Journal of Physiology (1990), **423**, 425–439
With 8 figures
Printed in Great Britain

MECHANISMS OF CAFFEINE ACTIVATION OF SINGLE CALCIUM-RELEASE CHANNELS OF SHEEP CARDIAC SARCOPLASMIC RETICULUM

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(*Received 22 September 1989*)

SUMMARY

1. Calcium-release channels of sheep cardiac junctional sarcoplasmic reticulum were incorporated into planar phospholipid bilayers. Single-channel current fluctuations were recorded under voltage clamp conditions.

2. Channels incorporate into the bilayer with a fixed orientation and channel open probability is regulated by the calcium concentration at the cytosolic face of the membrane.

3. Addition of caffeine (0·5–2·0 mM) to the cytosolic side of the membrane increased the open probability of the calcium-activated calcium-release channel by increasing the frequency of opening without significant alteration to the durations of open events. This effect was observed at both 0·1 and 10 μM-activating cytosolic calcium.

4. Caffeine (0·5–2·0 mM) did not activate the channel at a subactivating cytosolic calcium concentration (80 pM).

5. At subactivating calcium concentrations, channels could be activated by higher concentrations of caffeine ($> 5{\cdot}0$ mM) revealing a second, calcium-independent, mechanism for channel activation. Channel openings induced by these high concentrations of caffeine at subactivating calcium concentrations displayed different kinetics from those observed with calcium as the sole activating ligand or with combinations of calcium and low concentrations of caffeine.

6. Activation of channel opening by caffeine in the presence of calcium did not affect single-channel conductance. Channel openings produced by caffeine at subactivating cytosolic calcium concentrations had identical conductance and relative permeability to those seen on calcium activation.

7. Channels activated by caffeine at both activating and subactivating calcium concentrations were characteristically modified by ryanodine, Ruthenium Red, ATP and magnesium, implying that the same channel is involved under both conditions.

INTRODUCTION

Evidence suggests that calcium release from the mammalian cardiac sarcoplasmic reticulum (SR) plays an important role in excitation–contraction coupling. Although

the exact mechanism of the coupling is unknown, it is thought that a transient rise in the intracellular free calcium concentration, resulting from calcium influx during the action potential, triggers a rapid release of calcium from the SR into the cytoplasm, a process described as calcium-induced calcium-release (CICR; Fabiato, 1985*a*). Calcium flux studies using isolated SR membrane vesicles indicate that rapid calcium efflux from the SR is mediated by a conduction pathway localized in the junctional regions of the membrane system, which come into close apposition with the sarcolemma both at the cell surface and at the transverse tubular invaginations of the sarcolemma. Calcium efflux is activated by calcium and ATP and inhibited by magnesium, Ruthenium Red and calmodulin (Rousseau, Smith, Henderson & Meissner, 1986; Meissner & Henderson, 1987) from the cytosolic side of the membrane. Recent experiments in which isolated junctional SR membrane vesicles have been incorporated into planar phospholipid bilayers have demonstrated a high-conductance calcium-selective channel, the open probability of which may be modified by the agents listed above in a manner consistent with the channel playing a role in excitation–contraction coupling (Rousseau *et al.* 1986; Williams & Ashley, 1989). Ryanodine, a plant alkaloid known to disrupt excitation–contraction coupling in cardiac muscle preparations, interacts with the channel protein 'locking' it into a permanently open, reduced conductance state (Rousseau, Smith & Meissner, 1987; Anderson, Lai, Liu, Rousseau, Erickson & Meissner, 1989).

Caffeine has been widely used as a pharmacological tool for releasing calcium from intracellular stores in the study of excitation–contraction coupling. However, the mechanism by which caffeine exerts its action has not been fully resolved. In skinned cardiac cells, Fabiato has demonstrated that caffeine induces calcium release from the SR (Fabiato, 1985*a*) and as with CICR, caffeine-induced calcium release can be inhibited by ryanodine (Fabiato, 1985*b*). Caffeine has also been reported to increase the sensitivity of the myofilaments to calcium (Fabiato & Fabiato, 1976; Endo & Kitazawa, 1978) and to inhibit calcium uptake by the SR (Blinks, Olson, Jewell & Braveny, 1972; Fabiato & Fabiato, 1973; Niedergerke & Page, 1981).

More recently, $^{45}Ca^{2+}$ flux measurements have demonstrated the ability of caffeine to release calcium from isolated cardiac and skeletal muscle SR vesicles (Meissner & Henderson, 1987; Rousseau, Ladine, Liu & Meissner, 1988). Moreover, caffeine has also been shown to activate the calcium-release channel of both skeletal and cardiac muscle SR in voltage clamp studies involving incorporation of isolated SR membrane vesicles into planar phospholipid bilayers (Rousseau *et al.* 1988; Rousseau & Meissner, 1989). Rousseau & Meissner (1989) reported that caffeine activated the channel by increasing both the frequency and duration of open events in the canine cardiac SR calcium-release channel.

