Activation of the sheep cardiac sarcoplasmic reticulum Ca^{2+} -release channel by analogues of sulmazole

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1 The effect of sulmazole and several structurally related analogues on cardiac sarcoplasmic reticulum (SR) Ca^{2+} -release channel gating and on [^3H]-ryanodine binding to isolated SR membrane vesicles has been investigated.

2 The optical isomers, (+)- and (–)-sulmazole, increased the open probability (P_o) of single Ca^{2+} -release channels incorporated into phospholipid bilayers held under voltage clamp by increasing the frequency and duration of open events. The respective EC_{50} s were $423\ \mu\text{M}$ and $465\ \mu\text{M}$ at $10\ \mu\text{M}$ activating cytosolic Ca^{2+} and the Hill coefficients for activation were approximately two, suggesting that at least two molecules of either enantiomer are required to bind for channel activation.

3 Similarly the related enantiomers, (+)- and (–)-isomazole, which differ from sulmazole in the position of the pyridine nitrogen (4,5b for sulmazole; 4,5c for isomazole), were approximately as potent as each other and as potent as the isomers of sulmazole with EC_{50} s of approximately $445\ \mu\text{M}$.

4 In contrast, EMD 46512 and EMD 41000, which are sulmazole and isomazole analogues respectively, each with the methylsulphonyl oxygen removed, increased single-channel P_o with EC_{50} s of $42\ \mu\text{M}$ and $40\ \mu\text{M}$. The open and closed lifetime distributions were similar to those of the less potent analogues and the Hill coefficients were the same, suggesting that these compounds act at the sulmazole site on the Ca^{2+} -release channel.

5 All of the compounds tested were able to increase the P_o of channels in the absence of activating Ca^{2+} but were less potent than in the presence of Ca^{2+} . The drugs were effective only when added to the cytosolic face of the channel. None of the drugs could fully activate the channel in the absence of Ca^{2+} , partly due to only one drug molecule binding in the absence of Ca^{2+} , which is in contrast to the situation when activating Ca^{2+} is present. This suggests a synergistic action of these drugs and Ca^{2+} in Ca^{2+} -release channel activation.

6 EMD 46512 and EMD 41000 increased [^3H]-ryanodine binding to HSR vesicles with Hill coefficients of approximately two and EC_{50} s of $25\ \mu\text{M}$ and $28\ \mu\text{M}$, respectively, at $10\ \mu\text{M}$ Ca^{2+} . These drugs also increased [^3H]-ryanodine binding to HSR vesicles at pM Ca^{2+} but with Hill slopes of only one and EC_{50} s of 112 and $133\ \mu\text{M}$ for EMD 46512 and EMD 41000, respectively. In addition, maximal binding was reduced at pM Ca^{2+} in comparison to $10\ \mu\text{M}$ Ca^{2+} .

7 These data show that analogues of sulmazole increase the P_o of the cardiac SR Ca^{2+} -release channel and this occurs as the result of an increase in the frequency and duration of open events. They also demonstrate that the activation of the channel by these drugs is not stereoselective and therefore the configuration of the oxygen atom or methyl group attached to the sulphur atom does not affect their ability to elicit their effect. Similarly, the results show that the nitrogen in the 4, 5b or 4, 5c position of the pyridine ring does not affect Ca^{2+} -dependent or Ca^{2+} -independent activation of the Ca^{2+} -release channel. However, removal of the methylsulphonyl oxygen in sulmazole and isomazole results in two drugs which display a ten fold increase in potency over their respective parent compound in the activation of the Ca^{2+} -release channel. It is apparent that minor modifications of the sulmazole or isomazole molecules around the terminal sulphur atom dramatically affect potency but not maximal attainable effect, suggesting that the area around the sulphur atom may be critically involved in channel activation.

Keywords: Sulmazole analogues; sarcoplasmic reticulum; cardiac Ca^{2+} -release channel; caffeine; structure-activity relationships

Introduction

The main pathway for the release of intracellular Ca^{2+} in cardiac muscle cells is the sarcoplasmic reticulum (SR) Ca^{2+} -release channel. The open probability (P_o) of the Ca^{2+} -release channel is modulated primarily by the concentration of Ca^{2+} on the cytosolic face of the channel. It is also affected by various secondary agents such as adenosine and ATP (Smith *et al.*, 1985; McGarry & Williams, 1994), caffeine (Rousseau *et al.*, 1986; Sitsapesan & Williams, 1990) and anthraquinones (Holmberg & Williams, 1990b; Pessah & Zimanyi, 1991), some of which may act independently of activating Ca^{2+} . Ryanodine and ruthenium red act to modify single-channel conductance and to cause channel closure, respectively (Meissner & Henderson, 1987). It has recently been

demonstrated that therapeutically relevant concentrations of cardiac glycosides increase the P_o of the cardiac Ca^{2+} -release channel in a Ca^{2+} -dependent manner, an effect which may underlie part of the positive inotropic effect of these drugs, providing further evidence for the role of the Ca^{2+} -release channel in excitation-contraction coupling (McGarry & Williams, 1993a).

