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Similarities in the Effects of DIDS, DBDS and Suramin on Cardiac Ryanodine Receptor Function

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Abstract. The mechanisms involved in 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS)- and 4,4′-dibenzamidostilbene-2,2′-disulfonic acid (DBDS)-modification of sheep cardiac ryanodine receptor (RyR) channel function have been investigated. DIDS (50–500 μM) exerts at least three effects on single channel function. With Ca^{2+} as the permeant ion, DIDS increases both channel open probability (P_o) and single channel conductance in a similar manner to the effects observed with suramin. Both effects occur immediately and are fully reversible. Similar effects were observed with DBDS (10 μM–2 mM), a compound with the 4,4′-NCS groups of DIDS replaced with $NHCOC_6H_5$. DIDS (500 μM) also caused irreversible modification to the fully open channel level in 74% of the channels. This effect was not observed with suramin or DBDS (10 μM-1 mM). Competition studies with DBDS and suramin coupled with the close similarities in the effects of DIDS, DBDS and suramin on gating and conduction suggest that these ligands may all bind to the same sites on RyR. The DIDS-induced irreversible modification to the fully open state may result from the binding of the isothiocyanate groups to positively charged amino acids at or near the suramin binding sites although it is possible that this modification is unrelated to its other effects on channel function.

Key words: Ryanodine receptor — Suramin — DIDS — DBDS — Sarcoplasmic reticulum — Ca^{2+}-release channel

Introduction

Although suramin, a polysulfonated napthylurea has been shown to act as a noncompetitive P_2-purinoceptor antagonist [6, 9], it does not act as an antagonist at the adenine nucleotide/nucleoside sites on mammalian cardiac and skeletal RyR [7, 8, 19]. Rather, suramin acts as an agonist at RyR [7, 8, 19] and evidence suggests that it does not act via the adenine sites but instead binds to unique sites on the channel [8, 19]. Hill coefficients for channel activation indicate that at least two molecules of suramin binds to cause maximum activation. We have shown that suramin increases the P_o of rabbit skeletal and sheep cardiac RyR incorporated into planar phospholipid bilayers but the cardiac isoform has approximately ten times higher affinity for suramin [19]. The mechanism for suramin-induced RyR activation is very different to that for agents binding to the adenine sites. Agonists at the adenine binding sites cause a large increase in the frequency of very brief openings and the more effective agents also increase the duration of the open lifetimes at concentrations which increase P_o above 0.3 [12, 13]. In contrast, although suramin does increase the frequency of channel opening, the predominant mechanism for increasing P_o is an increase in the duration of open lifetimes [19]. In addition to an increase in P_o, we have also shown that, with Ca^{2+} as the permeant ion, suramin can increase single channel conductance by approximately 20% [19].

4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) is normally regarded as a chemical probe for the study of anion transporters [3], however, as it possesses structural similarities to suramin it has also been used as a ligand for purinoceptors [5]. The ability of both suramin and DIDS to interact with P_2-purinoceptors suggests that DIDS may also bind to the suramin sites on RyR. DIDS has been reported to activate mammalian cardiac and frog skeletal muscle RyR [10, 14, 23] but the sites of action have not been elucidated. The present study therefore examines the possibility that DIDS can activate the cardiac RyR channel by binding to suramin sites. If this is the case, it would be expected that DIDS would

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increase both P_o and single channel conductance and that the mechanism for the increase in P_o would be similar to that of suramin. The effect of DIDS, and a related compound, 4,4′-dibenzamidostilbene-2,2′-disulfonic acid (DBDS), on single channel function was therefore investigated using Ca^{2+} as the permeant ion. The results demonstrate that DIDS and DBDS both increase P_o and conductance in the manner expected for agents binding to the suramin sites on RyR. In addition, DBDS tends to reduce rather than potentiate the effects of suramin suggesting that the two ligands may compete for the same sites on RyR. DIDS also produces a third effect on channel function which is an irreversible modification to the fully open channel level which may be the result of isothiocyanate groups binding to positively charged amino acids on RyR which possibly form part of the suramin binding domains.

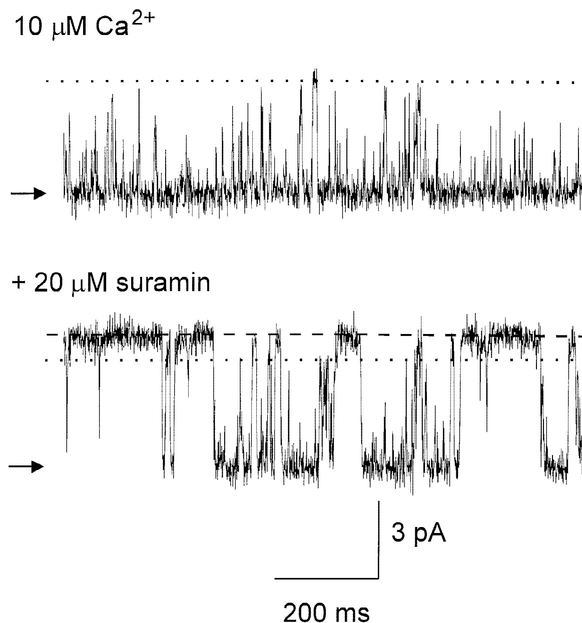

Fig. 1. The effects of suramin on current fluctuations through a typical single native sheep cardiac RyR incorporated into a planar phospholipid bilayer held at 0 mV. Ca^{2+} is the permeant ion and current flows from the luminal to the cytosolic side of the channel. The arrows indicate the closed channel level and the dotted lines indicate the fully open channel level in the absence of suramin. The dashed line indicates the fully open channel level in the presence of suramin. In the top trace, the channel is activated solely by 10 μM cytosolic Ca^{2+} and the P_o is 0.119 and current amplitude is 4.25 pA. Subsequent addition of 20 μM suramin to the cytosolic side of the channel increased P_o to 0.699 and current amplitude to 5.1 pA.

Materials and Methods

Preparation of SR Membrane Vesicles and Planar Lipid Bilayer Methods

Heavy SR membrane vesicles were prepared from sheep cardiac muscle as previously described by Sitsapesan et al. [16]. Heavy SR membrane vesicles were frozen rapidly and stored in liquid nitrogen. Vesicles were fused with planar phosphatidylethanolamine lipid bilayers as previously described [16]. The vesicles incorporated into the bilayer in a fixed orientation such that the *cis* chamber corresponded to the cytosolic space and the *trans* chamber to the SR lumen. The *trans* chamber was held at ground and the *cis* chamber was held at potentials relative to ground. After fusion of vesicles, the *cis* chamber was perfused with a solution containing 250 mM N-2-hydroxyethylpiperazine-N′-2-ethanesulphonic acid (HEPES), 125 mM Tris(hydroxymethyl)-methylamine (Tris), 10 μM free Ca^{2+}, pH 7.2. The *trans* chamber was perfused with a solution of 250 mM glutamic acid, 10 mM HEPES, pH to 7.2 with $Ca(OH)_2$ (free [Ca^{2+}] was approximately 50 mM). In some experiments symmetrical 250 mM CsPIPES, pH 7.2 was used as the recording solution. All experiments were performed at 23 ± 1°C. Additions of suramin, DIDS and DBDS were made to the *cis* chamber. The free [Ca^{2+}] and pH of the solutions in the absence and presence of suramin, DIDS and DBDS were measured at 23°C with a Ca^{2+} electrode (93–20, Orion Research, Boston, MA) and Ross-type pH electrode (Orion 81–55) as previously described in detail [16]. The reversible and irreversible effects of DIDS were tested by leaving DIDS in the *cis* chamber for 3 min, perfusing away the DIDS and recording for a further 3 min to check for reversibility.