In the present paper, we have extended this work by investigating the possible mechanisms of caffeine activation of the SR calcium-release channel from sheep cardiac muscle, in particular, whether cytosolic calcium is required for caffeine to increase channel open probability. We believe that at least two distinct modes of action are responsible for caffeine-induced calcium-release channel activation and hence caffeine-induced calcium release from the SR: a calcium-dependent mechanism seen with relatively low concentrations of caffeine (0·5–2·0 mM) and a calcium-independent mechanism requiring higher (> 5·0 mM) concentrations.

A preliminary report of this work has been communicated to the Physiological Society (Sitsapesan & Williams, 1989).

METHODS

Isolation of SR membrane vesicles

The methods used for the isolation of SR membrane vesicles were based on the procedure described by Meissner & Henderson (1987) for the isolation of canine cardiac SR vesicles. Sheep hearts were obtained from a local abattoir and transported to the laboratory in cold modified cardioplegic solution (Tomlins, Harding, Kirby, Poole-Wilson & Williams, 1986). Ventricular tissue was stripped of fat and minced prior to homogenization in a solution containing 1 mM-phenylmethyl-sulphonide fluoride (PMSF), 300 mM-sucrose and 20 mM-potassium piperazine-*N'N'*-bis-2-ethanesulphonic acid (PIPES), pH 7·4. The homogenate was centrifuged at 8000 r.p.m. in a Sorvall GSA rotor for 20 min and the pellet discarded. The supernatant was then centrifuged at 28000 r.p.m. for 60 min in a Sorvall A641 rotor. The mixed membrane population sedimented by this step was resuspended in a solution containing (in mM): 400 KCl, 0·5 $MgCl_2$, 0·5 $CaCl_2$, 0·5 EGTA, 25 PIPES, pH 7·0 plus 10% sucrose w/v and subfractionated on discontinuous sucrose-density gradients. The membrane suspension was layered over identical salt solutions containing 20, 30 and 40 % sucrose w/v and sedimented at 28000 r.p.m. for 120 min in a Sorvall AH629 rotor. Junctional SR membrane vesicles collecting at the 30–40 % interface were diluted in 400 mM-KCl and pelleted by centrifugation at 28000 r.p.m. for 60 min in a Sorvall A641 rotor before resuspension in a solution containing 400 mM-sucrose, 5 mM-*N'*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid (HEPES) titrated to pH 7·2 with tris(hydroxymethyl)-methylamine (Tris). Membrane vesicles were frozen rapidly in liquid nitrogen and stored at −80 °C.

Planar lipid bilayer methods

Lipid bilayers containing phosphatidylethanolamine (Avanti Polar Lipids, Birmingham, AL, USA) in decane (30 mg/ml) were formed across a 200 μm diameter hole in a polystyrene partition separating two fluid-filled compartments, referred to as the *cis* chamber (volume 0·5 ml) and the *trans* chamber (1·0 ml). Vesicles were added to the *cis* chamber and on fusion, incorporated into the bilayer with a fixed orientation so that the *cis* chamber corresponded to the cytosolic space and the *trans* chamber to the SR lumen (Miller & Rosenberg, 1979; Tomlins, Williams & Montgomery, 1984). The *trans* chamber was held at ground and the *cis* chamber could be clamped at various potentials relative to ground. Current flow through the bilayer was measured using an operational amplifier as a current–voltage converter as described by Miller (1982).

Planar bilayers were formed with the following solution in both the *cis* and *trans* chambers: 50 mM-choline chloride, 5 mM-$CaCl_2$ and 10 mM-HEPES/Tris, pH 7·2. Following bilayer formation, SR vesicles were added to the *cis* chamber and the choline chloride concentration of this chamber raised to 500 mM. Fusion of SR vesicles with the bilayer was marked by the appearance of Cl^--selective channels (Smith, Coronado & Meissner, 1985, 1986; Holmberg & Williams, 1989). The *cis* chamber was then perfused out with a solution containing 250 mM-HEPES, 125 mM-Tris and 10 μM-free calcium, pH 7·2 and the *trans* chamber was perfused with 250 mM-glutamic acid, 10 mM-HEPES, titrated to pH 7·2 with $Ca(OH)_2$ (free calcium concentration 80 mM). The free calcium concentration on the *cis*/cytosolic side of the bilayer could be altered by adding varying amounts of $CaCl_2$ and EGTA (Bers, 1982). Unless otherwise stated, drugs were added to the cytosolic side of the bilayer. In the presence of the solutions described above, current flow through calcium-release channels would be observed if they had been incorporated into the bilayer along with the Cl^- channels. A single fusion event usually corresponded with the incorporation of zero to four calcium-release channels. Experiments were performed at room temperature (23 ± 1 °C).

Data acquisition and analysis

Channel data were displayed on an oscilloscope and recorded on FM tape. Current recordings were replayed, low-pass filtered, at 1·0 kHz and digitized at 2·0 kHz using either an AT-based system (Intracel, Cambridge) or a PDP 11/73 based system (Indec, Sunnyvale, CA, USA). Channel open probability (P_o) and the lifetimes of open and closed events were monitored by 50 % threshold analysis at a holding potential of 0 mV. Lifetimes, accumulated from 3 min steady-state

recordings, were stored in sequential files and displayed in non-cumulative histograms. Individual lifetimes were fitted to a probability density function using the method of maximum likelihood (Colquhoun & Sigworth, 1983). Lifetimes lasting less than 1 ms were not fully resolved under the conditions of data acquisition described here 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).

