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Sulmazole (AR-L 115BS) Activates the Sheep Cardiac Muscle Sarcoplasmic Reticulum Calcium-Release Channel in the Presence and Absence of Calcium

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Summary. The properties of calcium-release channels of sheep cardiac muscle junctional sarcoplasmic reticulum (SR), have been investigated under voltage-clamp conditions following the fusion of isolated membrane vesicles with planar phospholipid bilayers. In the presence of activating calcium on the cytosolic side of the membrane, additions of the benzimidazole derivative sulmazole (AR-L 115BS) increased the open probability (P_o) of the channel reaching saturating values of 1.0 at 3 mM sulmazole. The drug did not affect single-channel conductance and activation was readily reversible. Analysis of channel open and closed lifetimes suggested that low concentrations of sulmazole (0.1 mM) may sensitize the channel to activating calcium, while at higher concentrations (1 mM and above), calcium and sulmazole act synergistically to produce a unique gating scheme for the channel. Millimolar concentrations of sulmazole also stimulate a degree of channel opening at subactivating (60 pM) calcium concentrations. Openings occurring under these conditions show very different kinetics to those of the calcium-activated channel but have an identical single-channel conductance and are modified by ATP, magnesium, ruthenium red and ryanodine in a similar manner to the calcium-activated channel. The release of calcium from the SR following the activation of the calcium-release channel by sulmazole may contribute to the positive inotropic action of this drug on mammalian cardiac muscle.

Key Words sarcoplasmic reticulum · calcium-release channel · sulmazole

Introduction

Sulmazole (AR-L 115BS) is a benzimidazole derivative which has been shown to produce positive inotropic effects in a range of mammalian cardiac muscle preparations [5, 6, 10, 29], and was at one time considered as a possible useful agent in the treatment of heart failure [23]. Sulmazole may achieve its positive inotropism via a number of mechanisms. Using chemically skinned preparations, it has been established that sulmazole increases the sensitivity of the contractile apparatus to calcium and that this is probably brought about by an increase in the affinity of Troponin I for calcium [29]. In intact preparations, sulmazole increased tissue cAMP levels, which is consistent with its action as an inhibitor of cardiac phosphodiesterase [8, 30]. The elevation of cAMP was preceded by increases in the magnitude of calcium transients associated with isometric contraction [8]. At concentrations of sulmazole above 0.3 mM these calcium transients were reduced in amplitude although force continued to increase [8].

In this report we describe the actions of sulmazole on the calcium-release channel of sheep cardiac muscle sarcoplasmic reticulum (SR) membranes. Contraction-initiating calcium is released from the SR membrane network of mammalian cardiac muscle cells in response to the depolarization of the sarcolemma during the action potential. In these tissues, significant quantities of calcium enter the cytoplasm during the plateau phase of the action potential via the dihydropyridine receptor/calcium channels, located in the transverse tubule invaginations and the surface sarcolemma. It is probable that calcium entering the cell in this way, stimulates the release of stored calcium from the SR via a process of calcium-induced calcium release (CICR) [9].

CICR has been demonstrated using isotope flux from isolated vesicles of junctional canine cardiac SR and the process has been shown to be regulated by a number of physiological and pharmacological agents. Calcium release is stimulated by calcium, ATP and caffeine and reduced by magnesium, calmodulin, ruthenium red and low pH [17]. Ryanodine activates release at low concentrations and inhibits it at higher concentrations [16].

Our understanding of the processes involved in calcium release from the SR have been extended by the demonstrations of single calcium-release channels in both skeletal [31, 32] and cardiac [2, 27, 36] SR membrane preparations. Single-channel current fluctuations have been resolved following the incorporation of isolated membrane vesicles, derived from junctional regions of the SR membrane net-

work, into planar phospholipid bilayers. Channels observed under these conditions display properties consistent with a role in SR calcium release, being activated by calcium, ATP [27, 36] and caffeine [26] added to the cytosolic face of the channel and showing inhibition by magnesium [2, 27], calmodulin [34] and ruthenium red [27]. The plant alkaloid ryanodine characteristically modifies both channel gating and conduction [28]. The specific, high affinity, interaction of ryanodine with the channel protein has permitted the purification of functional calcium channels from both skeletal [12, 15, 33] and cardiac [1, 14, 24] SR and their identification as the electron dense "feet" proteins seen linking the SR with the sarcolemmal membrane at triads [1, 13, 15].

Data presented in this study demonstrate that in addition to its actions of elevating cardiac cytoplasmic cAMP and sensitizing the contractile apparatus to calcium, sulmazole may also produce an elevation in cytosolic calcium via an activation of the calcium-release channel of the SR. Channel activation appears to be brought about by both a calcium-dependent and a calcium-independent activation of the channel. Sulmazole-stimulated calcium release from the SR may well contribute to the positive inotropic action of this compound on mammalian cardiac muscle.

Materials and Methods

Membrane Preparation

Sheep hearts were obtained from a local abbatoir. Freshly excised material was immersed in cold modified cardioplegic solution containing (in mM): NaCl 102, Nalactate 29, KCl 20, $MgCl_2$ 16, $CaCl_2$ 2, ethyleneglycolbis-(aminoethylether) tetra-acetic acid (EGTA) 2, for transportation to the laboratory. Junctional SR membrane vesicles were isolated using a modification of the procedure described by Meissner and Henderson [17]. Ventricular muscle was dissected free of fatty and connective tissue, minced and homogenized in four volumes of a solution containing 300 mM sucrose, 1 mM phenylmethyl sulphonylfluoride (PMSF), 20 mM potassium piperazine-N,N'-*bis*-2-ethane sulphonic acid (PIPES), pH 7.4. The homogenate was centrifuged at 8,000 rpm for 20 min in a Sorvall GSA rotor. A mixed membrane fraction was sedimented from the supernatant by centrifugation at 28,000 rpm for 60 min in a Sorvall A641 rotor. The pellet was resuspended in a solution composed of 400 mM KCl, 0.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.5 mM EGTA, 25 mM PIPES, pH 7.0, containing 10% sucrose wt/vol. The mixed membrane population was subfractionated on discontinuous sucrose gradients. The suspension was layered over identical salt solutions containing 20, 30 and 40% sucrose wt/vol and sedimented at 28,000 rpm for 120 min in a Sorvall AH629 rotor. The fraction that appeared at the 30/40% interface was collected, diluted in 400 mM KCl and pelleted by centrifugation at 28,000 rpm for 60 min in a Sorvall A641 rotor before resuspension in a solution containing 400 mM sucrose, 5 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) titrated to pH 7.2 with tris(hydroxymethyl)-methylamine (Tris). Membrane vesicles were snap-frozen in liquid N_2 and stored at −70°C.