Williams & Holmberg (1990) have shown that the caffeine analogue, (+)-sulmazole, activates the Ca^{2+} -release channel by both a Ca^{2+} -dependent and a Ca^{2+} -independent mechanism and that there is a synergistic action of Ca^{2+} and sulmazole on single-channel activity. Sulmazole isomers and several structurally related compounds (including caffeine) appear to exert similar effects in the cell. They increase the sensitivity of the contractile proteins to Ca^{2+} (Blinks & Endoh, 1984), inhibit type III phosphodiesterase and inhibit

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the sarcolemmal Na^+/K^+ ATPase (van Meel *et al.*, 1988; Honerjager *et al.*, 1989). Sulmazole has also been shown to be an adenosine A_1 antagonist and to be a functional antagonist of an inhibitory G-protein (Parsons *et al.*, 1988). All of these effects may contribute to the cardiotonic actions of these drugs.

Van Meel *et al.* (1988) showed that (+)- and (–)-sulmazole were equipotent in their ability to inhibit adenosine 3':5'-cyclic monophosphate (cyclic AMP) and guanosine 3':5'-cyclic monophosphate (cyclic GMP) phosphodiesterase (PDE) but that the (+)-isomer was a more potent Ca^{2+} -sensitizing agent, a more potent inhibitor of the Na^+/K^+ ATPase and a more potent inotropic compound (see Figure 1 for structures). They concluded that the increased myofibrillar sensitivity to Ca^{2+} induced by (+)-sulmazole underlay its increased inotropic effect. This would appear to suggest that the configuration of the methyl and oxygen groups around the sulphur atom of sulmazole mediate the increased potency of the compound over its (–)-enantiomer.

The effects on cardiac function of some of the sulmazole analogues used in this study were examined by Honerjager *et al.* (1989). In their study, they found that the position of the pyridine nitrogen (4,5b in sulmazole and EMD 46512 and 4,5c in isomazole and EMD 4100), was crucial for inhibition of the sarcolemmal Na^+/K^+ ATPase, since EMD 41000 and isomazole had little inhibitory activity. The presence of a methylsulphonyl oxygen also affected the ability of each compound to inhibit this enzyme, as sulmazole was approximately forty times less potent than its reduced analogue EMD 46512. The pyridine nitrogen position also affected the inhibition of type III PDE, as isomazole and EMD 41000 were approximately two and three times as potent as EMD 46512 and sulmazole, respectively. The reduced analogues were slightly more potent inhibitors of type III PDE than

their respective oxidised parent compounds. Not surprisingly, the reduced analogue EMD 46512 was found to be a more potent cardiotonic drug than sulmazole.

The effects of these compounds on the cardiac SR Ca^{2+} -release channel, with the exception of (+)-sulmazole, have not been directly investigated. An investigation of their action on the channel may help elucidate the structural features of compounds that are responsible for activity at the caffeine/sulmazole site on the release channel and may also shed light on a new action of these drugs in cardiac muscle cells.

A preliminary account of this work has been presented to the British Pharmacological Society (McGarry & Williams, 1993b).

Methods

Preparation of SR membrane vesicles

The membrane preparation was as described by Sitsapasan & Williams (1990). Fresh sheep hearts were obtained from the abattoir and transported to the laboratory in cold modified cardioplegic solution (Tomlins *et al.*, 1986). Approximately 100 g of left ventricle and septum were homogenized in a solution containing 300 mM sucrose, 20 mM potassium piperazine- $\text{N}'\text{N}'$ -bis-ethanesulphonic acid (PIPES) and 1 mM phenylmethanesulphonyl chloride (PMSF), pH 7.4. The homogenate was centrifuged at 8000 g_{av} in a Sorvall GSA rotor for 20 min at 4°C. The supernatant was centrifuged at 100,000 g_{av} in a Sorvall T647.5 rotor for 40 min. The resulting membrane pellet was resuspended in a solution containing (mM): KCl 400, MgCl_2 0.5, CaCl_2 0.5, 1,2-di(2-aminoethoxy)ethane- N,N,N',N' -tetraacetic acid (EGTA) 0.5, PIPES 25 and 10% w/v sucrose, pH 7.0. The membrane suspension was layered onto identical salt solutions containing 20, 30 and 40% w/v sucrose and centrifuged at 100,000 g_{av} for 120 min in a Sorvall AH-629 rotor at 4°C. Heavy SR (HSR) membrane vesicles collecting at the 30–40% interface were 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-sulphonic 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.

Single-channel 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* and *trans*. 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 with an operational amplifier as a current-voltage converter (Miller, 1982). Initially, both chambers contained 50 mM choline chloride, 10 mM HEPES and 5 mM CaCl_2 , with the pH adjusted to 7.4 with Tris. HSR vesicles were added to the *cis* chamber. The chamber was stirred and the choline chloride concentration increased to give a 7:1 gradient *cis-trans* to promote vesicle fusion with the bilayer, fusion being marked by the appearance of Cl^- -selective channels (Smith *et al.*, 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 solution allowing resolution of only Ca^{2+} -release channel currents. The *cis* chamber was perfused with 250 mM HEPES, 125 mM Tris, pH 7.4; and the *trans* with 250 mM glutamic acid, 10 mM HEPES, with the pH adjusted to 7.4 with $\text{Ca}(\text{OH})_2$, giving a $[\text{Ca}^{2+}]$ in the *trans* chamber of

Figure 1 The structures of sulmazole and its reduced analogue, EMD 46512. The position of the chiral sulphur atom is highlighted in sulmazole. The difference between sulmazole and isomazole is the position of the pyridine nitrogen. Its position in isomazole and its reduced analogue EMD 41000 is shown by the arrow. The benzimidazole numbering system is used. In the lower part of the figure, the position of the asymmetry in sulmazole is shown.

