DIDS Modifies the Conductance, Gating, and Inactivation Mechanisms of the Cardiac Ryanodine Receptor

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ABSTRACT The effects of the covalent modifier of amino groups, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) on the single-channel properties of purified sheep cardiac ryanodine receptors (RyR) incorporated into planar phospholipid bilayers were investigated. DIDS increased single-channel conductance and open probability (P_o) and induced unique modifications to the voltage-dependence of gating. The effects of DIDS on conduction and gating were irreversible within the time scale of the experiments, and both effects were dependent on the permeant ion. DIDS induced a greater increase in conductance with Ca^{2+} (20%) compared with K^+ (8%) as the permeant ion. After modification by DIDS, all channels could be rapidly inactivated in a voltage-dependent manner. The open probability of the DIDS-modified channel decreased with increasing positive or negative transmembrane potentials; however, inactivation was only observed at negative potentials. Our results demonstrate that inactivation of RyR channels is dependent on the ligand activating the channel, and this will have consequences for the control and termination of sarcoplasmic reticulum Ca^{2+} release in cardiac cells.

INTRODUCTION

4,4′-Diisothiocyanostilbene-2,2′-disulfonic acid (DIDS) has been shown by various authors to be a potent activator of ryanodine receptor (RyR) channels (Sitsapesan, 1999; Zahradníková and Zahradník, 1993; Oba et al., 1996; Kawasaki and Kasai, 1989). With Ca^{2+} as the permeant ion, it has also been demonstrated that the DIDS-induced increase in open probability (P_o) is accompanied by a simultaneous increase in single-channel conductance (Sitsapesan, 1999). This property of DIDS is shared by suramin and structurally related ligands, and evidence suggests that DIDS increases P_o and conductance by binding to the suramin receptors on RyR (Sitsapesan and Williams, 1996; Sitsapesan, 1999). Understanding the mechanisms leading to the DIDS-induced changes in RyR function is important, as these ligands have been suggested to interact with sites involved in voltage-dependent channel regulation (Zahradníková and Zahradník, 1993), pH-dependent gating changes (Zahradníková and Zahradník, 1993), and calmodulin binding (Klinger et al., 1999).

Unfortunately, there is much confusion over the exact effects that DIDS exerts on RyR function. Although we observed an increase in conductance with this ligand (Sitsapesan, 1999), other investigators reported that DIDS did not alter conductance (Zahradníková and Zahradník, 1993; Oba et al., 1996; Kawasaki and Kasai, 1989). Our experiments were performed with Ca^{2+} as the permeant ion while other studies used a monovalent cation, and therefore the different ionic conditions may explain the divergent results. Some investigators report that the effects of DIDS are irreversible (Kawasaki and Kasai, 1989; Zahradníková and Zahradník, 1993), others that the effects are reversible (Oba et al., 1996). Zahradníková and Zahradník (1993) reported that the effects of DIDS were voltage-dependent, while Oba et al. (1996) reported that they were not. In the present study we have therefore investigated the effects of DIDS on the purified cardiac RyR channel and used K^+ as the permeant ion. The large conductance of K^+ in the channel provides maximum resolution of the single-channel events, thereby optimizing our ability to monitor changes in conductance and gating. The use of symmetrical solutions also allows us to investigate in detail the voltage-dependent effects of DIDS. To distinguish between reversible and irreversible actions of DIDS we have carried out experiments only when a single channel has incorporated into the bilayer, and have perfused away the DIDS after any observed changes to channel function. Finally, the effects of 4,4′-dibenzamidostilbene-2,2′-disulfonic acid (DBDS), a structural analog of DIDS that does not possess the isothiocyanate groups, were investigated to establish whether other ligands without the isothiocyanate groups could also induce similar changes in conduction and voltage-dependence of gating.

Our results demonstrate that the interaction of DIDS with the purified cardiac RyR channel leads to an irreversible increase in P_o and conductance and a marked change in the voltage-dependence of gating. The results provide insight into the basic mechanisms that may be involved in the voltage-dependence of RyR gating and how ligands interacting at the same sites on RyR as DIDS may modify RyR function.