Planar Bilayer Methods

Isolated junctional cardiac SR membrane vesicles were incorporated into planar phospholipid bilayers formed across a 200-μm diameter hole in a polystyrene partition separating two solution-filled chambers designated *cis* and *trans*. Bilayers were formed using dispersions of phosphatidylethanolamine (Avanti Polar Lipids, Birmingham, AL) in *n*-decane (30 mg/ml). The *trans* chamber was held at ground and the *cis* chamber could be clamped at a range of holding potentials relative to ground. Current flow through the bilayer was monitored using an operational amplifier as a current-voltage converter [18]. SR membrane vesicle fusion was achieved essentially as described by Smith et al. [31]. Initially, both chambers contained 50 mM choline chloride, 5 mM $CaCl_2$ and 10 mM HEPES titrated to pH 7.4 with Tris. Following vesicle addition to the *cis* chamber the choline chloride concentration in this chamber was increased by the addition of aliquots of a 3 M stock solution to give a 7:1 gradient. The formation of an osmotic gradient across the bilayer led to vesicle fusion and the appearance of Cl^--selective channels [31].

Following vesicle fusion, both *cis* and *trans* chambers were perfused with the solutions required for the resolution of calcium-release channels. SR membrane vesicles incorporate into planar phospholipid bilayers with a fixed orientation so that the solution in the *cis* chamber is equivalent to the cytosolic medium and the solution in the *trans* chamber corresponds to the SR luminal solution [19, 35]. Therefore, the *cis* solution was replaced by a solution containing 250 mM HEPES and 125 mM Tris, pH 7.4 which had a contaminant free calcium concentration of approximately 10 μM as monitored with a calcium-sensitive electrode (Orion EA 920/93-20. Orion, Boston, MA) and the *trans* solution was replaced with a solution consisting of 250 mM glutamic acid, 10 mM HEPES, titrated to pH 7.4 with $CaOH_2$ (free calcium concentration 67 mM). Under these ionic conditions calcium current fluctuations were observed with positive charge flow from *trans* to *cis* corresponding to negative current.

Experiments were carried out at room temperature (22°C). General reagents were AnalaR grade supplied by BDH, U.K. Ryanodine was purchased from Cambridge BioScience, Cambridge, U.K. Ruthenium red and adenosine triphosphate (ATP) were from Sigma Chemical, U.K. Sulmazole (AR-L 115BS) was a generous gift from Boehringer Ingelheim. Solutions were prepared using de-ionized water produced by a Milli-Q water purification system (Millipore, U.K.).

Channel Data Acquisition and Analysis

Current fluctuations were displayed on an oscilloscope and recorded on FM tape. For analysis, data were filtered using a 4-pole RC-mode filter at a front panel setting of 1 kHz (unless otherwise indicated) and digitized using either an Indec PDP 11/73 based lab system (Indec, Sunnyvale, CA) or an AT-based system (Intracel, Cambridge, U.K.). Single-channel current amplitudes were obtained from digitized data. Single-channel open and closed lifetimes and open probabilities were determined by 50% amplitude threshold analysis of data digitized at 2 kHz. Lifetimes were stored in sequential files and displayed in noncumulative histograms. Individual times were fitted to a probability

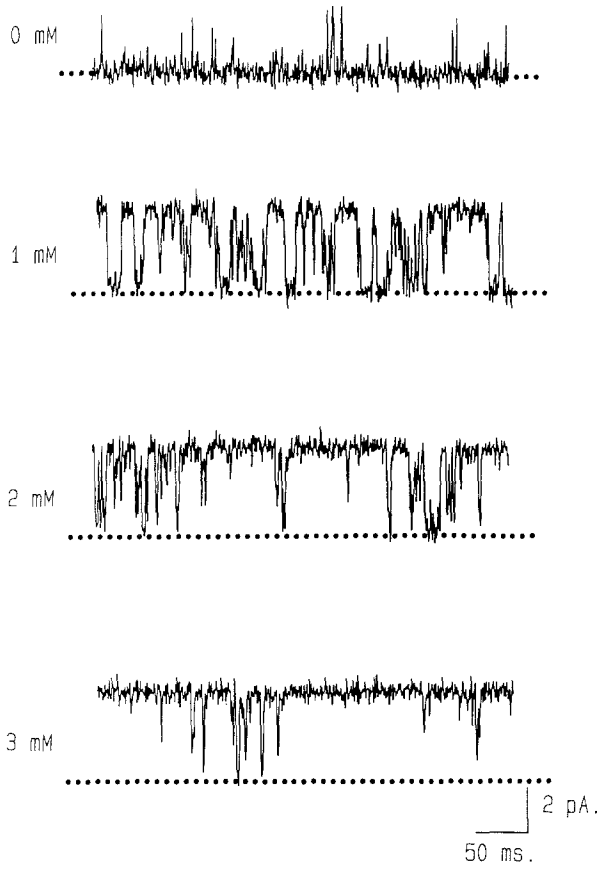

Fig. 1. Representative portions of data from a single-channel activated with 10 μM calcium and the indicated concentrations of sulmazole added to the cytosolic side of the bilayer. The dotted lines indicate the closed level. Holding potential was 0 mV. Open probabilities (P_o), monitored as described in the text were as follows:—0 mM sulmazole, 0.04; 1 mM sulmazole, 0.42; 2 mM sulmazole, 0.87; 3 mM sulmazole, 0.92

density function (pdf) using the method of maximum likelihood [7]. Under the conditions used for data acquisition, lifetimes with durations of less than 1 msec were not fully resolved and were therefore excluded from the fitting procedure. The missed events correction described by Colquhoun and Sigworth [7] was employed. Fits to double and triple exponentials were compared using the likelihood ratio test [4, 11].