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 each were calculated using EQCAL (Biosoft, Cambridge). The concentrations of free Ca^{2+} used in this study (10 μM and 80 μM) were attained by the addition of 1 mM EGTA and 1.005 mM or 0.15 μM CaCl_2 , respectively, to the cytosolic face of the channel. Experiments were performed at room temperature ($22 \pm 1^\circ\text{C}$).

Data acquisition, analysis and display

Single-channel data were displayed on an oscilloscope and recorded on video tape. Current recordings were replayed, low-pass filtered with an 8-pole Bessel filter (Frequency Devices 902) at 1 kHz and digitised at 2 kHz using an AT based system, Satori (Intracel, Cambridge). Channel P_o and open and closed lifetimes were determined by 50% threshold analysis at a holding potential of 0 mV. Single-channel P_o was determined from $P_o = t_{\text{open}} / (t_{\text{open}} + t_{\text{closed}})$ where t_{open} and t_{closed} are the mean open and closed lifetime durations, respectively. Digitized single-channel traces were transferred from Satori as plot files into CorelDraw 4 (Corel Corporation) and labelled for display. Lifetimes, accumulated from 3 min of steady state recordings, were stored in sequential files and

Figure 2 A single cardiac sarcoplasmic reticulum Ca^{2+} -release channel held at 0 mV. The channel was activated by 10 μM Ca^{2+} added to the cytosolic face of the channel (a). Single-channel P_o determined from 3 min of continuous recordings was increased on addition of 0.25 (b), 1 (c) and 3 mM (d) (–)–sulmazole: P_o increased to 0.353, 0.786 and 0.989 respectively. A full dose-response curve for all the data revealed an EC_{50} of 465 μM ($n=4$). Single-channel P_o was calculated as described in the Methods section. Single-channel conductance was unaffected by addition of the drug. Channel open level is shown above each respective portion of trace.

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 incompletely resolved under these data acquisition conditions and were therefore excluded from the fitting procedures. 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 were obtained from extrapolations of these lines. The $\text{Ca}^{2+}/\text{Tris}^+$ permeability ratio was calculated from the equation given by Fatt & Ginsborg (1958).

Binding assay

Binding of [^3H]-ryanodine to HSR membranes was conducted essentially as described by Holmberg & Williams (1990a). For equilibrium binding, HSR membranes (100 μg protein) were incubated with 1 nM [^3H]-ryanodine (76 Ci mmol^{-1}) for 90 min at 37°C in a solution containing 1 M KCl and 25 mM PIPES, pH 7.4, in a final volume of 1 ml. The appropriate analogue was added at the beginning of the assay. Non-specific binding was determined by the addition of 1 μM unlabelled ryanodine. Binding was terminated by the addition of 5 ml ice cold binding buffer followed by immediate filtration through a Whatman GF/B filter pre-soaked in binding solution. The filter was then washed with three further 5 ml volumes of buffer before being placed in 10 ml Packard MV scintillant and the remaining radioactivity on the filter counted in a liquid scintillation counter.

The data points from each single-channel and binding experiment for each drug were averaged and are displayed graphically as the mean $\pm$ standard error of the mean. The data were fitted to a four parameter logistic equation using INPLOT4 (GraphPad Software):

$$Y = A + \frac{(B-A)}{1 + (10^c/10^x)^d}$$

where A is the bottom of the curve; B is the maximum, constrained to 1; c is the log EC_{50} ; d is the Hill slope; and x is the ligand concentration.

Molecular modelling of drug structures

The structures of the different sulmazole analogues and also caffeine were modelled using the molecular modelling package HyperChem (release 3 for Microsoft Windows), from Autodesk Inc. on a Viglen 486 DX33. The structures were built and minimized in the MM+ force field using the Fletcher-Reeves algorithm (conjugate gradient method). The minimized structures were compared to those obtained using the Polak-Ribiere algorithm, to ensure a minimum had been reached, and in all cases were found to be identical.

Drugs

(+)- and (–)-sulmazole were gifts from Boehringer Ingelheim and were dissolved in distilled water. (+)- and (–)-isomazole were generously donated by E. Merck Pharmaceuticals and were also soluble in distilled water. EMD 46512 and EMD 41000, also donated by E. Merck, were dissolved in dimethylsulphoxide (DMSO). The concentration of DMSO never exceeded 2% v/v and this did not affect either the single-channel or binding experiments. Ryanodine was purchased from Cambridge Bioscience (U.S.A.) and [^3H]-ryanodine (76 Ci mmol^{-1}) from NEN (Stevenage, Herts). Phosphatidylethanolamine was from Avanti Polar Lipids, Alabama, U.S.A.