Data Acquistion and Analysis

Single-channel recordings were displayed on an oscilloscope and the analog voltage signal was stored in digital form on Digital Audio Tape (DAT) (Biologic, Intracel, Cambridge). All steady state recordings were carried out at 0 mV with Ca^{2+} as the permeant ion and at ±40 mV with Cs^+ as the permeant ion. Current recordings were filtered at 500 Hz (–3 dB attenuation) and digitized at 2 kHz with Ca^{2+} as the permeant ion and filtered at 1 kHz and digitized at 4 kHz with Cs^+ as the permeant ion. Channel open probability (P_o) and the lifetimes of the open and closed events were determined over 3 min of steady state recording using the method of 50% threshold analysis [4]. Lifetime analysis was carried out only when a single channel incorporated into the bilayer. Events <1 or 0.35 msec in duration were not fully resolved when Ca^{2+} and Cs^+ respectively was the permeant ion and were therefore excluded from lifetime analysis. Open and closed lifetimes accumulated from approximately 3 mins of recording were stored in sequential files and displayed in noncumulative histograms. Individual lifetimes were fitted to a probability density function (pdf) using the method of maximum likelihood [4] according to the equation: $f(t) = a_1(1/T_1)\exp(-t/T_1) + \ldots + a_n(1/T_n)\exp(-t/T_n)$ where T is the time constant in milliseconds and a is the relative area. A missed events correction was applied as described by Colquhoun and Sigworth [4]. A likelihood ratio test [2] was used to compare fits of up to four exponentials by testing twice the difference in $\log_e$ (likelihood) against the chi-squared distribution at the 1% level. Single-channel current amplitudes were measured from digitized data using manually controlled cursors.

Suramin and DIDS were obtained from Calbiochem (Nottingham, UK). DBDS was obtained from Molecular Probes (Leiden, The Netherlands) and ryanodine from Agri Systems (Wind Gap, PA). All solutions were prepared using MilliQ deionized water (Millipore, Harrow, UK) and filtered through a Millipore membrane filter before use.

The mean value ± SEM is given where $n \geqslant 4$. For $n = 3$, standard deviation (SD) is given. Where appropriate, Student's t test was used to assess the difference between mean values. A P value of <0.05 was taken as significant.

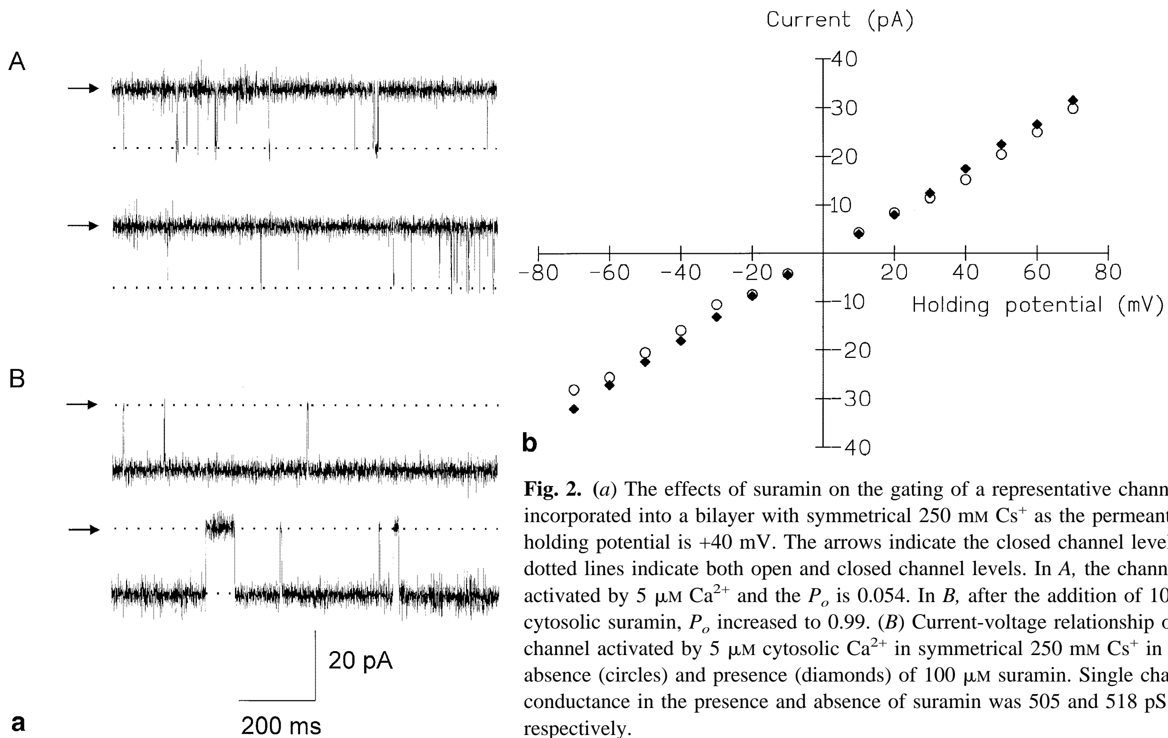

Fig. 2. (*a*) The effects of suramin on the gating of a representative channel incorporated into a bilayer with symmetrical 250 mM Cs^+ as the permeant ion. The holding potential is +40 mV. The arrows indicate the closed channel level; the dotted lines indicate both open and closed channel levels. In *A*, the channel is activated by 5 μM Ca^{2+} and the P_o is 0.054. In *B*, after the addition of 100 μM cytosolic suramin, P_o increased to 0.99. (*B*) Current-voltage relationship of a typical channel activated by 5 μM cytosolic Ca^{2+} in symmetrical 250 mM Cs^+ in the absence (circles) and presence (diamonds) of 100 μM suramin. Single channel conductance in the presence and absence of suramin was 505 and 518 pS, respectively.