Results

Effects of Sulmazole on Calcium-Activated Channels

Addition of sulmazole to single sheep cardiac SR calcium-release channels results in an increase in single-channel open probability. Representative current fluctuations from a single calcium-activated channel in the absence and in the presence of sequential additions of sulmazole to the cytosolic face of the membrane are shown in Fig. 1. The bilayer was held at 0 mV throughout and data were accumulated for 3 min at each concentration; values of open probability (P_o) were calculated from the entire 3-min recordings. Mean P_o values from a number of experiments at sulmazole concentrations ranging from 0.1 to 3.0 mM are shown in Fig. 2. Single-channel P_o rises from an initial level of 0.046 (±0.016 SEM) with 10 μM calcium as the sole activating ligand to a value of 0.96 (±0.023 SEM) in the presence of calcium plus 3 mM sulmazole on the cytosolic side of the membrane. Activation was readily reversed by perfusion and sulmazole was equally effective when added to the luminal face of the membrane (*data not shown*).

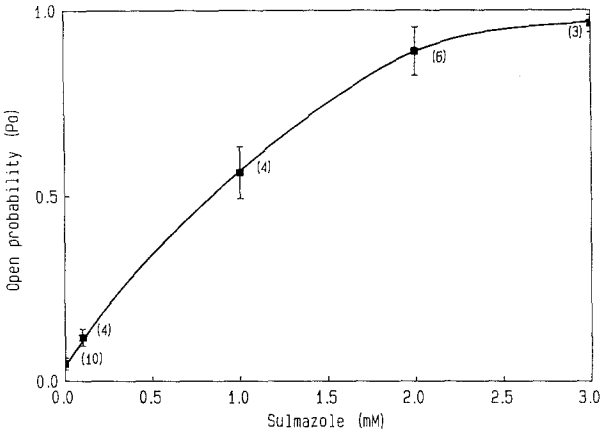

Fig. 2. Effects of increasing sulmazole concentrations on the open probability of channels activated with 10 μM calcium. Points are means (±SEM; numbers of experiments are indicated in brackets by each point). P_o values were determined as described in the text. Holding potential was 0 mV. The solid line has no theoretical significance

Information on the mechanisms involved in the activation of the sheep cardiac SR calcium-release channel by sulmazole may be obtained from an inspection of channel open and closed lifetimes. In the presence of 10 μM calcium as the sole ligand, most likely fits were obtained with two exponentials for the open time distribution and three exponentials for the closed time distribution [2, 3]. At low sulmazole concentrations (0.1 mM) the observed increase in P_o results mainly from an increased frequency of channel opening; the duration of opening events is virtually unaffected. In a typical experiment, in which P_o increased from 0.037 in the presence of 10 μM calcium to 0.095 following the addition of 0.1 mM sulmazole to the cytosolic face of the membrane, most likely fits were obtained with probability density functions such that

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

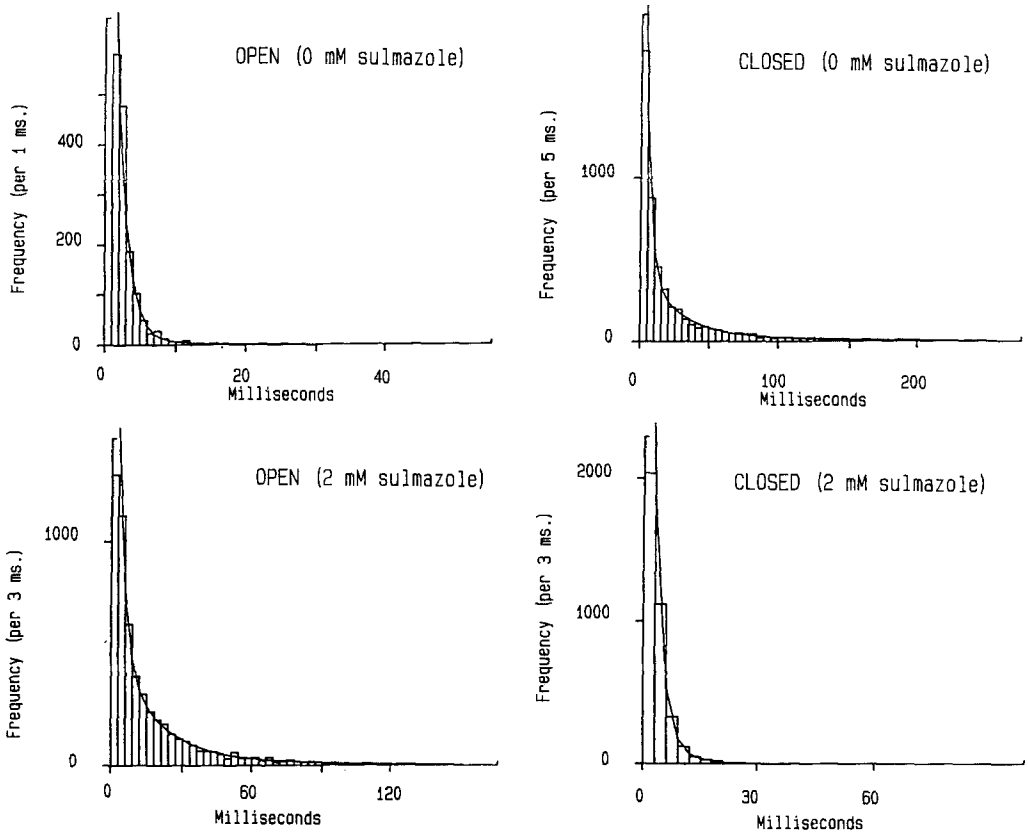

Fig. 3. Single-channel lifetimes in the presence of either 10 μM activating calcium (0 mM sulmazole) or 10 μM calcium plus 2 mM sulmazole. Lifetimes were determined as described in Materials and Methods. Lifetime histograms are shown together with probability density functions fitted by the method of maximum likelihood. Details of the pdfs, together with information for other sulmazole concentrations are given in the Table. The holding potential was 0 mV

For open lifetimes most likely fits were obtained to double exponentials with (*i*) 10 μM calcium: a_1 = 0.98, a_2 = 0.02, τ_1 = 1.47, τ_2 = 14.09 and (*ii*) 10 μM calcium + 0.1 mM sulmazole: a_1 = 0.97, a_2 = 0.03, τ_1 = 1.34, τ_2 = 9.84. For closed lifetimes most likely fits were obtained to triple exponentials with (*i*) 10 μM calcium: a_1 = 0.58, a_2 = 0.27, a_3 = 0.15, τ_1 = 5.08, τ_2 = 33.82, τ_3 = 159.66 and (*ii*) 10 μM calcium + 0.1 mM sulmazole: a_1 = 0.66, a_2 = 0.24, a_3 = 0.11, τ_1 = 4.83, τ_2 = 18.86, τ_3 = 42.28. Time constants are quoted in msec.