Results

Lack of stereoselectivity at the sulmazole activation site: (+)- and (-)-sulmazole

The P_o of single cardiac SR Ca^{2+} -release channels incorporated into lipid bilayers was increased dose-dependently by both (+)- and (-)-sulmazole. Figure 2 shows the activation of a single Ca^{2+} -release channel by increasing concentrations of (-)-sulmazole at $10\text{ }\mu\text{M}$ activating Ca^{2+} . The increase in P_o was seen only when the drugs were added to the cytosolic face of the channel. Analysis of the open and closed lifetime durations of these single-channels shows that the increase in P_o induced by (-)-sulmazole ($250\text{ }\mu\text{M}$ – 3 mM) occurred by an increase in the frequency and duration of open events. The increase in P_o at 0.25 mM and 0.5 mM was mainly due to the increased frequency of channel opening. This is demonstrated in Figure 3, which shows the open and closed lifetime durations of a single Ca^{2+} -release channel activated by 0.5 mM (-)-sulmazole. At $10\text{ }\mu\text{M}$ Ca^{2+} , the gating of the channel is best described by at least two open and three closed states. After addition of 0.5 mM (-)-sulmazole, the gating is best described by three open and two closed states. Occasionally, however, gating may be best described by three open and three closed states.

The activation of the channel by (+)-sulmazole has been described by Williams & Holmberg (1990). This enantiomer was also able to increase the P_o of single channels and this

also occurred by an increase in the frequency and duration of open events. The EC_{50} s for activation of the Ca^{2+} -release channel were $423\text{ }\mu\text{M}$ ($n = 4$) for (+)-sulmazole (see Figure 5) and $465\text{ }\mu\text{M}$ ($n = 4$) for (-)-sulmazole. The Hill slope for the activation was approximately two for both drugs, suggesting that at least two molecules bind to the Ca^{2+} -release channel. Both enantiomers displayed the ability to cause almost maximal activation of the channel in the presence of submaximally activating Ca^{2+} ($10\text{ }\mu\text{M}$) and the responses showed no signs of desensitization or fade, even over periods in excess of 20 min.

The position of the nitrogen in the pyridine ring is unimportant for activation of the Ca^{2+} -release channel

The structurally-related compounds (+)- and (-)-isomazole, which differ from the sulmazole isomers in the position of the pyridine nitrogen (4,5b in sulmazole; 4,5c in isomazole), also dose-dependently increased the P_o of single Ca^{2+} -release channels in the presence of $10\text{ }\mu\text{M}$ activating Ca^{2+} . Figure 4 shows the increase in single-channel P_o induced by (+)-isomazole. In common with the sulmazole compounds, the activation occurred when the drugs were added to the cytosolic face of the channel protein. The increase in P_o caused by both drugs was achieved by increasing the frequency and duration of open events and yielded EC_{50} s of $445\text{ }\mu\text{M}$ ($n = 4$) for both enantiomers. The sigmoid dose-response curves also had Hill slopes of two.

Figure 3 Open and closed lifetime histograms, with probability density functions obtained by maximum likelihood fitting to individual lifetimes for a single sheep cardiac sarcoplasmic reticulum (SR) Ca^{2+} -release channel activated by (a) $10\text{ }\mu\text{M}$ Ca^{2+} and (b) $10\text{ }\mu\text{M}$ Ca^{2+} + $500\text{ }\mu\text{M}$ (-)-sulmazole. Lifetimes, obtained from 3 min recordings, are displayed in non-cumulative histograms. Probability density functions, obtained by the method of maximum likelihood are drawn according to: $f(t) = a_1(1/\tau_1)\exp(-t/\tau_1) + \dots + a_n(1/\tau_n)\exp(-t/\tau_n)$, using Efit (Intracel). Open and closed lifetimes at $10\text{ }\mu\text{M}$ Ca^{2+} were best fitted by two and three exponentials, respectively, indicative of at least two open and three closed states. After addition of $500\text{ }\mu\text{M}$ (-)-sulmazole, the open and closed lifetime durations were best described by three open and two closed states. The areas and time constants are shown for each respective exponential component.

Removal of the methylsulphonyl oxygen of sulmazole or isomazole increases potency by ten fold

In both sulmazole and isomazole isomers, the terminal sulphur atom is connected by a double bond to an oxygen. Removal of this oxygen atom creates two reduced compounds: the sulmazole analogue, EMD 46512 and the isomazole congener, EMD 41000. EMD 46512 increases the P_o of single SR Ca^{2+} -release channels by a similar mechanism to its parent compounds (+)- and (-)-sulmazole. However, the reduced compound is approximately ten times as potent in comparison (Figure 5). EMD 46512 is able almost to activate fully the channel at $10\ \mu\text{M}\ \text{Ca}^{2+}$, with an EC_{50} of $42\ \mu\text{M}$ and a Hill slope of two ($n = 3$), by increasing the frequency and duration of open events (Figure 6).