Drugs

Stock solutions were prepared using deionized water and diluted in *cis* or *trans* solutions as required. ATP and caffeine were obtained from Sigma (Dorset), ryanodine from Cambridge Bioscience (Cambridge) and Ruthenium Red from BDH (Essex).

RESULTS

Caffeine activation in the presence of activating calcium

The cardiac SR calcium-release channels observed in the present study exhibited the characteristic sensitivity to the free calcium concentration on the cytosolic side of the bilayer described in previous studies (Ashley & Williams, 1988; Rousseau & Meissner, 1989; Ashley & Williams, 1990). Typical current fluctuations of a single sheep cardiac SR calcium-release channel in the presence of 10 μM-activating calcium are illustrated in Fig. 1*A*. Channel open probability at this concentration of calcium was in the range 0·02–0·045 ($n = 13$).

The addition of caffeine (0·5–2·0 mM) to the solution on the cytosolic side of the channel in the presence of 10 μM-calcium caused an activation of the channel within a few seconds and was fully reversible when caffeine was removed by perfusion. Figure 1*B* illustrates channels activation by 2 mM-caffeine with a resultant increase in P_o from 0·029 to 0·39. Calcium-release channel activation by caffeine is more obvious in channels displaying the lower P_o values seen at 0·1 μM-activating calcium. Figure 1*C* and *D* demonstrates activation under these conditions with P_o increasing from effectively zero to 0·25 following the addition of 1 mM-caffeine to the solution on the cytosolic side of the membrane. Caffeine produces no effect on the conductance of the cardiac SR calcium-release-channel. Figure 2 shows a single-channel current–voltage relationship for a channel in the absence and presence of caffeine. No significant differences exist between the two conditions; slope conductance and $Ca^{2+}/Tris^{+}$ permeability ratios (Smith *et al.* 1985) obtained from regression lines fitted to the data were as follows: in the absence of caffeine, 93 pS and 15·44; following the addition of 2 mM-caffeine, 89 pS and 13·32.

Details of the mechanisms involved in caffeine's activation of the cardiac SR calcium-release channel may be obtained from an inspection of the influence of low concentrations of the drug (0·5–2·0 mM) on single-channel lifetimes. Figure 3 shows the non-cumulative open and closed lifetime distributions of the SR calcium-release channel, activated by 10 μM-calcium, before and after the addition of 2 mM-caffeine to the cytosolic side of the membrane (current fluctuations of the same channel are shown in Fig. 1*A* and *B*), together with probability density functions obtained by the method of maximum likelihood. As we have previously demonstrated (Ashley & Williams, 1988; Williams & Ashley, 1989), with calcium as the sole activating ligand, most probable fits were obtained with two exponentials for the open-time distribution and three exponentials for the closed-time distribution. In the presence of 2 mM-

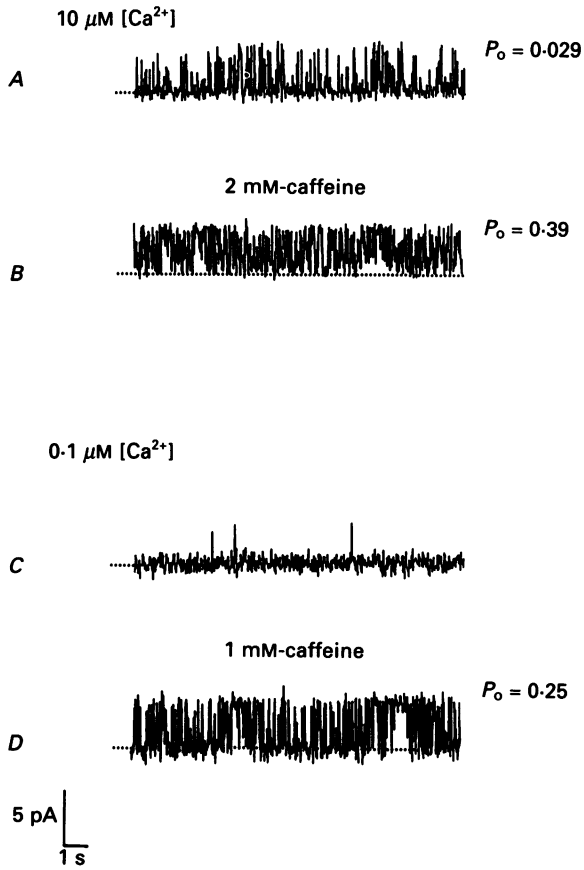

Fig. 1. Effects of caffeine on single cardiac muscle calcium-release channels in 10 μM- (*A* and *B*) and 0·1 μM-activating calcium (*C* and *D*). The dotted line indicates the zero current level. Holding potential was 0 mV. Channel open probabilities (P_o) were calculated as described in the Methods section and are indicated above each portion of recording.

caffeine on the cytosolic side of the membrane, the frequency of channel opening is increased with virtually no alteration to the duration or distribution of the open events. Increased P_os result from a reduction in the duration of the closed events.