Open and closed lifetime distributions taken from the experiment shown in Fig. 1 with either 10 μM calcium as the sole activating ligand or 10 μM calcium plus 2 mM sulmazole are plotted in Fig. 3. The probability density functions fitted by the method of maximum likelihood for 0, 1, 2, and 3 mM sulmazole are given in the Table. The increased channel P_o seen when these higher concentrations of sulmazole were added to the cystolic side of the membrane resulted from a progressive increase in both frequency of channel opening and the duration of open events. Following the addition of between 1 and 3 mM sulmazole, most likely fits to the open time distributions were obtained with three rather than two exponentials. The proportion of openings occurring to the shortest lifetime distribution was progressively reduced as the sulmazole concentration was raised and P_o increased. The duration of the longest open time distribution was markedly extended as the sulmazole concentration was increased. Closed time distributions in the presence of calcium plus 1 to 3 mM sulmazole were best fitted with two rather than three exponentials and the proportion of closures occurring to the shortest lifetime distribution increased significantly as the sulmazole concentration was raised and P_o increased.

Sulmazole has no significant effect on single calcium-release channel conductance or the relative permeabilities of calcium and Tris (Fig. 4). The accumulated control data, obtained from channels activated solely by 10 μM cytosolic calcium, produced a slope conductance of 100 pS and a reversal potential of 40.7 mV so that $pCa^{2+}/pTris^{+}$ = 13.9 [31]. In the presence of 10 μM calcium plus sulmazole (between 2 and 4 mM added to the cytosolic side of the

Table Lifetime distributions with increasing sulmazole in the presence of 10 μM calcium

Sulmazole (mM)	P_o	Open lifetime distribution		Closed lifetime distribution	
		Time constant (msec)	Area (%)	Time constant (msec)	Area (%)
0	0.04	1.43	0.95	3.6	0.49
		7.31	0.05	25.65	0.37
		—	—	123.09	0.14
1	0.42	2.47	0.68	3.46	0.78
		9.94	0.25	19.55	0.22
		22.15	0.07	—	—
2	0.87	2.94	0.44	2.03	0.87
		17.35	0.44	5.89	0.13
		63.84	0.12	—	—
3	0.92	3.05	0.31	1.88	0.94
		16.39	0.56	5.39	0.06
		121.20	0.13	—	—

membrane) the single-channel current-voltage relationship yielded a slope conductance of 101 pS and a reversal potential of 38.8 mV giving a $pCa^{2+}/pTris^{+} = 12.0$.

Effects of Sulmazole at Subactivating Calcium Concentrations

As we have previously demonstrated, the sheep cardiac SR calcium-release channel is extremely sensitive to the level of activating calcium at the cytosolic face of the channel protein; single-channel P_o is approximately 0.01 at 10 nM calcium and rises to approximately 0.25 at 100 μM calcium [3]; above 1 mM calcium P_o tends to decline as cytosolic calcium is further increased [36]. Lowering the calcium concentration on the cytosolic side of the membrane to below nanomolar levels usually abolishes channel opening. An example of this is given in Fig. 5. Fig 5*A* shows representative channel openings with an activating free calcium concentration of 10 μM (i.e., contaminating calcium in the bathing solution of 250 mM HEPES, 125 mM Tris, pH 7.4). Under these conditions we observed characteristic brief opening events with occasional simultaneous openings suggesting the presence of more than one channel in the membrane. Figure 5*B* shows a representative portion of trace following the addition of 12 mM EGTA to the cytosolic medium which lowered the free calcium concentration to a subactivating level of approximately 60 pM (calculated assuming an apparent binding constant (log K'Ca) of 7.1 at pH 7.4 with 10 μM calcium monitored as described in Materials and Methods). No opening events were detected during 2-min observation at this free calcium concentration. However,

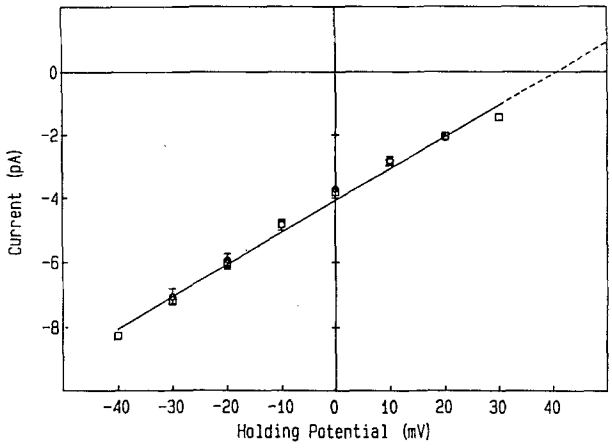

Fig. 4. Single-channel current-voltage relationships with either 10 μM calcium (□) or 10 μM calcium plus sulmazole (2–4 mM) (○) on the cytosolic side of the membrane. In both cases points are mean values (±SEM, n = 5), error bars smaller than the symbols are not displayed. The line drawn through the points is a regression line fitted to the 10-μM calcium data. Details of slope conductances and reversal potentials are given in the text

the addition of 2 mM sulmazole to the medium bathing the cytosolic face of the membrane (Fig. 5*C*) initiated opening with up to three channels simultaneously open. The opening events produced by sulmazole at these extremely low calcium concentrations were well defined and were obviously of a considerably longer duration than those observed with calcium as the sole activating ligand.