Results

The Effects of Suramin with Ca^{2+} as the Permeant Ion

Our previous work suggests that suramin modifies both the gating and the ion handling of sheep cardiac and skeletal RyR [19]. In the presence of micromolar cytosolic Ca^{2+}, suramin can almost fully activate the channels with EC_{50} values of 22.4 and 250 μM for sheep cardiac and rabbit skeletal channels respectively indicating a marked difference in potency for the two isoforms. Suramin also increased the conductance of the channels from approximately 103.4 pS to approximately 124 pS [19]. Both effects were completely reversible even at high concentrations [19]. Figure 1 illustrates the typical effects of suramin on the current amplitude and P_o of a single sheep cardiac RyR incorporated into a planar phospholipid bilayer with Ca^{2+} as the permeant ion. The characteristic long open events and increased current amplitude can be seen clearly.

Effects of Suramin with Cs^+ as the Permeant Ion

Although other investigators have recently shown activation of RyR with DIDS or suramin, no increase in conductance has been reported [14, 23]. It is of interest that these experiments were performed with a monovalent cation as the permeant ion. In contrast, our experiments with suramin [19] were carried out with Tris/HEPES in the cytosolic chamber and Ca^{2+} as the permeant ion. We therefore investigated if changing the ionic conditions could influence the effects of suramin on cardiac RyR function. Figure 2*a* demonstrates that with symmetrical 250 mM Cs^+ as the permeant ion, suramin (100 μM) is very effective at increasing the P_o of the sheep cardiac RyR and can almost fully activate the channel, as occurs with Ca^{2+} as the permeant ion. In contrast, Figure 2*b* demonstrates that, with Cs^+ as the permeant ion, no suramin-induced increase in conductance was observed. Conductance was 467 ± 21 pS (SEM; $n = 4$) in the absence and 487 ± 21 pS (SEM; $n = 4$) in the presence of suramin (100 μM). These experiments demonstrate that the suramin-induced conductance changes are very dependent on the ionic conditions. The effects of DIDS and DBDS on channel function were therefore examined using Ca^{2+} as the permeant ion in order to detect if DIDS and related compounds affect channel function in the same manner as suramin.

The Effects of DIDS

Figure 3 demonstrates that DIDS has three distinct effects on the single channel function of RyR. The first trace (*A*) shows a cardiac RyR channel gating with 10 μM cytosolic Ca^{2+} as the sole activator. In (*B*) 500 μM DIDS has been added to the cytosolic channel side and current amplitude and P_o have increased. Both effects occurred

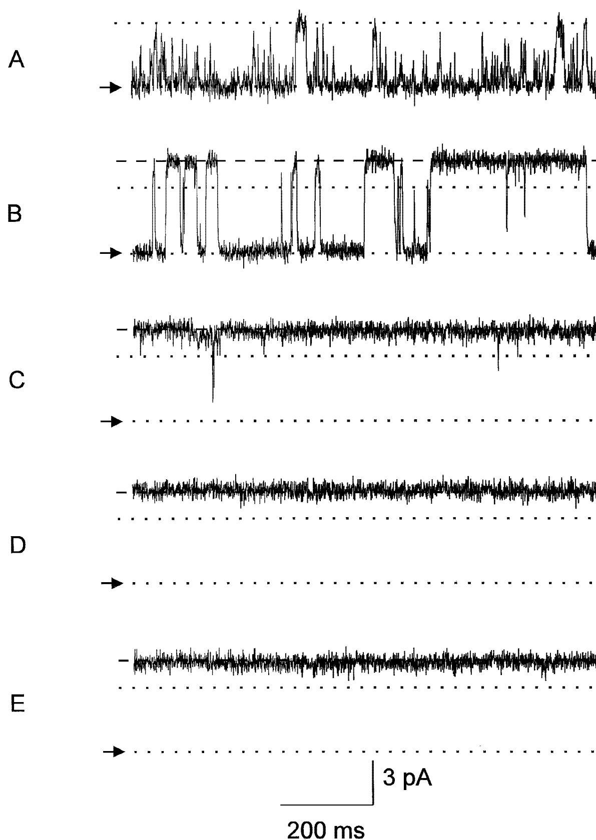

Fig. 3. The reversible and irreversible effects of DIDS on the function of a single representative channel. Ca^{2+} is the permeant ion. The arrows indicate the closed channel level and the dotted lines indicate the closed and open channel levels before addition of DIDS. The dashed line indicates the fully open channel level after addition of DIDS. In *A,* the channel is activated solely by 10 μM cytosolic Ca^{2+} and channel openings are characteristically brief. In *B,* 500 μM DIDS was added to the *cis* chamber and an increase in P_o and current amplitude occurred. *C* demonstrates the last closing events that were observed with this channel and *D* illustrates the channel locked into the fully open state. After 3 min DIDS was removed by perfusion of the *cis* chamber *E,* the channel remained fully open.

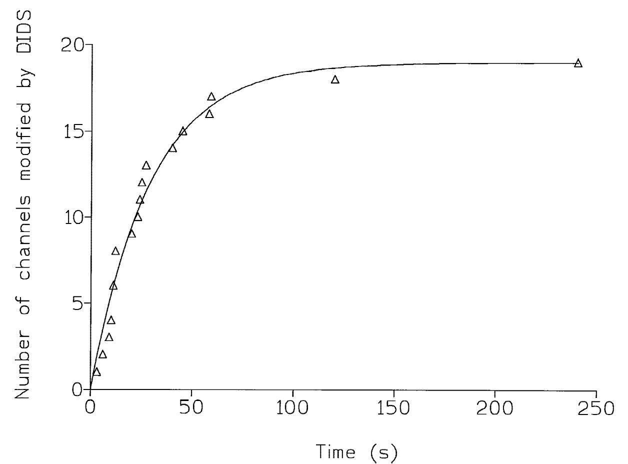

Fig. 4. Time course of the modification of sheep cardiac RyR channels to the fully open level by 500 μM DIDS. The figure shows the time after addition of DIDS to the *cis* chamber at which the channels were irreversibly opened. The time to the last observable closing was taken as the time of modification. The curve was fitted according to the equation $y = 19(1 - \exp)–0.034t))$ where t is the time in sec, 19 is the number of channels and 0.034 is the rate constant. The time for half the channels to be irreversibly opened was 20.2 sec.