Similarly, the reduced isomazole analogue, EMD 41000, was as potent as EMD 46512, with an EC_{50} of $40\ \mu\text{M}$ ($n = 5$). In comparison to its parent compounds it is also approximately ten times as potent (Figure 7).

None of the sulmazole congeners affected the single Ca^{2+} -release channel conductance at $10\ \mu\text{M}\ \text{Ca}^{2+}$ ($90 \pm 3\ \text{pS}$ ($\pm$ s.e. mean), $n = 7$) or the $\text{Ca}^{2+}/\text{Tris}^+$ permeability ratio (12.8 ± 0.2 , $n = 7$). These values are similar to those obtained by Williams & Holmberg (1990). The P_o of channels activated by any of the sulmazole analogues in the presence of activating Ca^{2+} was decreased by the addition of ruthenium red or Mg^{2+} (not shown).

Figure 4 The increase in single-channel P_o induced by addition of *cis*-(+)-isomazole at $10\ \mu\text{M}$ activating Ca^{2+} . At $10\ \mu\text{M}\ \text{Ca}^{2+}$ the P_o was 0.031 (a). This was increased to 0.267, 0.701 and 0.991 on addition of 0.25 (b), 1 (c) and 3 mM (d) (+)-isomazole to the *cis* chamber. The holding potential was 0 mV. The P_o was calculated from 3 min of steady state recording of single-channel activation. The EC_{50} obtained for this compound was $445\ \mu\text{M}$ ($n = 4$). The channel open level is indicated above each portion of data.

Ca^{2+} -independent activation of the Ca^{2+} -release channel by sulmazole analogues

All of the sulmazole analogues tested were able to increase the open probability of SR Ca^{2+} -release channels at sub-activating Ca^{2+} concentrations. In the effective absence of cytosolic Ca^{2+} (pM Ca^{2+}) the Ca^{2+} -release channel is closed. Figure 8 shows the increase in P_o when $100\ \mu\text{M}$ EMD 46512 was added to the cytosolic face of a single-channel in the presence of $80\ \text{pM}\ \text{Ca}^{2+}$. The P_o increased from zero to 0.56, at $10\ \mu\text{M}\ \text{Ca}^{2+}$, this concentration of EMD 46512 would maximally activate the channel. None of the compounds tested was able to cause full activation of the channel, even at up to 3 mM. The results show that there is a synergistic action of the sulmazole analogues and Ca^{2+} in increasing the P_o of single Ca^{2+} -release channels, in agreement with the results obtained by Williams & Holmberg (1990) for (+)-sulmazole.

Interestingly, single channels activated by the sulmazole congeners at pM Ca^{2+} were still modified to a characteristic sub-conductance state by addition of 200 nM ryanodine to the cytosolic face of the channel protein (not shown). This would appear to suggest that the binding of ryanodine to the Ca^{2+} -release channel may occur independently of Ca^{2+} , merely requiring the channel to be in an open state. This was also found by Williams & Holmberg (1990).

Effect of EMD 46512 and EMD 41000 on [^3H]-ryanodine binding to SR membranes

[^3H]-ryanodine binding is often used as a marker for Ca^{2+} -release channel open probability (Chu *et al.*, 1990; Holmberg & Williams, 1990a). Williams & Holmberg (1990) have shown that (+)-sulmazole increases the binding of [^3H]-ryanodine to HSR membranes. This is achieved by increasing the affinity of the Ca^{2+} -release channel for ryanodine.

EMD 46512 and EMD 41000 increased [^3H]-ryanodine binding to HSR membranes with EC_{50} s of $25\ \mu\text{M}$ and $25.12\ \mu\text{M}$, respectively, similar to the values for the increase in the P_o of the Ca^{2+} -release channel by these drugs (Figure 9). Both compounds increase equilibrium binding of 1 nM [^3H]-ryanodine from approximately $1\ \text{pmol}\ ^3\text{H}\ \text{mg}^{-1}$ protein to $2\ \text{pmol}\ ^3\text{H}\ \text{mg}^{-1}$ protein ($n = 5$) with Hill slopes of approximately

Figure 5 Comparison of the dose-response curves for (+)-sulmazole ($\square$) and its reduced analogue EMD 46512 (Δ). The EC_{50} for each compound was $423\ \mu\text{M}$ ($n = 4$) and $42\ \mu\text{M}$ ($n = 3$), respectively. The Hill slopes for the activation were 1.98 and 2.04 for EMD 46512 and (+)-sulmazole. Both compounds were able to activate almost maximally the Ca^{2+} -release channel but with different potencies. Each data point was obtained from a single sarcoplasmic reticulum SR Ca^{2+} -release channel activated in the presence of $10\ \mu\text{M}\ \text{Ca}^{2+}$ by either compound and held at 0 mV. Data points are shown $\pm$ s.e. mean. The curves were fitted to the data with INPLOT (GraphPad Software).

two for both drugs. This is also in agreement with the Hill slope for the increase of channel P_o by these drugs at $10 \mu\text{M}$ Ca^{2+} .