At subactivating calcium concentrations, sulmazole was equally effective from both sides of the membrane. Activation was reversible, but in contrast to calcium-activated channels, no openings were seen with 0.1 mM sulmazole and steady-state

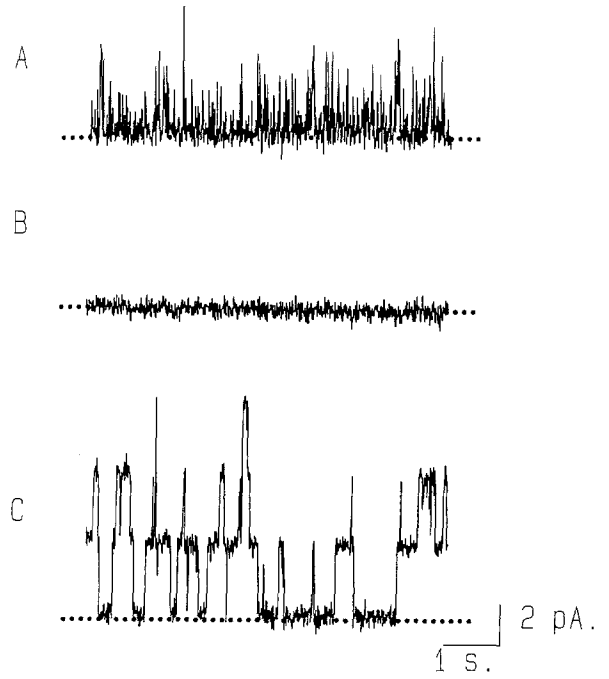

Fig. 5. (*A*) Current fluctuations from a bilayer containing at least three channels activated with 10 μM calcium on the cytosolic side of the membrane. (*B*) Opening events are completely abolished by the addition of 12 mM EGTA to the solution bathing the cytosolic face of the membrane, so lowering the free calcium concentration to 60 pM. (*C*) Activation of channel opening following the addition of 2 mM sulmazole to the medium on the cytosolic side of the bilayer at 60 pM calcium. In all cases the holding potential was 0 mV and the dotted line indicates the current level at which all channels in the bilayer are closed

current and single-channel P_o did not saturate at concentrations of sulmazole up to 5 mM (*data not shown*).

Figure 6 shows representative current fluctuations of a single sheep cardiac muscle SR calcium-release channel at 0 mV, with a free calcium concentration of 60 pM and 6 mM sulmazole in the solution at the cytosolic face of the membrane. The lower portion of the figure shows noncumulative open and closed lifetime distributions collected under these conditions for the same channel over a period of approximately 15 min, together with probability density functions obtained by the method of maximum likelihood. For the open times the most likely fit was obtained with a triple exponential with the following pdf:

$$f(t) = 0.24(1/16.14)\exp(-t/16.14) + 0.67(1/201.54)\exp(-t/201.54) + 0.08\,(1/1628.35)\exp(-t/1628.35). \quad (2)$$

Closed times were also best fitted to a triple exponential with the pdf:

$$f(t) = 0.27(1/140.23)\exp(-t/140.23) + 0.649(1/1254.60)\exp(-t/1254.60) + 0.09(1/3384.13)\exp(-t/3884.13). \quad (3)$$

In both cases time constants are quoted in msec. P_o for this experiment was 0.2. As was implied from inspection of single sulmazole-activated channel fluctuations at subactivating calcium concentrations (Figs. 5 and upper panel of Fig. 6), lifetime analysis confirmed that under these conditions mean open

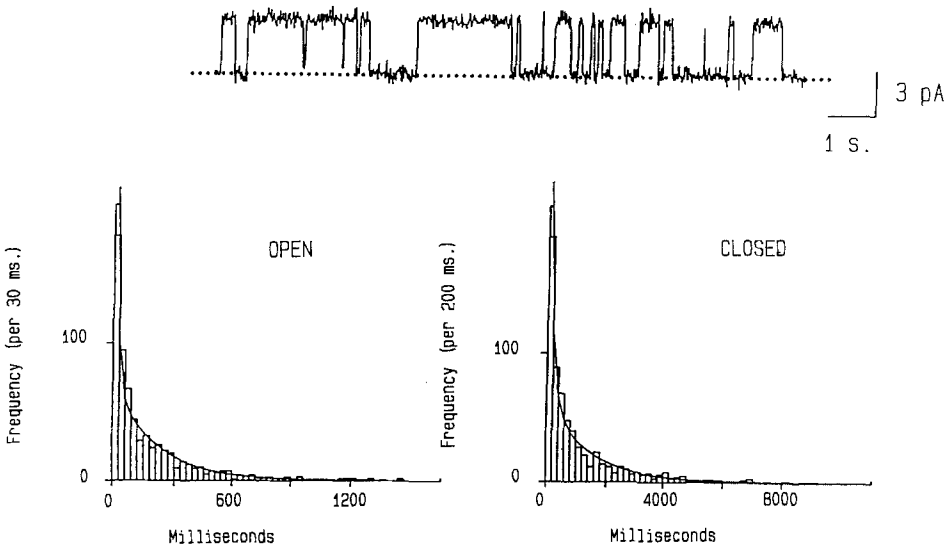

Fig. 6. Sulmazole-activated channel openings at 60 pM calcium. The top section of the figure shows a representative portion of data for a single channel activated with 6 mM sulmazole at a free calcium concentration of 60 pM on the cytosolic side of the channel. Holding potential was 0 mV and the dotted line represents the closed level of the channel. The lower section of the figure shows lifetime histograms accumulated over a period of 15 min together with pdfs fitted by the method of maximum likelihood. Details of the pdfs are given in the text

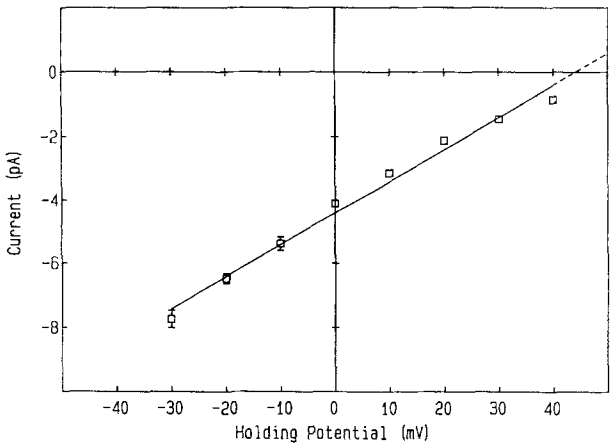

Fig. 7. Single-channel current-voltage relationship for sulmazole-activated (4–6 mM) channels at 60 pM calcium. Points are means (±SEM, $n = 4$), error bars smaller than the symbol are not displayed. The slope conductance and reversal potential obtained from the regression line are in the text