immediately and resemble very closely the effects of suramin illustrated in Figure 1. As was observed with suramin, the increase in P_o is characterized by long open events. Current amplitude at 0 mV was increased from 4.37 ± 0.12 pA (SEM; $n = 17$) in the presence of 10 μM cytosolic Ca^{2+} alone to 5.52 ± 0.17 (SEM; $n = 12$) in the presence of DIDS. The third effect of DIDS can be observed in traces *C, D* and *E* of Fig. 3. Trace *C* illustrates the last resolvable closings of the channel after which the channel remained fully open (*D*). DIDS (500 μM) was present in the *cis* chamber for 3 min and was then removed by perfusion after which the channel remained open (*E*). This irreversible effect of DIDS where the channel is locked into the fully open state at a current amplitude equal to that of the DIDS-activated channel, was not observed with suramin. Figure 4 illustrates that the progression to the irreversibly fully opened state occurred quickly after addition of 500 μM DIDS to the *cis* chamber (For 19 channels, the time for half the channels to become modifed ($t_{1/2}$) was 20.2 sec). 26% (7 of 27) of channels were not irreversibly modified by DIDS indicating at least two distinct steps in the effects of DIDS on the P_o of the cardiac RyR. Figure 5 shows a representative channel where DIDS increased P_o and current amplitude but did not irreversibly fully open the channel. Removal of DIDS from the *cis* chamber, after 3 min of recording, reversed the increases in P_o and current amplitude, as occurs with suramin [19]. A lower concentration of DIDS (50 μM) irreversibly opened only 1 of 4 channels after 3 min in the *cis* chamber although the characteristic long open states could still be observed with all the channels. Figure 6 illustrates the gating of a typical channel in the presence of low concentrations of DIDS. P_o increased from 0.012 ± 0.010 ($n = 3$; SD) in the presence of 10 μM Ca^{2+} alone to 0.378 ± 0.322 ($n = 3$; SD) after addition of 50 μM DIDS. The increase in P_o was predominantly caused by an increase in duration of open lifetimes. Mean open time increased from 0.79 ± 0.144 ms to 9.99 ± 9.32 msec ($n = 3$; SD) in the presence of 50 μM DIDS whereas the mean closed times were 109 ± 77 msec before and 148 ± 242 msec ($n = 3$; SD) after addition of DIDS. This mechanism for activation of the cardiac RyR is similar to that of suramin [19]. The progression to the irreversibly activated channel state, predominantly observed with 500 μM DIDS, does not occur

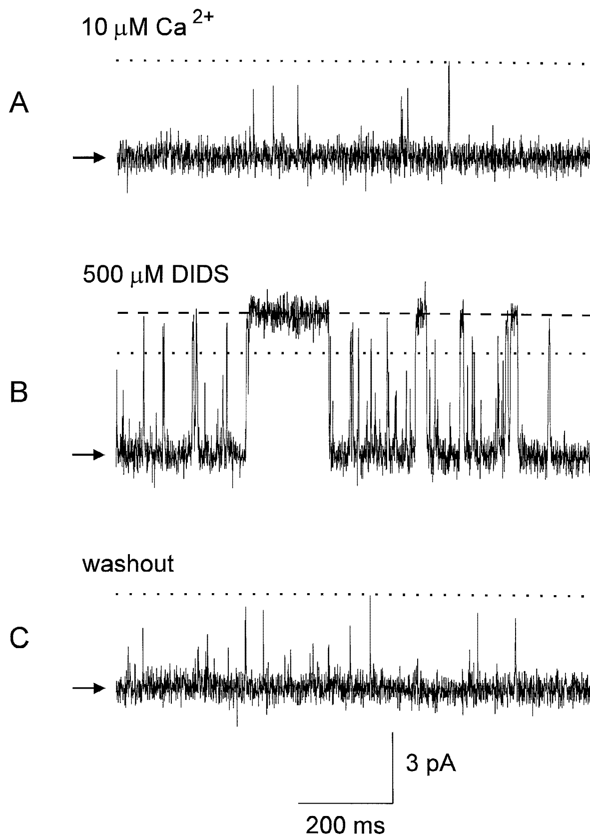

Fig. 5. The reversible effects of DIDS (500 μM) on a representative channel. Ca^{2+} is the permeant ion. The arrows indicate the closed channel level and the dotted lines indicate the open channel level in the absence of DIDS. The dashed line indicates the fully open channel level in the presence of DIDS. In *A* the channel is activated by 10 μM cytosolic Ca^{2+} alone. *B* illustrates the increase in P_o and current amplitude that occurs after the cytosolic addition of DIDS and *C* demonstrates that the effects of DIDS have not proceeded to the irreversibly opened channel state. DIDS was left for 3 min in the *cis* chamber and then perfused away to bring current amplitude and P_o back to control levels.

with suramin and it is possible that this is the result of the covalent binding ability of the 4,4′-NCS groups in DIDS. The effects of DBDS on RyR channel function were therefore investigated. DBDS has the 4,4′-NCS groups replaced with $NHCOC_6H_5$.

The Effects of DBDS

Figure 7 shows the typical effect of DBDS on channel function. DBDS increased P_o in a concentration-dependent manner from 0.006 ± 0.009 (n = 5, SD) in the presence of 10 μM Ca^{2+} alone to 0.078 ± 0.105 (n = 5; SD), 0.520 ± 0.288 (n = 3; SD) and 0.759 ± 0.389 (n = 3; SD) in the presence of 10 μM, 100 μM and 1 mM DBDS, respectively. As observed with suramin and DIDS, DBDS also increased current amplitude. Current amplitude at 0 mV was 4.37 ± 0.12 pA (SEM; n = 17) in the presence of 10 μM cytosolic Ca^{2+} alone and 5.16 ± 0.93 pA (SEM; n = 5) in the presence of DBDS. Figure 8 illustrates the effects of DIDS and DBDS on current amplitude over a range of holding potentials and shows that both compounds are effective at increasing single channel conductance.

The conductance of the cardiac RyR activated by 10 μM Ca^{2+} alone was 105.8 ± 2.5 pS (SEM; n = 5) and was 118.8 ± 4.13 pS (SEM; n = 4) and 125.5 ± 1.66 pS (SEM; n = 4) in the presence of 500 μM DBDS and DIDS respectively. Although DIDS appeared to cause a greater increase in conductance than DBDS, the effects of the two agents were not significantly different from each other. Unlike in the presence of DIDS, irreversible modification to the fully open state was never observed (n = 18) even in concentrations up to 2 mM (n = 3). After washing out DBDS from the *cis* chamber, current amplitude and P_o always returned to control values (n = 7).

As observed with suramin [19] and DIDS, DBDS increased P_o primarily by increasing the duration of open lifetimes. For example, the mean open and closed lifetime durations in the presence of 10 μM Ca^{2+} alone were 0.75 ± 0.18 and 647 ± 559 msec (n = 3; SD) respectively compared to 16.74 ± 6.82 and 65.32 ± 73.22 msec (n = 3; SD), respectively after addition of 100 μM DBDS. Figure 9 illustrates the effect of DBDS on the open and closed lifetime durations of a typical channel. The figure demonstrates that DBDS increases both the frequency and duration of open lifetimes but that the main cause of the increase in P_o is an increase the duration of the open lifetimes. DBDS causes an increase in the duration of the two brief open states that are observed in the presence of Ca^{2+} alone but in addition a third long open state occurs. The Table compares the effects of DBDS and DIDS on open and closed lifetime parameters and illustrates the similarities in the activation mechanism of these two agents. This mechanism for channel activation is the same as that for suramin [19] and leads to the characteristic long open events seen with all three agents.

