the same site as adenine nucleotides, such as ATP. The activation of the Ca^{2+}-release channel by ATP has already been demonstrated by others (Meissner & Henderson, 1987; Sitsapesan & Williams, 1991). In the experiments described here, we used the ATP analogue α,β-methylene-ATP, which is a more potent activator of the channel than ATP (*unpublished observations*). At 10 μM activating cytosolic Ca^{2+}, 100 μM α,β-methylene-ATP alone increased P_o to 0.701 ± 0.063 (n = 3). At an activating [Ca^{2+}] of 10 μM, 1 mM adenosine increased P_o to 0.682 ± 0.091 (n = 3) (Fig. 7). Further addition of 100 μM α,β-methylene-ATP failed to increase P_o; indeed, P_o on average fell to 0.655 ± 0.044 (n = 3).

Discussion

Adenosine activates the SR Ca^{2+}-release channel with an approximate EC_{50} of 0.75 mM. Since the activation does not affect the conductance of the channel or the $Ca^{2+}/Tris^{+}$ permeability ratio, it is apparent that the drug does not interact with the conduction pathway of the channel. That the drug only increases P_o when added from the cytosolic face of the channel suggests that it binds to specific sites (possibly two) on the protein. Additionally, the effect of the drug is concentration dependent and there is a synergistic effect of Ca^{2+} and adenosine on single channel P_o. This is demonstrated by the action of adenosine at both 10 μM activating cytosolic Ca^{2+} and at maximally activating [Ca^{2+}] (100 μM), where adenosine causes a further increase in single channel P_o.

The activation mechanism of the Ca^{2+}-release channel by adenosine is different from the activation by caffeine and sulmazole (Williams & Holmberg, 1990; Sitsapesan & Williams, 1991), which itself may suggest that adenosine and these compounds act at different sites on the channel. Caffeine and sulmazole, at low concentrations, seemingly sensitize the channel protein to the effects of Ca^{2+}. That is, they increase P_o by increasing the frequency of open events without affecting the channel open lifetime durations. At higher concentrations, however, the mechanism of activation is similar to that of adenosine in that they increase both the frequency and duration of open events. In contrast, adenosine increases the frequency and duration of open events at all concentrations tested. This further supports the suggestion that caffeine and sulmazole may activate the SR Ca^{2+}-release channel by acting at a different site from adenosine but at the same site as each other, on the cytosolic face of the channel.

These results also show that adenosine is more potent than caffeine in activating the cardiac SR Ca^{2+}-release channel. The EC_{50} for activation by adenosine at 10 μM activating cytosolic Ca^{2+} is approximately 0.75 mM, whereas the EC_{50} for activation by caffeine, although not calculated here, is expected to be much higher (Sitsapesan & Williams, 1991). Since both compounds are able to fully activate the Ca^{2+}-release channel in the presence of activating concentrations of Ca^{2+},

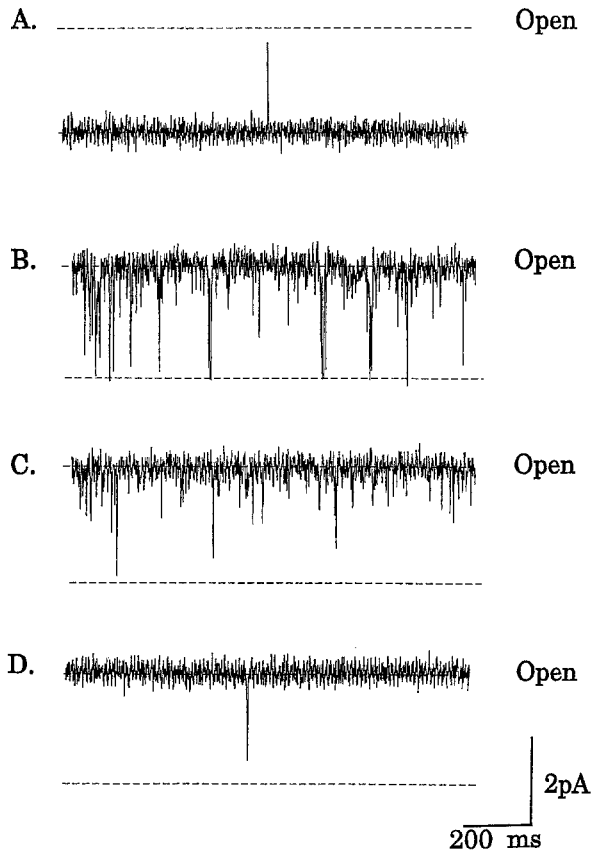

Fig. 5. A single Ca^{2+}-release channel activated by 10 μM Ca^{2+} (*A*) at a holding potential of 0 mV. Addition of 1 mM caffeine + 0.5 mM adenosine to the cytosolic face of the channel (*B*) increased P_o from 0.022 to 0.760. Increasing the cytosolic concentration of adenosine to 1 and 3 mM (*C* and *D*) in the continued presence of 1 mM caffeine further increased P_o to 0.951 and 0.992. The P_o values obtained with adenosine in the presence of 1 mM caffeine are higher than those obtained with adenosine when caffeine is not present. The simultaneous addition of adenosine and caffeine did not affect the conductance of the Ca^{2+}-release channel.