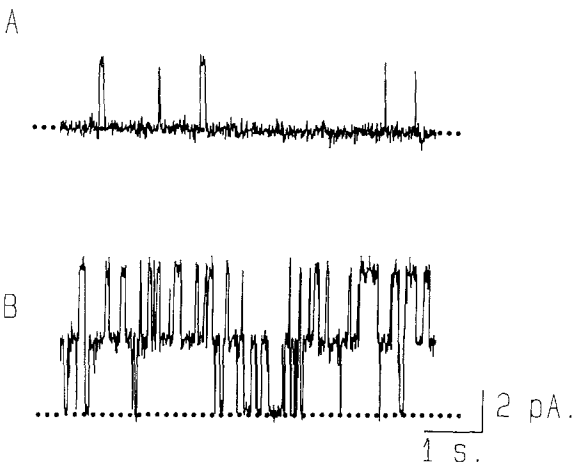

Fig. 8. Further activation of the sulmazole-activated channel by ATP. (*A*) Representative current fluctuations from a bilayer with 60 pM calcium plus 2 mM sulmazole in the solution bathing the cytosolic side of the membrane. (*B*) Current fluctuations from the same bilayer following the addition of 1 mM ATP to the solution on the cytosolic side of the membrane. In both cases, holding potential was 0 mV and the dotted line indicates the current level with all channels closed

and closed durations were considerably longer than those observed with calcium as the activating ligand.

The mean single-channel current-voltage relationship, obtained from four sheep cardiac muscle SR calcium-release channels with 60 pM calcium plus either 4 or 6 mM sulmazole in the solution bathing the cytosolic side of the membrane, is shown in Fig. 7. This relationship yields a single-channel slope conductance of 100.0 pS and a reversal potential of 44.1 mV, with a consequent $pCa^{2+}/pTris^{+}$ of 17.8. The slope conductance is virtually identical to that obtained with 10 μM calcium as the sole activating ligand or in the presence of 10 μM calcium plus sulmazole (Fig. 4).

SR calcium-release channels from both skeletal [21, 22, 25, 31, 32, 34] and cardiac [26, 27, 34] muscle are modulated by a number of physiological and pharmacological agents. We have examined the influence of a number of these agents on the activity of the sulmazole-activated cardiac SR calcium-release channel at subactivating calcium concentrations. An example of the action of ATP added to the cytosolic side of the membrane is displayed in Fig. 8. Figure 8*A* shows current fluctuations from a bilayer which contained two calcium release channels with 60 pM calcium plus 2 mM sulmazole on the cytosolic side of the membrane. The addition of 1 mM ATP to the solution on the cytosolic side of the bilayer (Fig. 8*B*) produced a marked activation of channel opening. At subactivating calcium concentrations such as these, 1 mM ATP was not found to activate the channel when added on its own (*data not shown*).

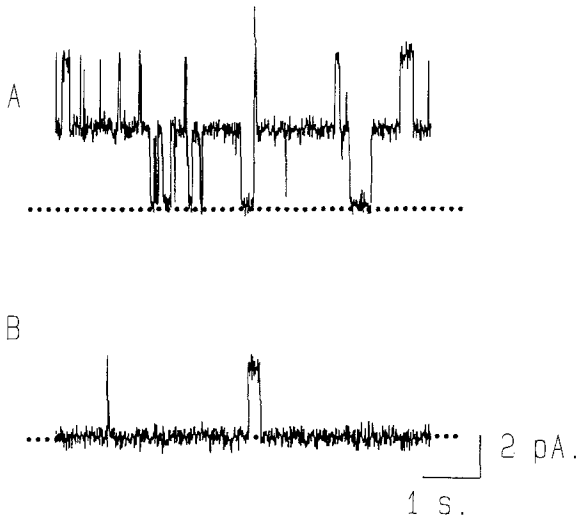

Fig. 9. Inhibition of sulmazole-activated channel by magnesium. (*A*) Representative current fluctuations from a bilayer with 60 pM calcium plus 4 mM sulmazole in the solution on the cytosolic side of the membrane. (*B*) Current fluctuations from the same bilayer following the addition of 2 mM $MgCl_2$ to the cytosolic solution. In both cases the holding potential was 0 mV and the dotted line indicates the current level with all channels closed

The influence of magnesium on the sulmazole-activated calcium-release channel at subactivating calcium concentrations is shown in Fig. 9. Figure 9*A* shows channel fluctuations with a solution containing 60 pM free calcium plus 4 mM sulmazole on

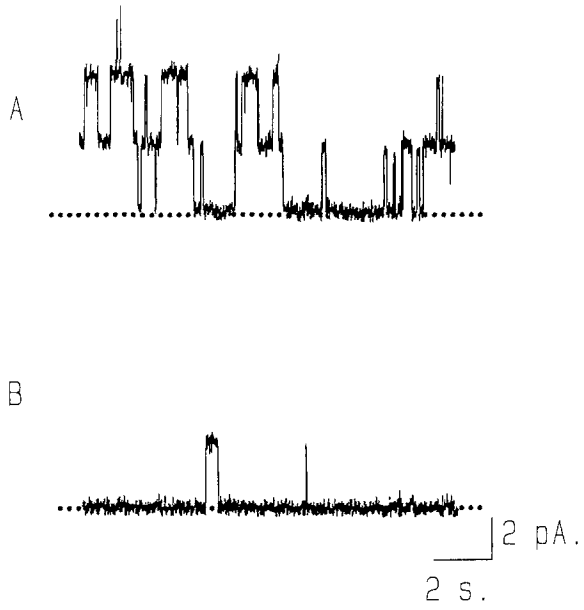

Fig. 10. Inhibition of the sulmazole-activated channel by ruthenium red. (*A*) Current fluctuations from a bilayer containing channels activated with 4 mM sulmazole at a calcium concentration of 60 pM. (*B*) Channel open probability is greatly reduced by the addition of 1 μM ruthenium red to the solution on the cytosolic side of the membrane. The holding potential was 0 mV and dotted lines represent the current level with all channels closed