Ca^{2+} -independent increase in [^3H]-ryanodine binding to HSR membranes by EMD 46512 and EMD 41000

As these drugs are able to increase the P_o of single Ca^{2+} -release channels in the effective absence of activating Ca^{2+} , their effect on equilibrium binding of [^3H]-ryanodine to HSR membranes in pM Ca^{2+} was investigated. At 80 pM Ca^{2+} , [^3H]-ryanodine binding is almost undetectable. However, pretreatment of membranes with $1 \mu\text{M}$ – 2 mM EMD 46512 dose-dependently increased binding to $111.36 \pm 4.51 \text{ fmol } ^3\text{H mg}^{-1}$ protein ($n = 5$) (Figure 10). This yielded an EC_{50} of $112.1 \mu\text{M}$ and an accompanying Hill coefficient of 0.96. Values of $139.5 \mu\text{M}$ and $0.83 \mu\text{M}$ ($n = 5$) were obtained for EMD 41000.

Site of action in relation to caffeine

The action of caffeine on cardiac SR Ca^{2+} -release channel P_o has been studied previously by Rousseau & Meissner (1989) and Sitsapasan & Williams (1990). Their results showed that the mechanism by which caffeine increases single-channel P_o is similar to that demonstrated here and by Williams & Holmberg (1990) for sulmazole and its analogues. As sulmazole and isomazole exert similar effects on contractile proteins, PDEs and on Ca^{2+} -release channel P_o to caffeine, it may be concluded that they act at the same sites on the release channel as

caffeine. Figure 11 demonstrates the superimposition of the energy minimized structures of (+)-sulmazole and caffeine. It clearly shows that the imidazopyridine group of sulmazole is almost exactly superimposable on the corresponding purine ring system of the caffeine molecule.

Discussion

The increase in the P_o of single Ca^{2+} -release channels caused by (+)- and (–)-sulmazole demonstrates that there is no stereoselectivity at this activation site on the channel protein, as both enantiomers are approximately equally potent. Both compounds have similar EC_{50} s and activate the channel with Hill coefficients of approximately two, suggesting that at least two molecules of either compound are required to bind to the Ca^{2+} -release channel to cause full activation in the presence of $10 \mu\text{M}$ activating Ca^{2+} . The drugs increased P_o by increasing the frequency and duration of open events. However, at lower concentrations, although the open lifetime durations were slightly increased, the single-channel P_o was increased mainly by an increase in the frequency of channel opening. This is broadly in accord with the data of Williams & Holmberg (1990).

The observation that there is no stereoselectivity at the sulmazole site on the Ca^{2+} -release channel is supported by the effects of the analogues, (+)- and (–)-isomazole. These drugs are equipotent in increasing the P_o of the channel at $10 \mu\text{M}$ Ca^{2+} , with EC_{50} s of $445 \mu\text{M}$. They are also approximately as potent as (+)- and (–)-sulmazole. As the sul-

Figure 6 Open and closed lifetime durations of a single cardiac sarcoplasmic reticulum Ca^{2+} -release channel activated by $10 \mu\text{M}$ Ca^{2+} (a) and $10 \mu\text{M}$ Ca^{2+} + $80 \mu\text{M}$ EMD 46512 (b). Lifetime durations are displayed in non-cumulative histograms. The probability density functions, obtained by the method of maximum likelihood, are fitted using Efit (Intracel) according to $f(t) = a_1(1/\tau_1)\exp(-t/\tau_1) + \dots + a_n(1/\tau_n)\exp(-t/\tau_n)$. At $10 \mu\text{M}$ Ca^{2+} , open and closed lifetime durations are best described by two open and three closed states; in the presence of $80 \mu\text{M}$ EMD 46512, these are best described by three open and two closed states, reflecting that EMD 46512 increases P_o by increasing the frequency of opening as well as the duration of open events.

mazole and isomazole enantiomers are equipotent, activate the Ca^{2+} -release channel with Hill coefficients of approximately two and increase single-channel P_o by similar mechanisms it seems unlikely that these two sets of drugs exert their effects by binding to distinct sites on the Ca^{2+} -release channel. It is therefore reasonable to conclude that they bind to the sulmazole sites.

The data obtained with both these sets of isomers also demonstrate that the position of the nitrogen in the pyridine ring, either in the 4,5b or 4,5c position, does not affect the potency of these drugs in activating the Ca^{2+} -release channel. The change in the position of the pyridine nitrogen changes the electron density in the ring system. As this has no effect, this may suggest that the nitrogen atom in the 4,5b or 4,5c positions in the pyridine ring in these compounds does not play a significant role in the binding of these compounds to their sites on the channel protein.

That there is no stereoselectivity at the sulmazole activation site together with our demonstration that the position of

Figure 7 Comparison of the dose-response curves for (+)-isomazole (□) and its reduced analogue EMD 41000 (Δ). Each point is the mean $\pm$ s.e. mean from four (isomazole) or five (EMD 41000) different experiments. In each experiment the channels were activated by these compounds in the presence of $10 \mu\text{M}$ Ca^{2+} and were voltage clamped at 0 mV. The EC_{50} s for (+)-isomazole and EMD 41000 were $445 \mu\text{M}$ and $40 \mu\text{M}$ respectively. The sigmoidal curves were fitted to the data by INPLOT (GraphPad Software).