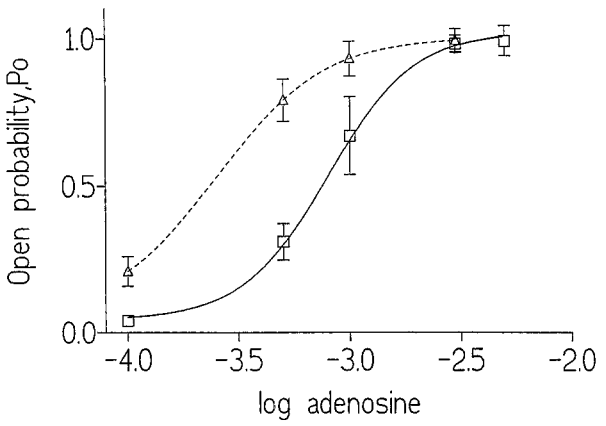

Fig. 6. Comparison of the effect of adenosine (□) and 1 mM caffeine + adenosine (△) at 10 μM activating Ca^{2+} on single Ca^{2+}-release channel P_o. The EC_{50} for activation by adenosine was 0.754 mM; the EC_{50} for activation in the presence of 1 mM caffeine was approximately 0.316 mM. The Hill coefficients, obtained from the linear slopes of both of the fitted binding curves were approximately two. INPLOT (GraphPad Software) was used to fit a curve to the data points.

it is apparent that although both drugs have equal efficacy, adenosine is more potent.

The estimated Hill slope of the adenosine activation of the channel in the presence of caffeine at 10 μM Ca^{2+} was approximately two, suggesting that caffeine increases the sensitivity of the Ca^{2+}-release channel to adenosine; it does not appear to increase the sensitivity by increasing the number of adenosine molecules binding to the channel. Although it should be noted that 1 mM caffeine increased P_o from control to approximately 0.21 alone, the increased P_o caused by adenosine in the presence of caffeine would appear not to be merely due to the channel being "primed" by caffeine, as the increased P_o is more than additive, which is apparent at lower adenosine concentrations.

Although not rigorously tested, that P_o was not increased when both 1 mM adenosine and 100 μM α,β-methylene ATP were added to the cytosolic face of the channel, suggests that both drugs at these concentrations have approximately equal efficacy and may compete for the same site on the SR Ca^{2+}-release channel. Alternatively, they may act at distinct sites and the activation caused by one drug functionally antagonizes the activation caused by the other. Although this possibility cannot be discounted, it appears unlikely, as agents that activate the release channel, for example sulmazole and caffeine, increase the activation caused by other ligands such as Ca^{2+} and adenosine. Hence, the adenosine activation site on the channel protein is probably the same as that for adenine nucleotides. That there is a common activation site for both adenine nucleosides and nucleotides is in contrast to cell surface receptors, where the adenine nucleosides are more selective for P_1 purinergic receptors and nucleotides are more selective for P_2 receptors (Ribeiro & Sebastiao, 1986).

As adenosine interacts with at least one of the open states and one of the closed states of the channel protein, it may be possible that more potent drugs binding to this site will increase single channel P_o in the absence of Ca^{2+} (by interaction with one of the closed states of the channel). Although adenosine does not activate the channel in the absence of Ca^{2+}, it is possible that much higher concentrations of adenosine could increase the P_o of SR Ca^{2+}-release channels at sub-activating Ca^{2+} (as is seen with caffeine). However, the low solubility of adenosine hinders our ability to test this hypothesis.