The similarities in the activation mechanisms of suramin, DIDS and DBDS indicate that all three agents may bind to common sites on RyR. The effects of a submaximal concentration of suramin in the presence of DBDS was therefore investigated to establish if any potentiation occurred. A typical example of this experiment is shown in Fig. 10. In the presence of 10 μM Ca^{2+}, 10 μM DBDS increased P_o to 0.078 ± 0.047 (n = 4; SEM). Subsequent addition of 10 μM suramin increased P_o to 0.118 ± 0.038 (n = 4; SEM). This is no greater than the effect of 10 μM suramin alone which gives a P_o of 0.254 ± 0.026 (n = 6; SEM) demonstrating that DBDS

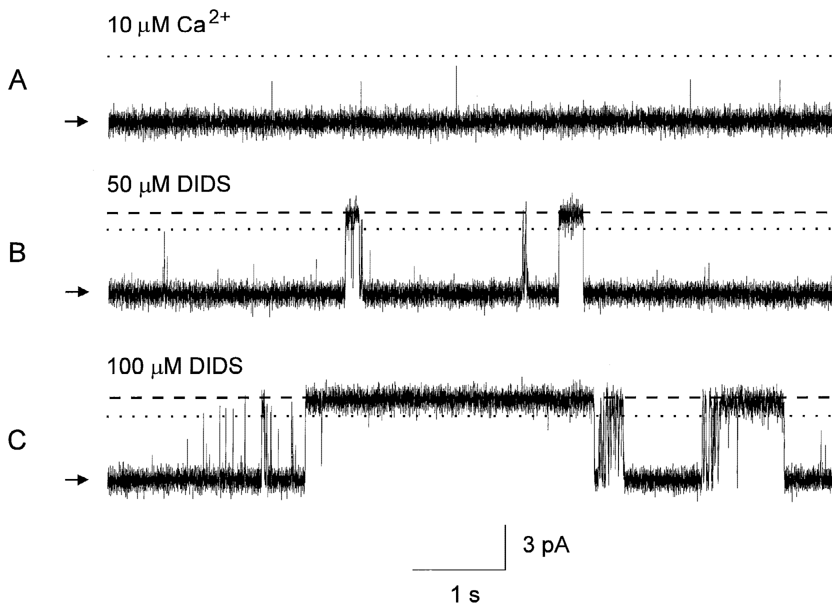

Fig. 6. The effects of low concentrations of DIDS on the gating of a representative cardiac RyR. The arrows indicate the closed channel level and the dotted lines indicate the open channel level in the absence of DIDS. The dashed line indicates the fully open channel level in the presence of DIDS. In *A* the channel was activated by 10 μM cytosolic Ca^{2+} alone (P_o = 0.008). DIDS was then added to the *cis* chamber to give concentrations of 50 μM (P_o = 0.120) *B* and 100 μM (P_o = 0.243) (*C*). Note the different timescale of the traces to allow visualization of the long open and closed events.

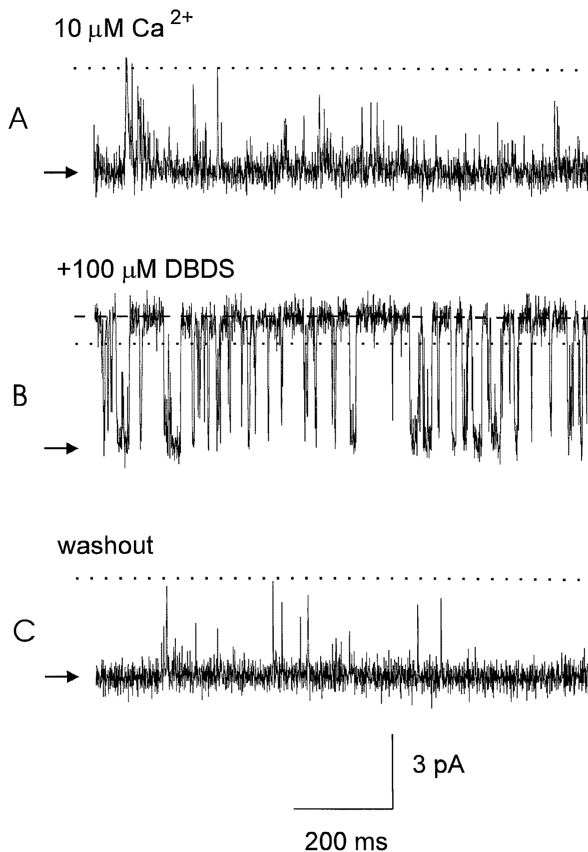

Fig. 7. The effects of DBDS (100 μM) on the current fluctuations through a typical single cardiac RyR. Ca^{2+} is the permeant ion. The arrows indicate the closed channel level and the dotted lines indicate open channel level in the absence of DBDS. The dashed line indicates the fully open channel level in the presence of DBDS. In *A* the channel is activated by 10 μM cytosolic Ca^{2+} alone. In *B* 100 μM DBDS results in an increase in P_o and current amplitude which is fully reversible after washout of DBDS from the *cis* chamber (C).

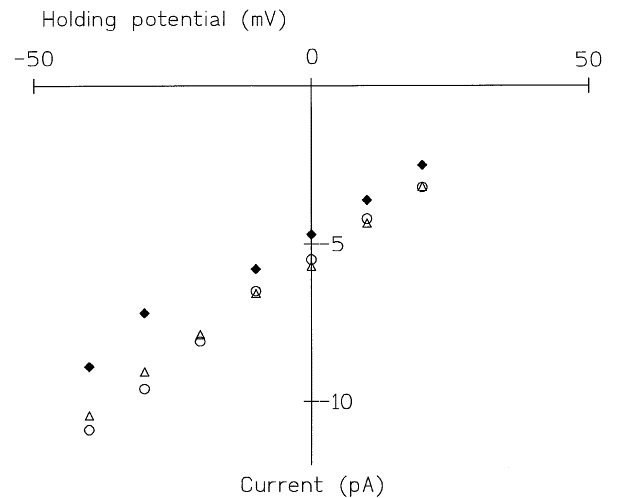

Fig. 8. Current-voltage relationships of typical channels activated by 10 μM cytosolic Ca^{2+} with Ca^{2+} as the permeant ion. The control is shown by the diamonds (104 pS). The circles show the effect of 500 μM DIDS (126 pS) and the triangles show the effect of 500 μM DBDS (121 pS).

does not potentiate the effects of suramin but instead may be competing for the same binding sites as suramin.

Are the Reversible and Irreversible Effects of DIDS Ca^{2+}-Sensitive?