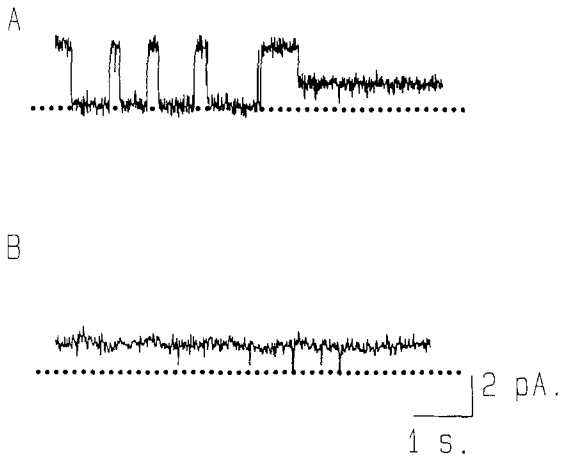

Fig. 11. Modification of the sulmazole-activated channel by ryanodine. (*A*) Current fluctuations of a single sulmazole-activated channel with a free calcium concentration of 60 pM plus 8 mM sulmazole in the solution on the cytosolic side of the membrane. Channel conductance and gating is modified 45 sec after the addition of 5 μM ryanodine to the cytosolic side of the bilayer. Brief closing events from the ryanodine-modified conductance level are seen here and in *B*. Holding potential was 0 mV and the dotted lines indicate the closed current level

the cytosolic side of the bilayer; the bilayer contained at least four channels. Following the addition of 2 mM $MgCl_2$ to the cytosolic solution the frequency of channel openings was lowered dramatically.

Figure 10 demonstrates the blocking action of the polycation ruthenium red on the sulmazole-activated cardiac SR calcium-release channel at subactivating calcium concentrations. Channel fluctuations in the presence of 60 pM cytosolic calcium plus 4 mM sulmazole are shown in Fig. 10*A*. Figure 10*B* demonstrates the reduced frequency of channel openings observed approximately 2 min after the addition of 1 μM ruthenium red to the solution on the cytosolic side of the membrane. Increasing the ruthenium red concentration to 2 μM completely abolished channel openings (*data not shown*).

The plant alkaloid ryanodine has been demonstrated to be a highly specific, high affinity ligand for the SR calcium-release channel. Single-channel studies have established that ryanodine interacts with the calcium activated channel "locking" it into an open state with reduced conductance. The data presented in Fig. 11 demonstrate that an identical effect of ryanodine is seen when this compound is added to the cytosolic solution bathing a sulmazole-activated calcium-release channel at a subactivating calcium concentration. Figure 11*A* shows the characteristic action of ryanodine. The channel was activated by 8 mM sulmazole added to a cytosolic solution with a free calcium concentration of 60 pM and 5 μM ryanodine was added to this solution 45 sec before the blocking reaction occurred. Brief closing events from the ryanodine-modified conductance level occur at a higher frequency for the sulmazole-activated channel at subactivating calcium concentrations than with ryanodine-modified calcium-activated channels.

Can the Degree of Channel Activation Observed with Sulmazole at Subactivating Levels of Calcium explain the Degree of Activation Seen in the Presence of Activating Levels of Calcium?

We investigated this possibility by monitoring P_o for single channels activated initially solely by 10 μM calcium, then following the addition of a saturating concentration of sulmazole and finally following the addition of 12 mM EGTA to lower the calcium concentration on the cytosolic face of the channel to a subactivating concentration. An example of such an experiment is given in Fig. 12. Figure 12*A* shows representative current fluctuations at 10 μM calcium. The P_o measured over a 3-min period at 0 mV was 0.048, the mean open time (T_o) was 1.35 msec and the mean closed time (T_c) was 27.03 msec. Fol-

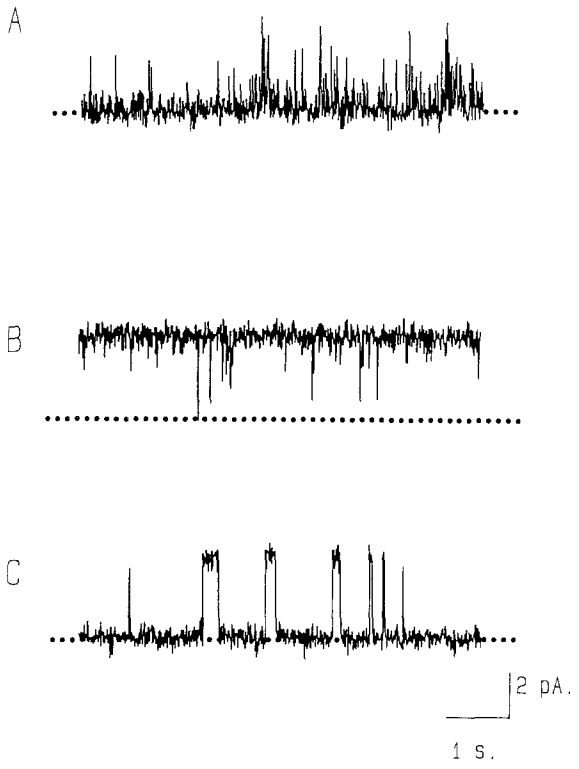

Fig. 12. (*A*) Representative current fluctuations of a single channel activated by 10 μM calcium in the medium bathing the cytosolic face of the bilayer. (*B*) The same channel following the addition of 2 mM sulmazole to the cytosolic solution and (*C*) after lowering the free calcium concentration to 60 pM by the addition of 12 mM EGTA. Holding potential was 0 mV. In all cases the dotted line represents the closed channel level

lowing the addition of 2 mM sulmazole to the solution bathing the cytosolic face of the membrane, P_o increased to 0.998, T_o was 299.46 msec and T_c was 1.14 msec (Fig. 12*B*). However, this dramatic increase in P_o was largely reduced by lowering the cytosolic calcium concentration to 60 pM; P_o fell to 0.051, with T_o equal to 39.42 msec and T_c equal to 732.84 msec (Fig. 12*C*). From this type of experiment it would appear that the calcium-independent activation of the sheep cardiac SR calcium-release channel by sulmazole seen at subactivating calcium concentrations, although significant, was not sufficient to explain the degree of activation observed in the presence of activating calcium concentrations.

Discussion

The activation of the SR calcium-release channel reported here is consistent with the increased calcium transients reported to occur in isolated cardiac muscle preparations in the presence of sulmazole [8] and suggests, in addition to a sensitization of the contractile proteins and an elevation of cytosolic cAMP, a role for increased SR calcium permeability in the positive inotropic effect of this drug.