A similar pattern emerges from the analysis of lifetimes of channels activated by caffeine in the presence of low activating concentrations of free calcium (0·1 μM; Fig. 1*C* and *D*). In the absence of caffeine, insufficient openings were obtained to allow lifetime analysis under these conditions; however, following the addition of 1 mM-caffeine to the solution bathing the cytosolic side of the membrane, lifetime analysis demonstrates that, as is the situation when the calcium-release channel is activated with calcium as the sole ligand, combinations of 0·1 μM-calcium and millimolar caffeine produce a pattern of gating which can best be described with at least two open and three closed states; the time constants and relative areas of which closely resemble those seen at comparable values of P_o with calcium as the sole activating ligand. For example the probability density functions for the open and closed times of the data shown in Fig. 1*D* are as follows: open times $f(t) =$

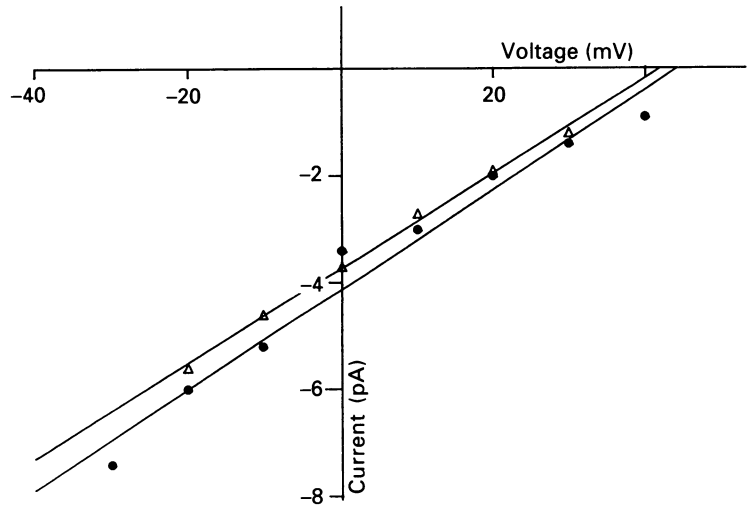

Fig. 2. Single-channel current–voltage relationship for a cardiac calcium-release channel activated by 10 μM-cytosolic calcium in the presence (●) and absence (△) of 2 mM-caffeine on the cytosolic side of the membrane.

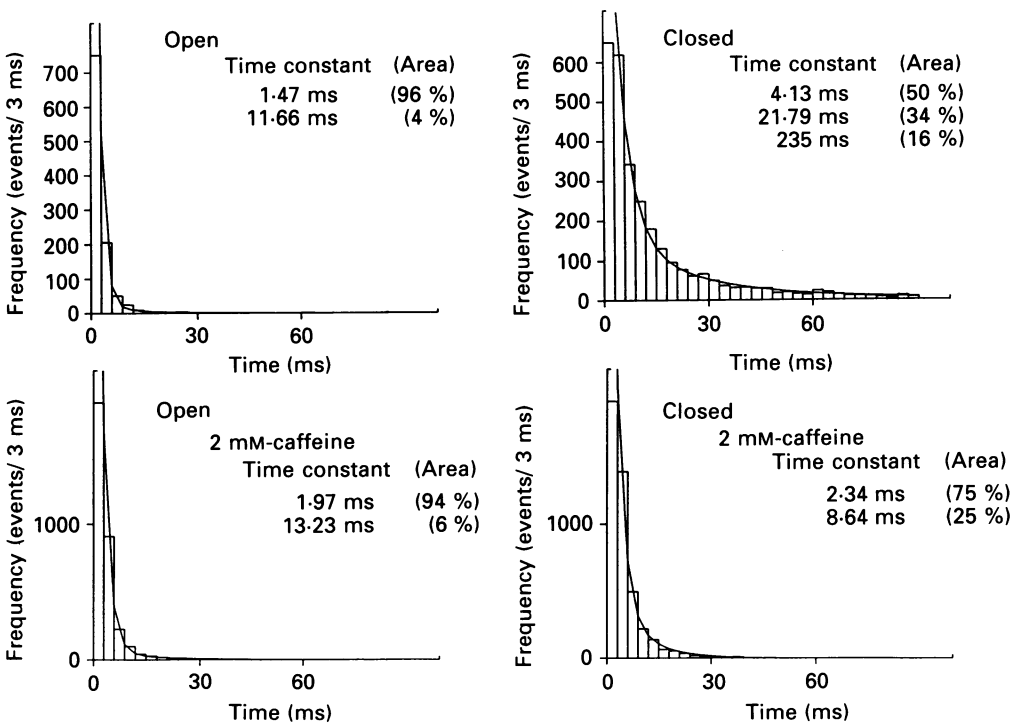

Fig. 3. The effect of a low concentration of caffeine on single-channel lifetimes. Lifetimes were determined as described in the Methods section. Lifetime histograms are shown together with probability density functions fitted by the method of maximum likelihood and are displayed for a channel activated by 10 μM-calcium in the absence and presence of 2 mM-caffeine on the cytosolic side of the membrane. Details of probability density functions are given in the figure. The holding potential was 0 mV.