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MATERIALS AND METHODS

DIDS was obtained from CN Biosciences (Beeston, UK) and Sigma-Aldrich (Poole, UK). DBDS was obtained from Molecular Probes (Leiden, The Netherlands). All other chemicals were obtained from Sigma-Aldrich and were best available grade. Solutions were prepared using MilliQ

deionized water (Millipore, Harrow, UK) and filtered through a Millipore membrane filter (0.45 μm pore diameter) before use.

Purification of the sheep cardiac RyR

Heavy sarcoplasmic reticulum (SR) membrane vesicles were prepared from sheep hearts as described by Sitsapesan et al. (1994a). Heavy SR vesicles were solubilized with 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS) and the sheep cardiac RyR was purified as previously described (Lindsay and Williams, 1991).

Planar lipid bilayer experiments

Proteoliposomes containing RyRs were incorporated into planar phospholipid bilayers as previously described (Sitsapesan and Williams, 1994b). Channel incorporation occurred 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 while the *cis* chamber was held at potentials relative to ground. For experiments where Ca^{2+} was the permeant ion, the *cis* chamber was perfused with 250 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), 125 mM tris(hydroxymethyl)-methylamine (Tris), 10 μM free [Ca^{2+}], pH 7.2, and the *trans* chamber was perfused with 250 mM glutamic acid, 10 mM HEPES, titrated to pH 7.2 with $Ca(OH)_2$ (free [Ca^{2+}], ~50 mM). In all other experiments the *cis* and *trans* chambers were perfused with 210 mM KCl, 20 mM HEPES, 10 μM free [Ca^{2+}], pH 7.2, to give identical *cis/trans* solutions. $CaCl_2$ was used to obtain the required free [Ca^{2+}]. All experiments were performed at 23 ± 1°C. Additions of DIDS or DBDS were made to the *cis* chamber. Stock solutions of DIDS and DBDS were made up in the *cis* (cytosolic) recording solution. The pH and free [Ca^{2+}] of the *cis* recording solutions were measured using a calcium electrode (Orion 93-20) and Ross-type pH electrode (Orion 81- 55) as previously described (Sitsapesan et al., 1991), and were not affected by the DIDS or DBDS.

Data acquisition and analysis

Single-channel data were displayed on an oscilloscope and stored in digital form on Digital Audio Tape (DAT) (Biologic, Intracel, Cambridge). A single-channel event-detection program, Satori (Intracel, Cambridge, UK), was used to analyze the data. Analysis was performed only when a single channel incorporated into the bilayer. Current recordings were filtered at 500 Hz (−3 dB frequency) with an 8-pole Bessel filter and digitized at 2 kHz. Single-channel current amplitudes were measured from the digitized data using manually placed cursors; P_o values and the lifetimes of the open and closed states were determined from ≥3 min of steady-state recording using 50% threshold analysis (Colquhoun and Sigworth, 1983). Voltage-dependent inactivation events were irreversible within the 30 s of the recordings until the holding potential was reversed. We therefore calculated P_o in two ways: 1) including voltage-dependent inactivating events (P_{oI}) and 2) excluding voltage-dependent inactivating events (P_{oE}). Voltage-dependent inactivation events were excluded from the analysis of open and closed dwell times. P_o, mean dwell times, and conductance values are quoted as mean ± SEM where $n \geq 4$. 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.