Suramin can increase the conductance and P_o of cardiac RyR in the absence of activating cytosolic [Ca^{2+}] [19] although the opening and closing events are of much longer duration. Figure 11*a* demonstrates how DIDS modifies channel function at subactivating [Ca^{2+}]. At

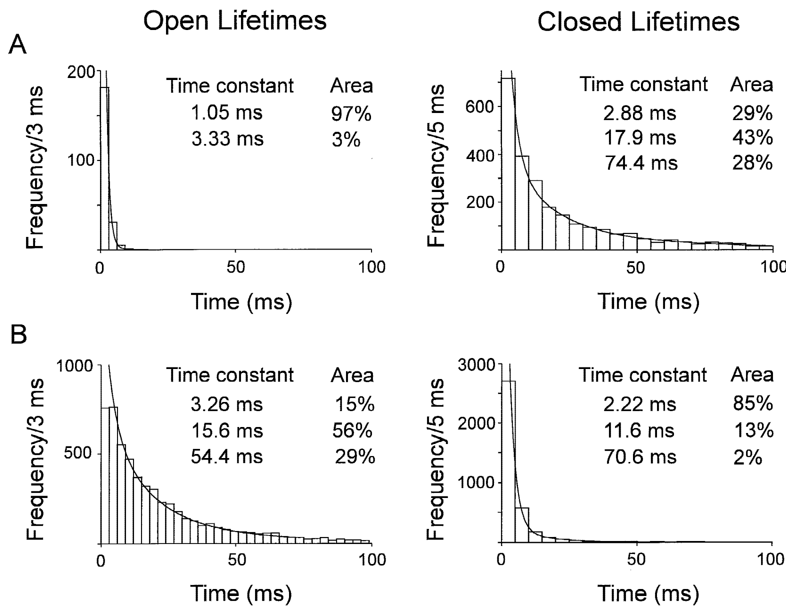

Fig. 9. Demonstrates the effects of DBDS on the open and closed lifetime distributions of a typical channel activated by 10 μM cytosolic Ca^{2+} alone *A* and after activation by DBDS (100 μM) in the presence of 10 μM Ca^{2+} (*B*). The solid lines are the pdfs showing the best fit to the data calculated by the method of maximum likelihood (described in *Materials and Methods*). The time constants and percentage areas are shown for each distribution.

Table. Effects of DBDS and DIDS on open and closed lifetime parameters

	T_1	Area	T_2	Area	T_3	Area
Open lifetime parameters						
10 μM Ca^{2+}	1.6 ± 0.5	95 ± 2	10.7 ± 6.9	5 ± 2		
100 μM DBDS	6.2 ± 6.9	60 ± 19	15 ± 15	31 ± 17	59 ± 70	9 ± 3
50 μM DIDS	2.7 ± 0.7	70 ± 8	21 ± 15	26 ± 8	152 ± 123	4 ± 0.6
Closed lifetime parameters						
10 μM Ca^{2+}	3.5 ± 0.6	27 ± 3	25 ± 6	46 ± 4	97 ± 27	27 ± 6
100 μM DBDS	3.8 ± 1.4	38 ± 30	15 ± 9	28 ± 11	62 ± 37	34 ± 39
50 μM DIDS	2.6 ± 0.6	66 ± 11	20 ± 14	28 ± 7	172 ± 133	6 ± 5

Time constants (T_1, T_2, T_3) and percentage areas were obtained from maximum likelihood fitting of the data as described in Materials and Methods. Each value is the mean ± SD of 3 observations.

picomolar free [Ca^{2+}] the channel is shut and no openings can be resolved. After adding DIDS to the *cis* chamber, very long openings and closings occur (note the different timescale of this figure) similar to the effects observed with suramin under identical conditions [19]. Activation can still proceed to the irreversible fully opened state and trace C shows the last closing event. After washout of DIDS the channel remained fully open.

Can Ryanodine Modify Channel Function after Irreversible Channel Opening by DIDS?

In Figure 11*b,* a single channel was irreversibly opened by 500 μM DIDS and the DIDS was then removed from the *cis* chamber. Addition of ryanodine (1 μM) resulted in an abrupt and irreversible change in current amplitude to 45 ± 3.6% (SD; $n = 3$) of the fully open channel level. This reduction in current amplitude is similar to that usually observed with ryanodine (approximately 40%) under identical ionic conditions [15, 21]. Ryanodine also modified channels activated by DIDS that did not proceed to the irreversible open state (*results not shown*) although this was not surprising as ryanodine can modify the gating of channels activated by suramin [19].

Discussion

Conductance Changes

Suramin (100 μM) was equally effective at increasing the P_o of sheep cardiac RyR channels when Ca^{2+} or Cs^+ was the permeant ion. Under both conditions, the characteristic very long open events were observed (*see* Figs. 1 and 2). With Cs^+ as the permeant ion, however, no increase in conductance was observed. A possible explanation for the results is that suramin causes conformational changes in the channel protein that affect the affinity of the conduction pathway for divalent cations more than that for monovalent cations. The study demonstrates that suramin-induced changes in conductance are very dependent on the ionic conditions of the experi-

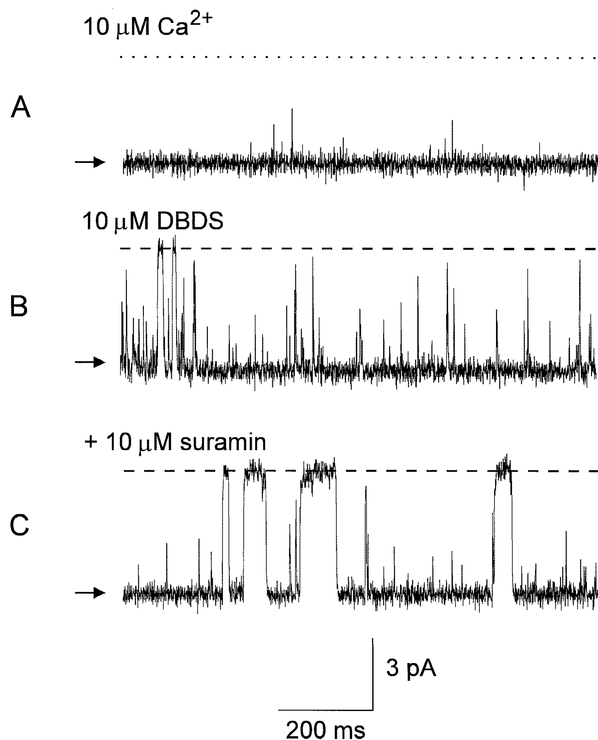

Fig. 10. The effects of suramin on a typical single channel already activated by 10 μM cytosolic Ca^{2+} and 10 μM DBDS. The arrows indicate the closed channel level and the dotted lines indicate the control open channel level. The dashed line indicates the fully open channel level in the presence of DBDS. In *A* the channel is activated by 10 μM cytosolic Ca^{2+} alone (P_o = 0.001). In *B* 10 μM DBDS is added to the *cis* chamber increasing P_o to 0.114 and in *C* 10 μM suramin is added increasing P_o to 0.177.

ments and explains why we observe an increase in conductance with suramin [19], DIDS and DBDS while other studies of the effects of suramin [8] and DIDS [14, 23] in which Cs^+ was the permeant ion, reported no change.