Figure 8 A single cardiac sarcoplasmic reticulum Ca^{2+} -release channel held at 0 mV at sub-activating (80 pM) Ca^{2+} . At this concentration, the channel is closed (a). On addition of $100 \mu\text{M}$ EMD 46512 to the cytosolic face of the channel, P_o increased from 0 to 0.560 (b), showing that these compounds are able to increase the P_o of the channel independently of activating Ca^{2+} and therefore must do so by binding to a closed conformation of the channel protein.

the pyridine nitrogen is unimportant for the activation of the Ca^{2+} -release channel by these analogues is in contrast to the situation with the sensitization of the myofilaments to Ca^{2+} and the inhibition of the Na^+/K^+ ATPase and type III PDE by these compounds. Van Meel *et al.* (1988) showed that (+)-sulmazole was a more potent Ca^{2+} -sensitizing agent than its (−)-enantiomer. Also, Honerjager *et al.* (1989) found that sulmazole compounds were more potent inhibitors of the ATPase and that isomazole compounds were slightly more potent PDE inhibitors. It could have been suggested that these analogues were binding to type III PDE which are known to be present in SR membranes (Kauffman *et al.*, 1989; Lugnier *et al.*, 1993), thereby modulating Ca^{2+} -release channel P_o . However, as the pharmacology of these compounds in activating the SR Ca^{2+} -release channel is different

Figure 9 Stimulation of [^3H]-ryanodine binding to cardiac sarcoplasmic reticulum vesicles by EMD 46512 (□) and EMD 41000 (Δ) at $10 \mu\text{M}$ Ca^{2+} . Incubation of vesicles with 1 nM [^3H]-ryanodine yielded specific equilibrium binding with Ca^{2+} of 1.117 ± 0.187 ($n = 6$) $\text{pmol } ^3\text{H mg}^{-1}$ protein. Both EMD 46512 and EMD 41000 caused an approximate doubling of specific binding. The EC_{50} s for activation were 25 and $28.2 \mu\text{M}$, respectively. Each point is the mean of six different experiments. The standard errors are omitted for clarity. The Hill coefficients were 1.84 and 2.00 for EMD 46512 and EMD 41000. The curves were fitted using INPLOT (GraphPad Software).

Figure 10 Stimulation of [^3H]-ryanodine binding by EMD 46512 (□) and EMD 41000 (Δ) at pM Ca^{2+} . At pM Ca^{2+} , specific binding was almost undetectable. However, addition of these analogues increased specific binding to approximately $112 \text{ fmol } ^3\text{H mg}^{-1}$ protein, significantly less than that obtained at $10 \mu\text{M}$ Ca^{2+} . The EC_{50} s obtained from the fitted sigmoidal curves for EMD 46512 and EMD 41000 were $112.1 \mu\text{M}$ and $139.5 \mu\text{M}$. Each point is the mean $\pm$ s.e. mean of five experiments. The Hill slopes were 0.96 and 0.83 respectively, suggesting that only one molecule of either drug binds to the closed state of the channel protein to increase [^3H]-ryanodine binding.

Figure 11 The superimposition of the minimized structure of caffeine and sulmazole. The structures of both compounds were minimized using both the Fletcher-Reeves and Polak-Ribiere algorithms in the MM+ force field, to ensure a minimum had been reached for each, using HyperChem (Autodesk Inc.). The structures were superimposed using the programme. Double bonds in the caffeine ring system have been omitted for clarity. The imidazopyridine ring of sulmazole (and the rest of the analogues tested here) was almost exactly superimposable on the purine ring of caffeine. This structural homology and that the ring systems adopt similar low energy conformations may indicate that they bind to common sites on the cardiac SR Ca^{2+} -release channel.

from that in the inhibition of the ATPase or PDE, it is apparent that these drugs are binding to sites, probably on the Ca^{2+} -release channel, distinct from either ATPase or PDE.

Reduction of either sulmazole or isomazole, thereby removing the methylsulphonyl oxygen, produces EMD 46512 and EMD 41000, which are approximately ten times as potent as their parent compounds in activating the Ca^{2+} -release channel. They increase P_o by increasing the frequency and duration of open events and the Hill slopes for the activation are two, suggesting that two molecules of either reduced compound are also required to bind to the channel for full activation at $10\text{ }\mu\text{M}$ Ca^{2+} .

The compounds tested here displayed the ability to increase [^3H]-ryanodine binding to cardiac HSR membranes at $10\text{ }\mu\text{M}$ Ca^{2+} . EMD 46512 and EMD 41000 increased equilibrium binding to approximately twice the level in the absence of either drug. The EC_{50} s were $25\text{ }\mu\text{M}$ and $25.12\text{ }\mu\text{M}$, close to those values obtained from single-channel experiments. The Hill slopes for the sigmoidal binding curves were two, also in agreement with the single-channel data and providing further confirmation that two molecules of either drug bind to the Ca^{2+} -release channel in the presence of activating Ca^{2+} .