RESULTS

Effects of DIDS on the purified RyR with Ca^{2+} as the permeant ion

It has previously been shown that DIDS, DBDS, and suramin cause similar changes to gating and conductance in native (incorporated from heavy SR membrane vesicles) sheep cardiac RyR under conditions where Ca^{2+} is the permeant ion (Sitsapesan, 1999). In addition to these effects, DIDS also modifies the channel irreversibly to an open state from which no closing events can be resolved. The conductance of the irreversible open state is equivalent to that of the suramin or DIDS reversibly activated channel. Fig. 1 illustrates the effect of DIDS (500 μM) on a purified sheep cardiac RyR using the same experimental conditions with Ca^{2+} as the permeant ion, and shows that the effects of DIDS on the purified channel are the same as those on the native channel (Sitsapesan, 1999). In essence, DIDS increased the current amplitude of the openings and increased P_o by causing long opening events, as shown in the top right trace of Fig. 1. While the DIDS-activated channels are gating with obvious open and closed events in this manner, the effects of DIDS are fully reversible. Perfusing away the DIDS at this stage brings current amplitude and P_o back to control levels. Subsequent irreversible modification to the DIDS fully open state also occurred (*middle trace*), 30–215 s after addition of DIDS to the *cis* chamber ($n = 3$). After modification, the channel did not close again even after perfusing away the DIDS (*bottom trace*). The effects were irreversible on the time scale of the experiments (up to 30 min of recording).

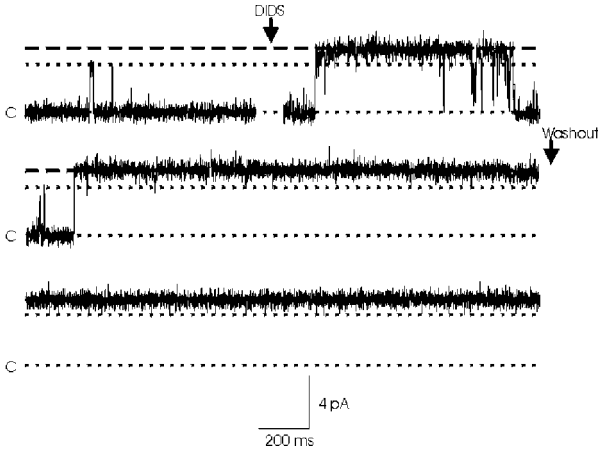


FIGURE 1 The effects of DIDS on a typical single purified RyR with Ca^{2+} as the permeant ion. The holding potential is 0 mV and Ca^{2+} current flows from the luminal to the cytosolic channel side. The closed channel level is indicated by "C," the dotted lines indicate the control closed and fully open levels, and the dashed lines indicate the DIDS-induced fully open channel level. The control P_o was 0.02. The arrows indicate when DIDS was added to the cytosolic chamber and when it was washed away by perfusion of the chamber with cytosolic solution. In the top left-hand trace the channel is activated by 10 μM Ca^{2+}. After addition of DIDS (500 μM) an increase in current amplitude and P_o can be observed. Irreversible modification to the fully open state occurs 30 s after addition of DIDS to the *cis* chamber (*middle trace*) as evidenced by the fully open channel of increased current amplitude even after washout of DIDS (*bottom trace*).

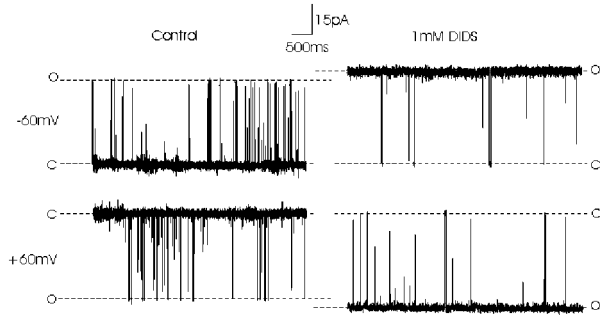


FIGURE 2 Effects of DIDS on a typical single purified cardiac RyR with symmetrical 210 mM K^+ as the permeant ion. Current fluctuations in the absence (*left panel*) and presence (*right panel*) of 1 mM cytosolic DIDS at holding potentials of −60 mV (*top traces*) and +60 mV (*bottom traces*). Dotted lines labeled "O" and "C" indicate the open and closed channel levels, respectively.