Reversible Effects of DIDS and DBDS

The increases in conductance caused by suramin and DBDS were completely reversible. The increase in conductance observed with DIDS was also reversible if progression to a fully open channel state with no observable closings did not occur. The effect of these ligands on conductance is unique and has not been observed with agents acting at other known binding sites on RyR and therefore, given the structural similarities of suramin, DIDS and DBDS, suggests that they may act via common binding sites. Further evidence that these ligands bind to the same sites is that the reversible effects on gating of suramin, DIDS and DBDS are also unique. The mechanism for the increase in P_o for these agents is

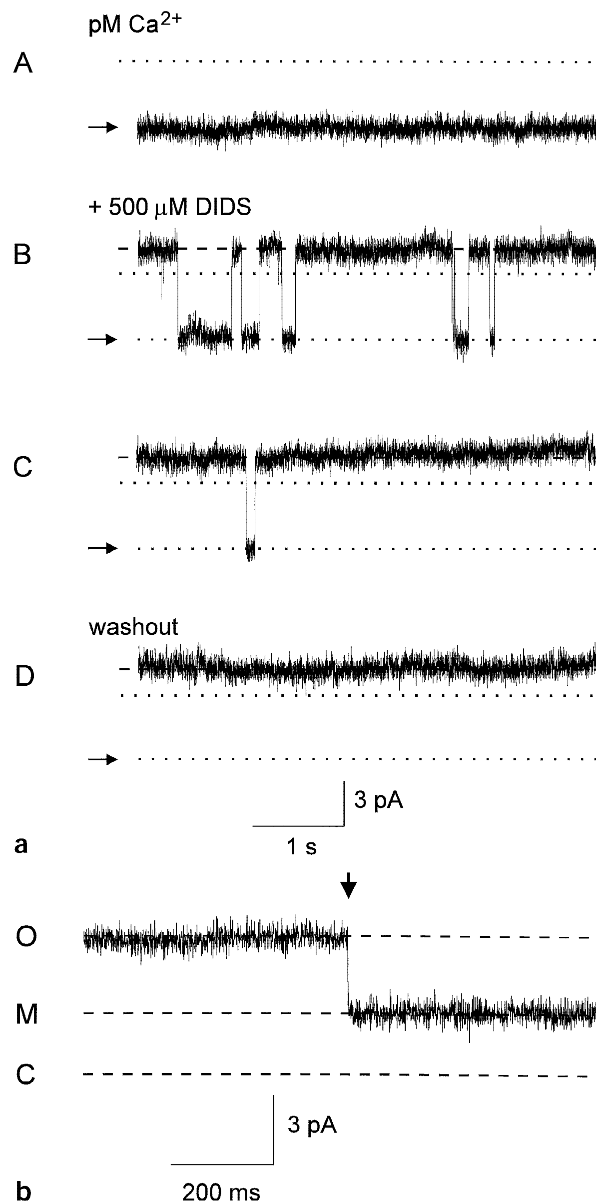

Fig. 11. (*a*) The effects of DIDS in the absence of activating levels of cytosolic Ca^{2+}. The arrows indicate the closed channel level and the dotted lines indicate the open and closed channel levels in the absence of DIDS. The dashed line indicates the fully open channel level in the presence of DIDS. A single representative RyR channel has incorporated into the bilayer but in *A* there are no openings because 12 mM EGTA has been added to the *cis* chamber bringing the free [Ca^{2+}] to approximately 100 pM. In *B* 500 μM DIDS has been added to the *cis* chamber and very long open events (note the time scale) of high current amplitude result. *C* shows the last closing event and *D* demonstrates that the channel is still fully open after removing the DIDS by perfusion of the cis chamber. (*b*) The effects of ryanodine on a single sheep cardiac RyR irreversibly activated by DIDS. 500 μM DIDS was added to the *cis* chamber and after the channel was locked in the open state the DIDS was removed from the channel by perfusing out the *cis* chamber. Ryanodine (1 μM) was then added to the *cis* chamber and the arrow indicates the time at which the fully open channel was modified to a lower conductance state.

predominantly an increase in the duration of the open times. Not only does the increase in duration of open lifetimes contribute more to the increase in P_o than the increase in frequency of channel opening at high ligand concentrations and high P_o values but this is also true for low ligand concentrations where P_o is only slightly increased ([19] and Table). This leads to the very distinctive pattern of gating seen with these agents caused by the long clear open and closed events. The gating is very different to the rapid flickery type of gating which is observed with cytosolic Ca^{2+} as the sole channel activator [1, 18, 20] or, for example, if the channel is activated by an adenine nucleotide in the presence of Ca^{2+} [12]. In fact, lifetime analysis has demonstrated that other secondary ligands, in the presence of 10 μM Ca^{2+}, including adenine nucleotides or caffeine related compounds, all increase the frequency of channel opening with little or no effect on the duration of open lifetimes at low concentrations where P_o is increased to ≤0.3–0.5 [11, 12, 17, 22]. Competition studies with suramin and DBDS provide further evidence for common binding sites. A submaximal concentration of suramin (10 μM) is less effective in the presence than in the absence of 10 μM DBDS indicating that the two ligands are competing for the same sites.