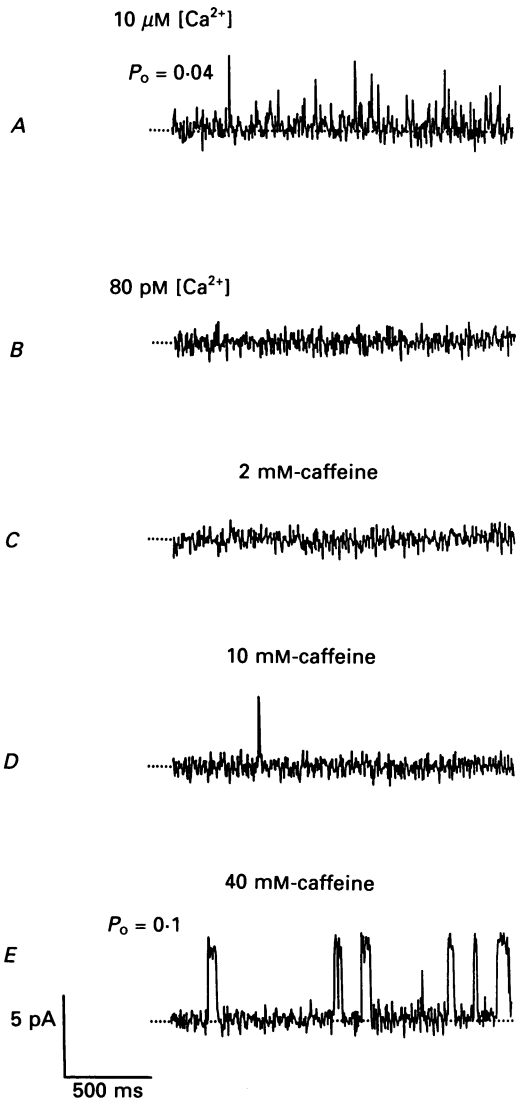

Fig. 4. Representative current fluctuations from a single calcium-release channel in the presence of: 10 μM-calcium (*A*), 80 pM-calcium (*B*), 80 pM-calcium plus 2 mM-caffeine (*C*), 80 pM-calcium plus 10 mM-caffeine (*D*) and 80 pM-calcium plus 40 mM-caffeine (*E*). The dotted lines indicate zero current levels. The holding potential was 0 mV.

$0{\cdot}90(1/1{\cdot}99)\exp(-t/1{\cdot}99)+0{\cdot}10(1/8{\cdot}56)\exp(-t/8{\cdot}56)$, closed times $f(t) = 0{\cdot}63(1/2{\cdot}81)\exp(-t/2{\cdot}81)+0{\cdot}32(1/10{\cdot}89)\exp(-t/10{\cdot}89)+0{\cdot}05(1/48{\cdot}51)\exp(-t/48{\cdot}51)$; in all cases time constants are quoted in milliseconds.

Caffeine activation at subactivating calcium concentrations

When the free calcium concentration in the solution on the cytosolic side of the bilayer was reduced to approximately 80 pM (calculated concentration following the addition of 12 mM-EGTA to the solution containing 10 μM-calcium), cardiac SR calcium-release channels invariably failed to open; P_o became zero. At these sub-

activating calcium concentrations low concentrations of caffeine (0·5–2·0 mM), which were effective in the presence of activating calcium, had no effect ($n = 15$); P_o remained zero. However, the channel could be induced to open by higher concentrations of caffeine added to the solution on the cytosolic side of the bilayer as is shown in Fig. 4. Concentrations of caffeine in the range 5–10 mM produced infrequent openings. Under these conditions, too few events occurred for the construction of lifetime histograms. At subactivating calcium concentrations, with 10 mM-caffeine on the cytosolic side of the channel, occasional channel openings were observed in 60% of the channels investigated ($n = 15$).

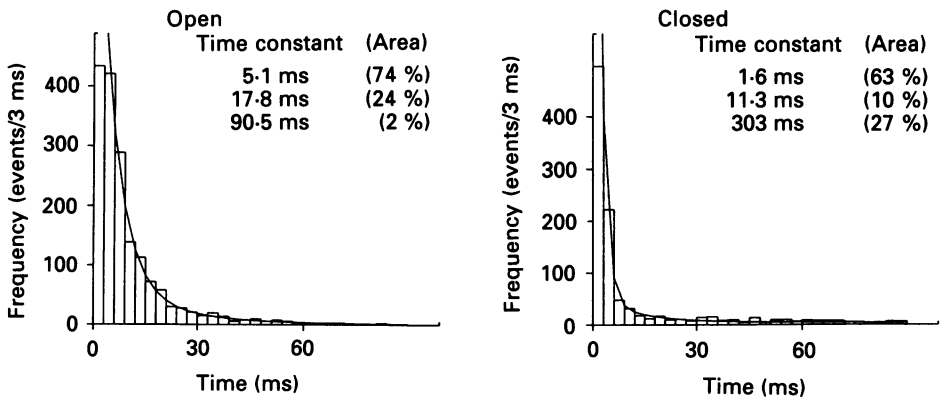

Fig. 5. Lifetime distributions of a calcium-release channel activated by 40 mM-caffeine at 80 pM-calcium. Lifetimes were determined as described in the Methods section. Lifetime histograms are shown together with details of probability density functions fitted by the method of maximum likelihood. The holding potential was 0 mV.