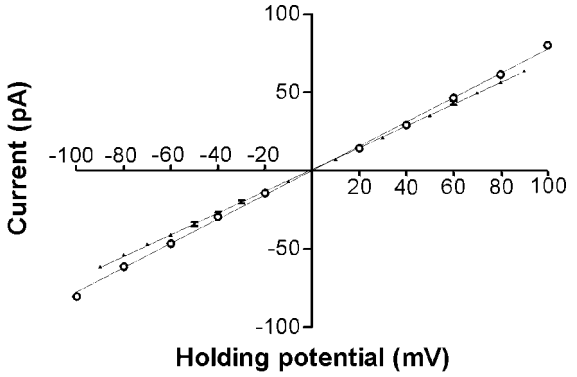


FIGURE 3 Comparison of the current-voltage relationship of the cardiac RyR in symmetrical 210 mM KCl solutions, before (*triangles*; 702 ± 4 pS) and after (*circles*; 760 ± 2 pS) addition of 1 mM cytosolic DIDS. The data points are the mean values from four experiments. Error bars are SEM. Where the error bars are not shown they are within the symbol.

Effects of DIDS on the purified RyR with K^+ as the permeant ion

Satisfied that DIDS could cause the same modifications to conductance and gating of the purified channel as was previously observed with the native channel, we then investigated the effects of DIDS on the purified channel in symmetrical 210 mM K^+ solutions. DIDS (1 mM) was added to the *cis* chamber when the holding potential was 0 mV. After stirring to equilibrate the cytosolic solution, the holding potential was switched to a positive potential and it was observed in all experiments ($n = 29$) that modification to channel function had already occurred. The control P_o values, with 10 μM cytosolic Ca^{2+} as the sole channel activator, were always <0.2 ($n = 9$) at holding potentials between −60 mV and +60 mV. In comparison, after DIDS modification, the channels were almost fully open ($n = 29$) at these holding potentials. Fig. 2 shows a typical recording of a single purified RyR at ±60 mV before and after addition of 1 mM DIDS and clearly shows the large increase in P_o. Unlike the DIDS modification observed with Ca^{2+} as the permeant ion where no closing events were resolved (see Fig. 1), there were brief closing events at all holding potentials monitored. Also apparent was the small but significant increase in current amplitude. Fig. 3 shows the current-voltage relationship of the channels before and after modification by DIDS. Conductance was increased from 702 ± 4 pS to 760 ± 2 pS (SEM; $n = 4$; $p < 0.05$). This amounts to an 8% increase in conductance in comparison to the 20% increase observed with Ca^{2+} as the permeant ion (see Fig. 1) (Sitsapesan, 1999).

The DIDS-modified channel exhibits voltage-dependent gating

Comparison of the gating of the DIDS-modified channel at ±60 mV suggested that there were more closing events at +60 mV than at −60 mV. We therefore compared the voltage-dependence of gating of purified cardiac RyRs activated solely by 10 μM cytosolic Ca^{2+} with that of channels modified by DIDS. The relationship between P_o and holding potential for purified channels activated solely by 10 μM Ca^{2+} is shown in Fig. 4. P_o increased as the transmembrane potential was increased toward either more positive or negative values. P_o was always <0.3 ($n \geq 4$) at holding potentials between +80 mV and −80 mV, increasing from 0.04 ± 0.05 and 0.02 ± 0.02 ($n = 4$; SEM) at +20 mV and −20 mV, respectively, to 0.22 ± 0.24 and 0.21 ± 0.19 ($n = 4$; SEM) at +80 mV and −80 mV, respectively.

Channel modification by DIDS produced a complete change in the voltage-dependence of gating such that the channels were virtually fully open between +20 mV and