Irreversible Effects of DIDS

An important finding is that DBDS, which differs from DIDS only in that the two reactive isothiocyanate groups have been replaced by $NHCOC_6H_5$, causes similar reversible effects as suramin and DIDS on gating and conductance. It is unlikely therefore that either of these reversible effects of DIDS can be attributed to the reactive effects of the isothiocyanate groups. By a similar argument, the subsequent irreversible locking of the channel into the fully open state several seconds after the addition of DIDS (500 μM) to the *cis* chamber can only be attributed to the isothiocyanate groups, possibly by covalently binding with reactive amino groups of lysine residues on RyR. It is not known whether the irreversible effects of DIDS result from the interaction of DIDS with the suramin binding sites or whether other domains of interaction are involved. The possibility that the irreversible effects of DIDS do result from a covalent interaction with the suramin binding sites is supported by the structural similarities of DIDS and DBDS and by the observation that the irreversibly fully opened state is the same conductance as the DIDS-reversibly-activated channel open state. In addition, irreversible modification to the fully open level is not observed without first observing an increase in mean open time and conductance. It is possible, however, that the irreversible effects of DIDS can only occur when the channel is in the open state and that increasing the P_o might increase the probability of the channel being in a conformation that exposes sites for DIDS to bind irreversibly. Even in the absence of activating cytosolic Ca^{2+}, irreversible modification by DIDS occurs although it is noticeable that DIDS first induces long openings of increased conductance. Irreversible modification of the channel is therefore not likely to depend on a change in the sensitivity of RyR to cytosolic Ca^{2+}, for example due to a change in the affinity of Ca^{2+} for the cytosolic Ca^{2+} activation or inactivation sites. Once the channel has become irreversibly opened, it appears that agents that modify gating become ineffective. For example, after irreverisbly opening a channel by DIDS in the absence of activating cytosolic Ca^{2+} (Fig. 11), perfusing out the *cis* chamber to a solution containing 10 μM Ca^{2+} (*results not shown*) and then lowering the free [Ca^{2+}] again to picomolar levels (Fig. 11*a* trace *D*) does not cause any change in P_o (which remains at 1). Interestingly, ryanodine can still modify the channel to a reduced conductance level and therefore can still bind. It is possible, however, that the binding of ryanodine to its site on RyR may be affected by the irreversible modification of RyR by DIDS, for example the on and/or off rates of ryanodine binding may be altered. A more quantitative evaluation of the effects of DIDS on the interaction of ryanodine with RyR needs to be performed using [^{3}H]ryanodine binding studies or by investigating the effects of a reversible ryanoid on channel function.

In summary, the results of this study suggest that DIDS and DBDS may interact with the sheep cardiac RyR by binding to the suramin binding sites. DIDS and DBDS exhibit reversible increases in conductance and P_o which closely resemble the effects of suramin. In the majority of channels, 500 μM DIDS also irreversibly opens the channels and this may result from DIDS binding irreversibly with positively charged amino acids at or near the suramin sites.

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References

1. Ashley R.H., Williams, A.J. 1990. Divalent cation activation and inhibition of single calcium release channels from sheep cardiac sarcoplasmic reticulum. *J. Gen. Physiol.* **95:**981–1005
2. Blatz, A.L., Magleby, K.L. 1986. A quantitative description of 3 modes of activity of fast chloride channels from rat skeletal muscle. *J. Physiol.* **378:**141–174
3. Cabantchik, Z.I., Greger, R. 1992. Chemical probes for anion transporters of mammalian cell membranes. *Am. J. Physiol.* **262:**C803–C827
4. Colquhoun, D., Sigworth, F.J. 1983. Fitting and statistical analysis of single-channel recording. *In:* Single-channel recording. B. Sakmann and E. Neher, editors pp. 191–263. Plenum, New York & London
5. Connolly, G.P., Harrison, P.J. 1995. Discrimination between UTP-

and P2-purinoceptor-mediated depolarization of rat superior cervical ganglia by 4,4′-diisothiocyanatostilbene-2,2′-disulphonate (DIDS) and uniblue A. *Br. J. Pharmacol.* **115:**427–432

6. Dunn, P.M., Blakeley, A.G.H. 1988. Suramin: a reversible P2-purinoceptor antagonist in the mouse vas deferens. *Br. J. Pharmacol.* **93:**243–245
7. Emmick, J.T., Kwon, S., Bidasee, K.R., Besch, K.T., Besch, H.R., Jr. 1994. Dual effect of suramin on calcium fluxes across sarcoplasmic reticulum vesicle membranes. *J. Pharm. Exptl. Ther.* **269:**717–724
8. Hohenegger, M., Matyash, M., Poussu, K., et al. 1996. Activation of the skeletal muscle ryanodine receptor by suramin and suramin analogs. *Mol. Pharmacol.* **50:**1443–1453
9. Hoyle, C.H.V., Knight, G.E., Burnstock, G. 1990. Suramin antagonizes responses to P2-purinoceptor agonists and purinergic nerve stimulation in the guinea-pig urinary bladder and taenia coli. *Br. J. Pharmacol.* **99:**617–621
10. Kawasaki, T., Kasai, M. 1989. Disulfonic stilbene derivatives open the Ca release channel of sarcoplasmic reticulum. *J. Biochem.* **106:**401–405
11. Kermode, H., Chan, W.M., Williams, A.J., Sitsapesan, R. 1998. Glycolytic pathway intermediates activate cardiac ryanodine receptors. *FEBS Lett.* **431:**59–62
12. Kermode, H., Williams, A.J., Sitsapesan, R. 1998. The interactions of ATP, ADP and inorganic phosphate with the sheep cardiac ryanodine receptor. *Biophys. J.* **74:**1296–1304
13. McGarry, S.J., Williams, A.J. 1994. Adenosine discriminates between the caffeine and adenine nucleotide sites on the sheep cardiac sarcoplasmic reticulum calcium-release channel. *J. Membrane Biol.* **137:**169–177
14. Oba, T., Koshita, M., Van Helden, D.F. 1996. Modulation of frog skeletal muscle Ca^{2+} release channel gating by anion channel blockers. *Am. J. Physiol.* **271:**C819–C824
15. Sitsapesan, R., McGarry, S.J., Williams, A.J. 1994. Cyclic ADP-ribose competes with ATP for the adenine nucleotide binding on the cardiac ryanodine receptor Ca^{2+}-release channel. *Circ. Res.* **75**(3):596–600
16. Sitsapesan, R., Montgomery, R.A.P., MacLeod, K.T., Williams, A.J. 1991. Sheep cardiac sarcoplasmic reticulum calcium release channels: modification of conductance and gating by temperature. *J. Physiol.* **434:**469–488
17. Sitsapesan, R., Williams, A.J. 1990. Mechanisms of caffeine activation of single calcium-release channels of sheep cardiac sarcoplasmic reticulum. *J. Physiol.* **423:**425–439
18. Sitsapesan, R., Williams, A.J. 1994. Gating of the native and purified cardiac SR Ca^{2+}-release channel with monovalent cations as permeant species. *Biophys. J.* **67:**1484–1494
19. Sitsapesan, R., Williams, A.J. 1996. Modification of the conductance and gating properties of ryanodine receptors by suramin. *J. Membrane Biol.* **153:**93–103
20. Smith, J.S., Coronado, R., Meissner, G. 1986. Single channel measurements of the calcium release channel from skeletal muscle sarcoplasmic reticulum. *J. Gen. Physiol.* **88:**573–588
21. Williams, A.J. 1992. Ion conduction and discrimination in the sarcoplasmic reticulum ryanodine receptor/calcium-release channel. *J. Muscle. Res. Cell Mot.* **13:**7–26
22. Williams, A.J., Holmberg, S.R.M. 1990. Sulmazole (AR-L 115BS) activates the sheep cardiac muscle sarcoplasmic reticulum calcium-release channel in the presence and absence of calcium. *J. Membrane Biol.* **115:**167–178
23. Zahradníková, A., Zahradník, I. 1993. Modification of cardiac Ca^{2+} release channel gating by DIDS. *Pfluegers Arch.* **425:**555–557