The primary agonist of the cardiac muscle SR calcium-release channel is calcium [27] and with this ion as the sole agonist we have previously described a basic gating scheme for the channel involving two open and three closed states. Increases in channel open probability occurring in response to elevations of the activating calcium concentration are brought about as a result of the interaction of calcium ions with at least one of the closed states of the channel, leading to a reduction in all three mean closed times and an increase in the frequency of channel opening. Open times and hence the frequency of channel closing remain unaffected over an activating calcium range of 0.01 to 100 μM [3]. Our evidence suggests that there is no need for cooperative binding of more than one calcium ion to the channel to elicit activation as has been reported for calcium activation of the skeletal muscle transverse-tubule potassium channel [20].

With increasing concentrations of activating calcium P_o reaches a maximum at values well below 1.0 and tends to decline at calcium concentrations above 1 mM [3]. Higher P_o values can be achieved using combinations of calcium and a second ligand such as millimolar ATP [36] or millimolar caffeine [26]. Lowering the calcium concentration on the cytosolic side of the channel to 60 pM abolishes channel opening and secondary agonists such as ATP are ineffective at these subactivating calcium concentrations. In contrast, millimolar concentrations of sulmazole will activate the sheep cardiac muscle SR calcium-release channel at subactivating calcium concentrations. Lifetime analysis of these openings revealed that sulmazole activation differed considerably from that seen with calcium as the sole ligand. Open times as well as closed times required three exponential components to fit distributions and the mean lifetimes of all components were considerably longer than those seen during calcium activation. Therefore, millimolar concentrations of sulmazole can directly activate the sheep cardiac muscle SR calcium-release channel, producing a gating scheme which is distinctly different from that seen upon calcium activation.

The single-channel slope conductance of the sulmazole-activated channel is not significantly different from that seen with calcium as the sole activating ligand, a finding which would suggest that the calcium-independent sulmazole-activated channel and the calcium-activated channel are the same species of channel.

The sulmazole-activated channel displays simi-

lar properties to the calcium-activated channel in response to a range of physiological and pharmacological modifiers. We interpret these observations as further support for our belief that sulmazole is activating the same channel protein as calcium. ATP, which as noted above, is itself unable to activate the channel at subactivating calcium concentrations, produced a marked further activation of the sulmazole-activated channel. Sulmazole and ATP appear to be acting synergistically to activate the channel; however, a more complete elucidation of this phenomenon would require a detailed examination of channel lifetimes in the presence of combinations of these two ligands. The decreased P_o values seen with the sulmazole-activated channel in the presence of ruthenium red appear to occur via a decrease in the frequency of channel opening. This would also seem to be the case when magnesium is added to the cytosolic face of the sulmazole-activated channel. This observation is consistent with the proposed mechanism of the inhibition of the calcium-activated channel by magnesium. We have previously demonstrated that magnesium, added to the cytosolic face of the membrane, decreases P_o by decreasing the frequency of channel opening and have proposed that magnesium ions compete for the calcium activation site on the closed channel [3]. If the magnesium-bound closed state were unable to open, the frequency of channel opening would decline. A consequence of this proposal is that the sulmazole-activated channel should be more sensitive than the calcium-activated channel to magnesium block as, in the absence of competing calcium, the probability of the occurrence of the magnesium-bound closed state would be high. This possibility is now under investigation. Ruthenium red could presumably act in a similar fashion; interacting with the closed channel, not necessarily at the calcium activation site, to produce a state which is unable to open.

The modification of the conduction and gating properties of the sulmazole-activated channel seen with ryanodine closely resembles the actions of this alkaloid on the calcium-activated cardiac and skeletal muscle SR calcium release channels [28]. Measurements of specific [^{3}H]ryanodine binding to sheep cardiac junctional SR vesicles have demonstrated that sulmazole is capable of stimulating significant binding at subactivating calcium concentrations, while ATP and comparable concentrations of caffeine produce no significant binding (*data not shown*). These findings support our contention that sulmazole is directly activating the same channel as calcium and are consistent with our observations on the varying effectiveness of these ligands to open the channel at subactivating calcium concentrations.

When a low concentration of sulmazole (0.1 mM) was added as a second ligand to cardiac SR calcium-activated calcium-release channels the observed increase in P_o was brought about in a manner which suggests that this concentration of sulmazole may be sensitizing a calcium-sensitive closed state of the channel to calcium. Under these conditions, activation was achieved via an increased frequency of opening; the rates of closing remained virtually unaltered as would be observed with an increase in the concentration of activating calcium. This concentration of sulmazole did not activate the channel at subactivating calcium concentrations.

With the addition of higher concentrations of sulmazole (1–3 mM) single-channel P_o progressively increased to saturating values of approximately 1.0 and inspection of open and closed lifetimes demonstrated that this resulted from a progressive increase in both the frequency of channel opening and a decrease in the rate of channel closing. Clearly, this degree of activation could not result purely from a sensitization of the channel to calcium, as calcium-activated channels saturate at P_o values well below 1.0 and calcium activation is brought about without modification of the frequency of channel closing. The possibility that the degree of activation produced by high concentrations of sulmazole could be explained by a combination of calcium sensitization of the channel and the calcium-independent sulmazole activation seen at these concentrations of sulmazole is excluded by the experiment described in Fig. 12. In this experiment calcium-independent sulmazole activation of the channel produced an overall mean open time of approximately 39 msec, whereas that seen with the same concentration of sulmazole plus 10 μM calcium was approximately 300 msec. As stated above calcium sensitization would increase P_o via a decrease in channel mean closed times with no effect on mean open times; therefore it is not possible for a combination of calcium sensitization and calcium-independent sulmazole activation to explain saturation of P_o and the observed channel lifetimes seen with combinations of calcium plus high concentrations of sulmazole. We believe that the synergistic interaction of calcium and sulmazole with the channel protein produces a unique pattern of activation. A similar scheme has been proposed for the activation of the skeletal muscle SR calcium-release channel by combinations of calcium and ATP [32].

In conclusion, we have demonstrated that sulmazole interacts with the calcium-release channel of sheep cardiac muscle SR and increases the prob-

ability of channel opening. Multiple mechanisms appear to be involved in the drug's action; at low concentrations sulmazole may sensitize the calcium receptor of the channel, while at higher concentrations sulmazole and calcium combine synergistically to create a unique gating scheme. At these concentrations, sulmazole can directly activate the channel in a calcium-independent fashion.

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