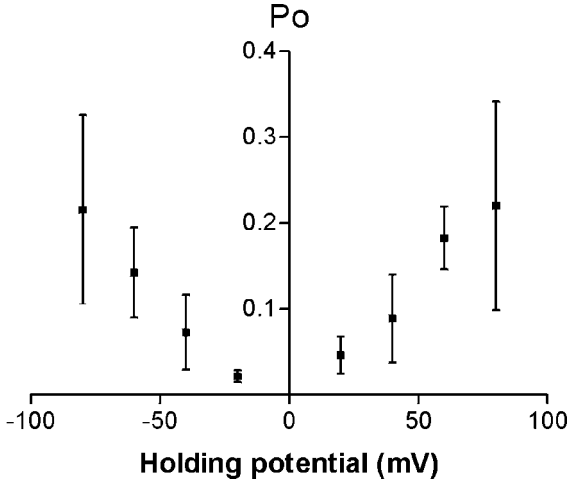


FIGURE 4 The effect of holding potential on the P_o of purified cardiac RyR channels in symmetrical 210 mM K^+ solutions. The only channel activator present is 10 μM cytosolic Ca^{2+}. Error bars are SEM for $n = 4$.

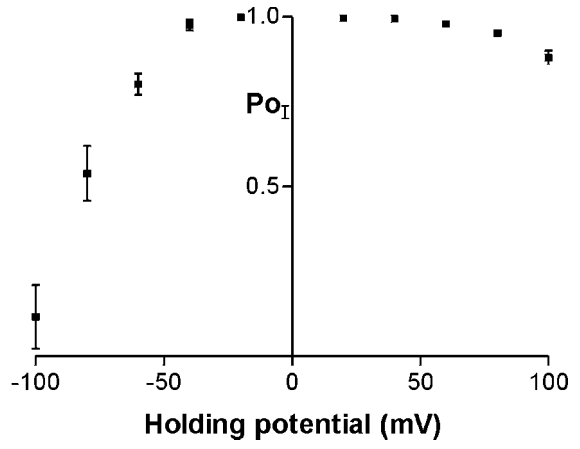


FIGURE 5 The effect of holding potential on the probability that the DIDS-modified RyR channels will be open in the first 30 s following the change in membrane potential (P_{oI}). Different channels were used for the results shown in Figs. 4 and 5. Symmetrical 210 mM K^+ solutions were used. Error bars are SEM for $n \geq 4$.

−20 mV, and open probability was *reduced* as the holding potential became more positive or negative (Fig. 5). In particular, we found that P_{oI} approached zero at very high negative potentials, whereas P_{oI} was reduced much more gradually at positive potentials. The reason for the asymmetry in the P_{oI}-voltage relationship was that the DIDS-modified channel exhibited two main types of closing events: 1) brief closing events at all holding potentials, and 2) closings at negative holding potentials from which the channel only re-opened (during the 30 s of measurement at a negative potential), if the polarity of the holding potential was reversed. We have termed this type of closing voltage-dependent inactivation. To investigate the relative contributions of the inactivation events and the brief closing events to the overall gating of the DIDS-modified channel we also calculated P_o and mean open and closed dwell times before the onset of inactivation. Fig. 6 illustrates the effect of holding potential on the brief closing events (for clarity, examples of inactivation are not included, but are shown in Fig. 9). Fig. 6 shows that as the holding potential becomes increasingly positive (Fig. 6 *A*) or negative (Fig. 6 *B*), the probability of the channel dwelling in the fully open state is reduced. This trend is illustrated in Fig. 7, which shows how (if inactivation events are excluded) P_{oE} decreases slightly as the magnitude of the holding potential is increased (0.953 ± 0.01 and 0.973 ± 0.01 (n = 4; SEM) at +80 and −80 mV, respectively).