Raising the caffeine concentration still further produced consistent channel activation at subactivating calcium concentrations (Fig. 4). At 40 mM-cytosolic caffeine ($n = 8$) we observed well-defined opening events which were of a markedly longer duration than those seen during calcium activation of the channel or with combinations of calcium and low concentrations of caffeine at comparable values of P_o. Lifetime analysis confirmed this observation. Figure 5 shows non-cumulative open and closed lifetime distributions collected from the channel shown in Fig. 4 *E*. Details of the probability density functions obtained by the method of maximum likelihood are given in the figure. The 40 mM-caffeine-activated channel at subactivating calcium concentrations requires at least three exponentials to fit both open and closed lifetime distributions.

Is the caffeine-activated channel the calcium-release channel?

The single-channel current–voltage relationship of a cardiac SR calcium-release channel activated by 40 mM-caffeine at a subactivating calcium concentration is illustrated in Fig. 6. The slope conductance and reversal potential obtained for the channel under these conditions are virtually identical to those obtained with channels activated solely by calcium or combinations of calcium and low concentrations of caffeine, suggesting that 40 mM-caffeine is activating the cardiac SR

calcium-release channel rather than recruiting another species of channel. This observation is confirmed by experiments with Ruthenium Red and ryanodine which are illustrated in Fig. 7. Ruthenium Red, which has been shown to dramatically reduce P_os of calcium-activated skeletal and cardiac muscle SR calcium-release channels (Smith *et al.* 1985; Rousseau *et al.* 1986; Nagasaki & Fleischer, 1988, 1989; Holmberg & Williams, 1989), completely abolished the activity of the caffeine-activated channel either in the presence of activating calcium (data not shown) or with high caffeine concentrations at a subactivating calcium concentration

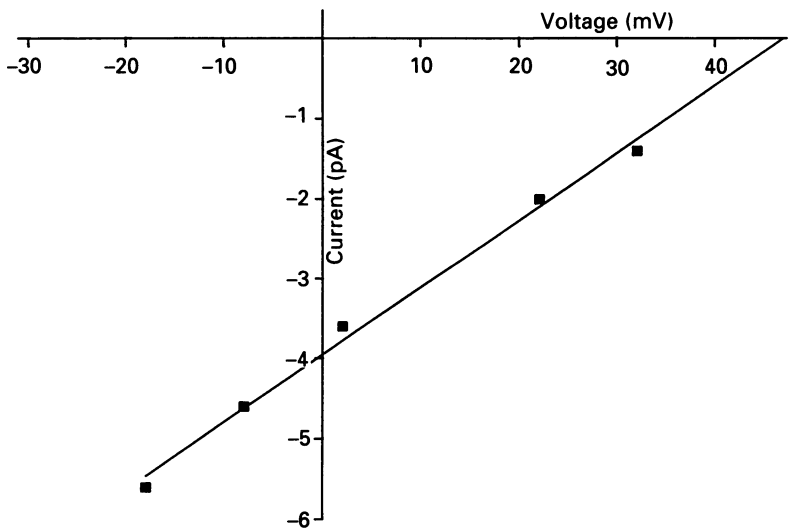

Fig. 6. Current–voltage relationship for a single calcium-release channel activated by 40 mM-caffeine in the absence of activating calcium (80 pM). Slope conductance = 84 pS; $Ca^{2+}/Tris^{+}$ permeability ratio = 15·44.

(Fig. 7*A*). Ryanodine is a specific high-affinity ligand for the SR calcium-release channel (Lai, Erickson, Rousseau, Liu & Meissner, 1988*a*, *b*; Rardon, Cefali, Mitchell, Seiler & Jones, 1989) and characteristically interacts with the calcium-activated skeletal or cardiac channel protein, locking it into a low-conductance open state (Rousseau *et al.* 1987). An identical action is seen when ryanodine is added either to the solution bathing the cytosolic side of a channel activated by 40 mM-caffeine at a subactivating calcium concentration (Fig. 7*B*), or if ryanodine is added following activation of the calcium-activated channel by low concentrations of caffeine (data not shown).

Calcium-activated cardiac SR calcium-release channels can be further activated by millimolar concentrations of ATP added to the cytosolic face of the channel (Williams & Ashley, 1989), while millimolar concentrations of magnesium decrease channel open probability by competing with activating calcium for a site on the cytosolic face of the channel protein (Ashley & Williams, 1988, 1990). Figure 8 demonstrates that calcium-independent caffeine-activated channels are similarly affected by these two ligands. Figure 8*A* is a representative portion of trace from a