All of the analogues are able to increase the P_o of single Ca^{2+} -release channels in the effective absence of activating cytosolic Ca^{2+} , however full activation (P_o approximately 1) could not be attained, in agreement with the earlier findings of Williams & Holmberg (1990). The compounds also increased [^3H]-ryanodine binding in the effective absence of activating Ca^{2+} (80 pM). EMD 46512 and EMD 41000 increased specific binding from almost zero to approximately $112\text{ fmol } ^3\text{H mg}^{-1}\text{ protein}$, with EC_{50} s of $112\text{ }\mu\text{M}$ and $139\text{ }\mu\text{M}$, respectively. The value for maximal binding is significantly less than that obtained in the presence of $10\text{ }\mu\text{M}$ Ca^{2+} and the EC_{50} s are significantly increased. Hence, both the potency and efficacy of these compounds are much reduced in the absence of Ca^{2+} .

Interestingly, the Hill slope for the increase in binding in the absence of Ca^{2+} was approximately one for both compounds. These data suggest that only one molecule of either isomer may bind to the closed conformation of the channel and that maximal [^3H]-ryanodine binding cannot be realised under these conditions. Further, it suggests that Ca^{2+} may stimulate the binding of a second molecule of EMD 46512 or

41000. When two drug molecules are bound to the channel protein and Ca^{2+} occupies its activation sites, maximal [^3H]-ryanodine binding may then occur. Hence the presence of activating Ca^{2+} increases the potency and efficacy of these drugs partly by stimulating the binding of a second drug molecule to the channel.

Extrapolating this to the single-channel data, suggests that when one molecule of one of these drugs binds to the channel, maximal P_o may not be attained and a different pattern of gating occurs. When Ca^{2+} is present, the binding of a second drug molecule is stimulated, and the presence of two molecules of activating drug together with Ca^{2+} allows full channel activation. This shows that there is a Ca^{2+} -dependent and a Ca^{2+} -independent action of these drugs to increase the P_o of the Ca^{2+} -release channel.

The differences in the structures of the sulmazole and isomazole enantiomers reside in the orientation of the double-bonded oxygen atom and the methyl group around the terminal sulphur atom, the sulphur being a chiral centre. As the enantiomers are mirror images of each other, it can be estimated that the oxygen in each protrudes $0.825\text{ \AA}$ above or below the plane of the molecule (which is flat) and the carbon of the methyl group protrudes $1.715\text{ \AA}$ above or below the plane. The reduced analogues, EMD 46512 and EMD 41000, are devoid of a double bonded oxygen attached to the sulphur and the remaining methyl group reorientates into the plane of the molecule. As these drugs are approximately ten times as potent as their respective parent compounds, it is possible that the presence of this oxygen in sulmazole and isomazole hinders the binding of the compounds to the Ca^{2+} -release channel. The lack of the physical bulk or lack of charge due to the oxygen may thus allow the reduced analogues better access to their sites, resulting in increased potency of the reduced compounds. However, it is also a possibility that the protruding methyl group in sulmazole or isomazole may similarly hinder proper access to binding sites and contribute to the lack of potency of these drugs in comparison to the reduced analogues. Regardless of this uncertainty, it is apparent that minor modifications around the sulphur atom dramatically affect potency, suggesting that the area around the sulphur may be critically involved in the channel activation. Further modifications in the area around this atom may result in drugs with greater ability to activate the Ca^{2+} -release channel or even antagonists of the compounds studied here. Notwithstanding this, it is not unreasonable to suggest that modifications at other points in the molecule may also affect the potency of the compound.

Modelling of the structures of the analogues used in this study and caffeine show that the imidazopyridine ring of the sulmazole analogues can be directly superimposed on the purine ring of caffeine. As these compounds exert similar effects by similar mechanisms (albeit with different potencies) on the SR Ca^{2+} -release channel, it is possible that they act by binding to the same sites on the channel protein. As the caffeine site on the channel has been demonstrated to be distinct from the adenosine sites (McGarry & Williams, 1993a) this suggests that adenosine and sulmazole also act at different sites on the channel. If the sulmazole analogues and caffeine act at the same sites on the Ca^{2+} -release channel then this suggests that although substituents in the area around the sulphur atom of the sulmazole compounds may influence potency, the imidazopyridine ring of the drugs is also involved in their binding to the protein.

In conclusion, this study shows that several analogues of sulmazole increase the P_o of the cardiac SR Ca^{2+} -release channel. The increase in P_o occurs by an increase in the frequency and duration of open events. The data show that there is no stereoselectivity at the sulmazole site and therefore, the positions of the oxygen and methyl groups attached to the sulphur atom of sulmazole and isomazole do not affect activity. Similarly, the position of the pyridine nitrogen, 4,5b or 4,5c positions, does not affect potency of the drugs. However, removal of the methylsulphonyl oxygen increases po-

tency by approximately ten fold. These results also suggest that activation of the Ca^{2+} -release channel by these compounds in the absence of Ca^{2+} occurs by only one drug molecule binding to the channel. Full activation occurs in the presence of Ca^{2+} partly due to Ca^{2+} stimulating the binding of a second drug molecule. Modelling the structures of the

drugs suggests that they act at the same activation sites as caffeine on the Ca^{2+} -release channel.

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