The failure of adenosine to increase the P_o of channels activated by (+)-sulmazole at sub-activating Ca^{2+} appears to suggest that adenosine requires the presence

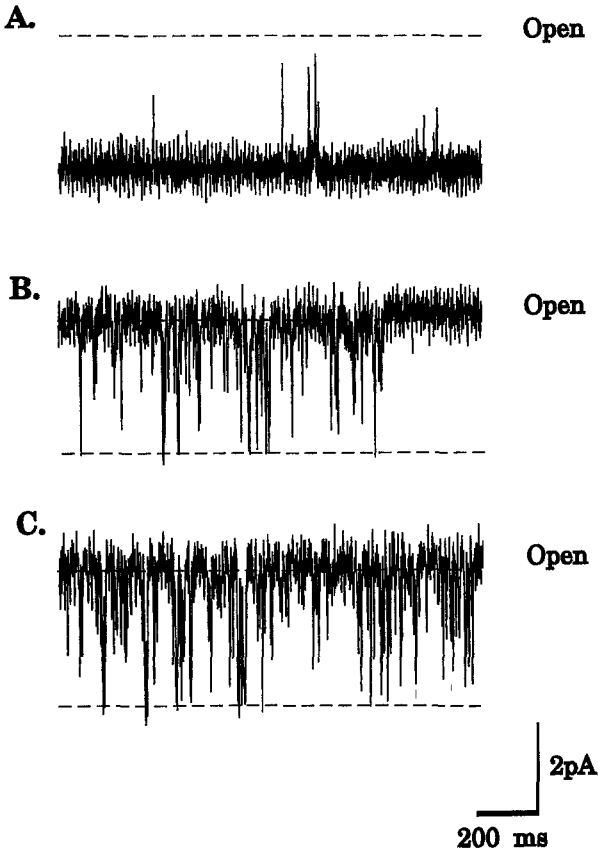

Fig. 7. A single SR Ca^{2+}-release channel held at 0 mV. The channel was activated by 10 μM cytosolic Ca^{2+}, resulting in a P_o of 0.033 (*A*). Addition of 100 μM α/β-methylene ATP to the *cis* chamber increased P_o to 0.719 (*B*). Further addition of 1 mM adenosine to the *cis* chamber decreased P_o to 0.614 (*C*). That there was no increase in P_o on addition of adenosine may suggest that both compounds compete for similar binding sites on the cardiac SR Ca^{2+}-release channel. This situation is in contrast to that seen when adenosine and caffeine activate Ca^{2+}-release channels at 10 μM Ca^{2+}, where a marked potentiation of P_o is observed in comparison to the activation by either adenosine or caffeine alone.

of Ca^{2+} to elicit its effects. This theory appears to be at odds with the action of ATP on (+)-sulmazole-activated channels in the absence of activating Ca^{2+}. In these experiments, Williams and Holmberg (1990) demonstrated that ATP augmented the increase in P_o induced by (+)-sulmazole at sub-activating Ca^{2+} concentrations, suggesting that more potent adenine compounds may require only an open channel to elicit their effects on single channel P_o. We propose that the failure of adenosine to increase the P_o of channels activated by (+)-sulmazole in similar conditions is due to the relative lack of potency of adenosine.

Since it is now clear that the adenosine and caffeine sites are distinct entities on the Ca^{2+}-release channel, we have begun to investigate the effects of a number of adenosine and caffeine analogues on the Ca^{2+}-release channel. Some of the adenosine compounds appear to be more potent than adenosine (*unpublished observations*), although their effect in the absence of activating Ca^{2+} has not yet been investigated; caffeine analogues, based on sulmazole, are more potent than caffeine, activate the channel in the absence of Ca^{2+} and potentiate the effects of adenosine at 10 μM Ca^{2+} (McGarry & Williams, 1993*b*).

The application of more potent (novel) analogues of adenosine to intact cardiac muscle, or conditions where adenosine concentrations are elevated such as in ischemia, are unlikely to result in an increased contraction (in contrast to xanthine analogues, which also inhibit cAMP phosphodiesterase and sarcolemmal Na^+/K^+ ATPase), since these compounds would have to compete with mM concentrations of ATP for the site on the Ca^{2+}-release channel. This also assumes the unlikely scenario that these drugs would not affect the high affinity cell-surface adenosine receptors. Hence, it is unlikely, for both these reasons, that adenosine plays a physiological role in the activation of the Ca^{2+}-release channel.

In conclusion, this report contains the first description of the mechanism of action of adenosine on single cardiac SR Ca^{2+}-release channels. Adenosine dose-dependently increases single Ca^{2+}-release channel P_o in the presence, but not the absence, of activating Ca^{2+}. The increase in P_o occurs as a result of an increase in the frequency and duration of open events with no effect on single channel conductance. The increase in P_o is potentiated by caffeine and decreased by adenine nucleotides, suggesting similar sites of action for adenine nucleosides and nucleotides and distinct activation sites for caffeine. The results also show that adenosine is a more potent activator of the cardiac SR Ca^{2+}-release channel than caffeine in these conditions.

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