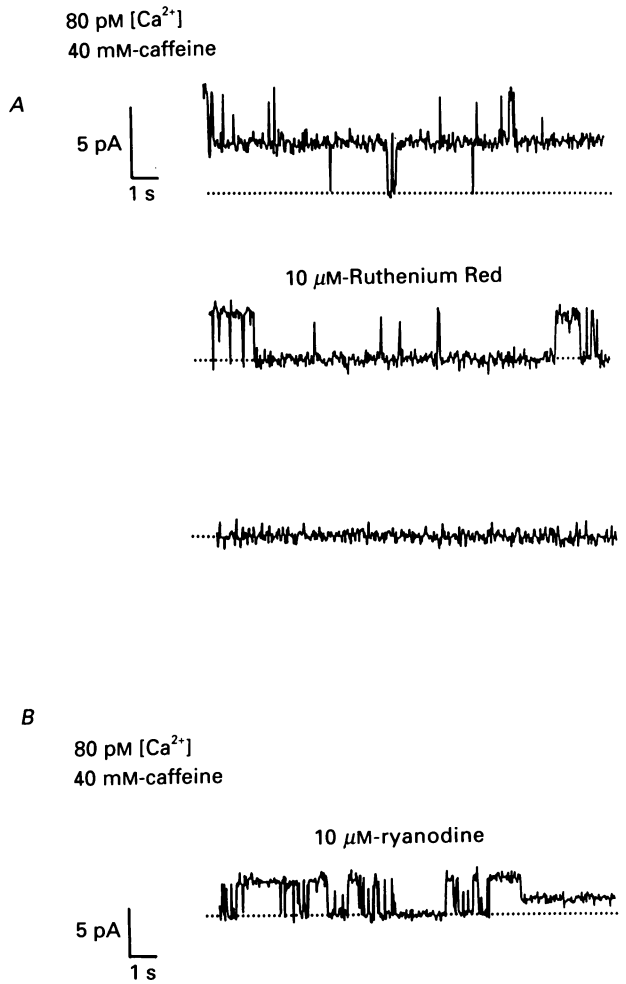

Fig. 7. Effects of Ruthenium Red and ryanodine on the cardiac calcium-release channel activated by 40 mM-caffeine at a free calcium concentration of 80 pM. *A*, upper trace: representative current fluctuations from a bilayer containing at least two channels prior to the addition of Ruthenium Red. Middle trace: fluctuations immediately following the addition of 10 μM-Ruthenium Red to the solution bathing the cytosolic face of the membrane. Lower trace: approximately 1 min after the addition of Ruthenium Red. No opening events were seen after this point. *B*, single-channel current fluctuations approximately 2 min following the addition of 10 μM-ryanodine to the solution bathing the cytosolic face of the membrane. The channel enters a characteristic low-conductance open state following the interaction of ryanodine. In all cases the holding potential was 0 mV and the dotted lines indicate zero current flow.

channel activated by 8 mM-cytosolic caffeine in the presence of 10 μM-calcium. After addition of EGTA to lower the free calcium concentration to 80 pM (Fig. 8 *B*), only the channel openings resulting from calcium-independent caffeine activation remain. The addition of 2 mM-ATP to the cytosolic side of the bilayer further activates the channel (Fig. 8*C*) and the subsequent addition of 2 mM-$MgCl_2$ to the solution on the cytosolic side of the membrane dramatically reduces the probability of channel opening (Fig. 8*D*).

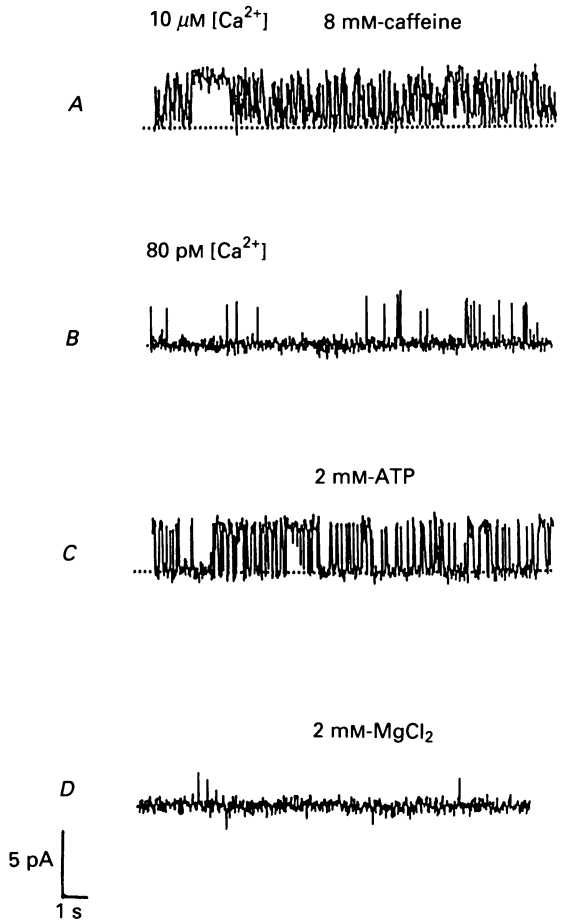

Fig. 8. Effects of ATP, magnesium and subactivating levels of calcium on a single caffeine-activated calcium-release channel. Caffeine (8 mM) in the presence of 10 μM-calcium produced a high P_o with pronounced long open events (*A*). After the addition of 12 mM-EGTA to lower the free calcium to a calculated level of 80 pM, occasional calcium-independent openings occurred (*B*). Addition of ATP (2 mM) to the cytosolic side of the membrane produced a further increase in the number of open events (*C*). Addition of magnesium (2 mM) led to an abolition of channel opening (*D*).

DISCUSSION

In this report we describe activation by caffeine of a calcium-selective channel located in a population of isolated membrane vesicles derived from the junctional regions of the sarcoplasmic reticulum network of sheep ventricular muscle. This channel displays properties consistent with a role in calcium-induced calcium release from the SR membrane network – the mechanism thought to be responsible for the contraction-initiating elevation of cytosolic calcium in response to excitation in the mammalian myocardium (Fabiato, 1985*a*; Nabauer, Callewaert, Cleemann & Morad, 1989). The probability of the channel being open is primarily regulated by the free calcium concentration in the solution bathing the cytosolic face of the channel protein (Ashley & Williams, 1988; Williams & Ashley, 1989; Ashley & Williams,

1990). Open probability may be further increased by ATP and is reduced by magnesium and Ruthenium Red (Williams & Ashley, 1989; Ashley & Williams, 1990).

Low concentrations of caffeine (0·5–2·0 mM) can activate the SR calcium-release channel by increasing the frequency of channel opening; this mechanism is calcium dependent. When the calcium concentration on the cytosolic side of the membrane is reduced to a subactivating level (80 pM), low doses of caffeine will not activate the channel. In this respect caffeine (0·5–2·0 mM) is similar to ATP which will further activate the cardiac SR calcium-release channel in the presence of activating concentrations of calcium, but is without effect at subactivating calcium concentrations (Rousseau *et al.* 1986). At subactivating concentrations of calcium, a second calcium-independent activation of the cardiac SR calcium-release channel is revealed with higher concentrations of caffeine. A gating system distinct from that observed in the presence of calcium alone or with combinations of low concentrations of caffeine and calcium is activated. It has previously been suggested that caffeine activation of the calcium-release channel can be explained in terms of an increased calcium sensitivity of the channel protein in the presence of the drug (Rousseau *et al.* 1988). Our results indicate that this cannot be the whole story.

The gating of the sheep cardiac SR calcium-activated calcium-release channel has been described by at least three closed and two open states. Elevating the calcium concentration on the cytosolic side of the membrane in the range 0·01–100 μM reduces the duration of all three closed lifetimes, without altering the distribution or duration of open lifetimes (Ashley & Williams, 1988; Williams & Ashley, 1989; Ashley & Williams, 1990). The resulting increase in channel open probability can be explained by the interaction of calcium with one or more of the closed states of the channel, leading to an increase in the frequency of channel opening. Increasing channel opening frequencies, without altering closing frequencies, can only increase P_o up to a certain level. This critical level of P_o appears to be lower than 0·5 and is attained at free calcium concentrations in the range of 100 μM–1 mM. For the channel to reach higher P_o values, the frequency of channel closing must be reduced. The effects observed on activation of the cardiac SR calcium-release channel by low concentrations of caffeine in the presence of activating concentrations of calcium are indistinguishable from merely raising the activating calcium concentration. Channel open probability is increased by a reduction of the durations of all three closed lifetimes. It therefore appears that the action of low concentrations of caffeine could be explained in terms of an increased affinity of the calcium-activation site of the calcium-release channel.

At higher caffeine concentrations a further mechanism is brought into action. An additional calcium-independent activation of the channel is seen. Our contention that the channel activation seen at 80 pM-free calcium plus high concentrations of caffeine is truly calcium independent and not the result of extreme calcium sensitization is supported by analysis of gating kinetics under these conditions. The probability density functions obtained from maximum likelihood fitting of the open lifetimes differ considerably from those obtained with calcium as the sole activating ligand, or with combinations of calcium and low concentrations of caffeine.

Ryanodine binds with high affinity to the calcium-release channel of cardiac and

skeletal muscle junctional SR (Pessah, Francini, Scales, Waterhouse & Casida, 1986; Lai *et al.* 1988*a*,*b*; Imagawa, Smith, Coronado & Campbell, 1987; Inui, Saito & Fleischer, 1987; Rardon *et al.* 1989) and this alkaloid characteristically modifies both the conduction and gating of the calcium-activated channel, producing a long-lasting, reduced-conductance, open state (Rousseau *et al.* 1987). Ryanodine produced identical modification of the calcium-independent caffeine-activated channel, thereby providing strong evidence that the channels activated by caffeine in the absence of activating calcium are SR calcium-release channels. In addition, the activity of the calcium-independent caffeine-activated channel can be regulated by ATP, magnesium and Ruthenium Red as can the calcium-activated channel.

In conclusion, we have demonstrated that the sheep cardiac muscle SR calcium-release channel may be activated by caffeine. Activation is achieved via an increase in the open probability of the channel with no effect on single-channel conductance and activation is freely reversible. At low concentrations, caffeine may sensitize the calcium activation site of the channel protein to calcium; the mechanism involved in calcium sensitization is unclear, but a similar mechanism has been proposed to explain the action of caffeine on contractile proteins. Higher concentrations of caffeine produce increased channel opening involving a direct calcium-independent mechanism.

The actions of caffeine reported here will no doubt contribute to the inotropic effects of this compound on mammalian cardiac muscle preparations.

This work was supported by grants from the British Heart Foundation.

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