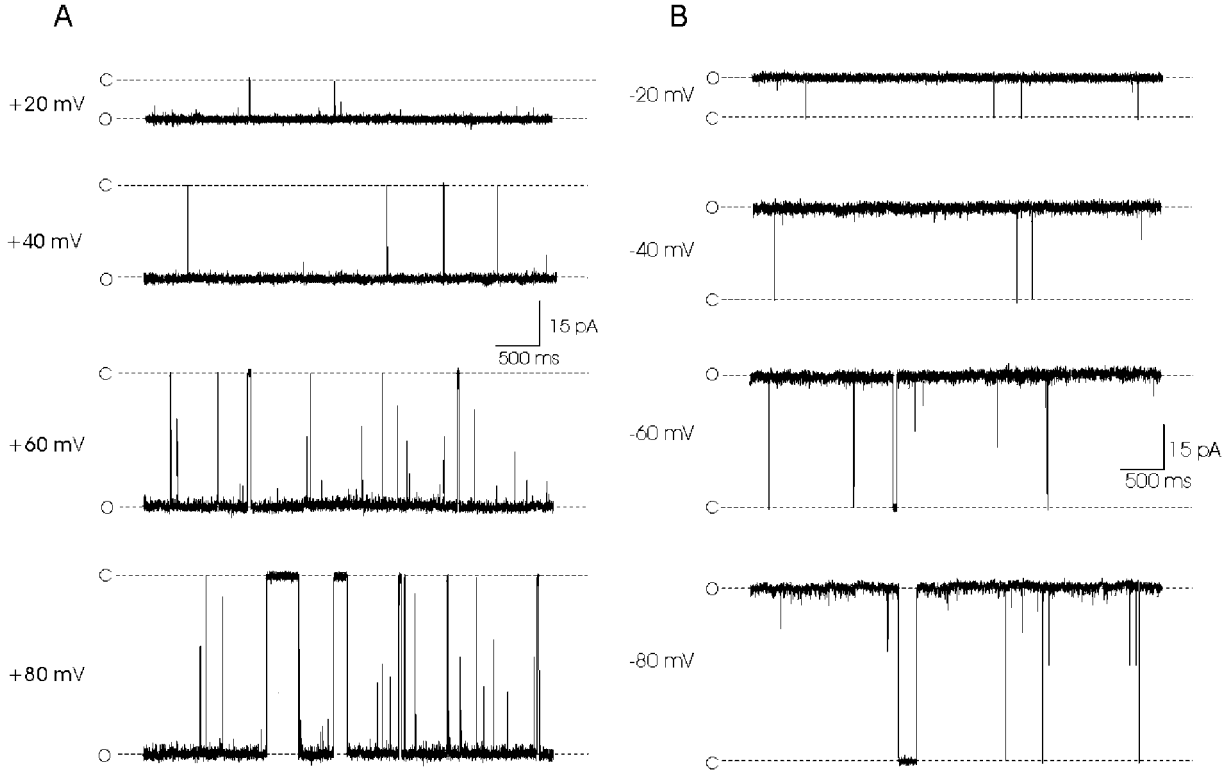


FIGURE 6 Voltage-dependent gating of the DIDS-modified RyR channel in symmetrical 210 mM KCl solutions. (*A*) and (*B*) illustrate current fluctuations through the channel at positive and negative holding potentials, respectively. Dotted lines labeled "O" and "C" indicate the fully open and fully closed levels, respectively. For clarity, examples of voltage-dependent inactivation events are not shown, but are illustrated in a separate figure (Fig. 9).

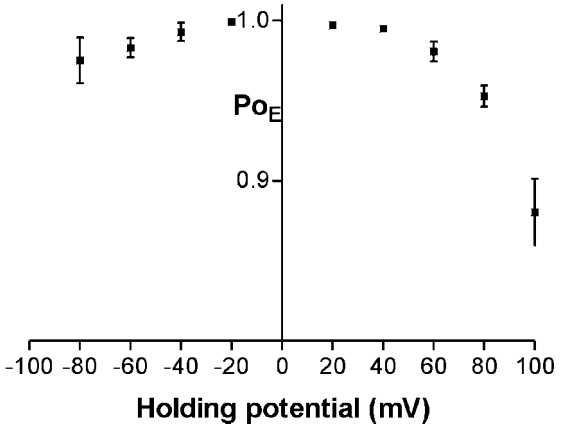


FIGURE 7 Voltage-dependence of the open probability (P_{oE}) of the DIDS-modified channel in the first 30 s following the switch in holding potential when the voltage-dependent inactivation events are excluded from the analysis. The mean values and SEM ($n = 4$) are shown.

Mechanisms underlying the voltage-dependent closings

If the inactivation events are excluded, P_{oE} decreases more with increasing positive holding potential than with increasing negative holding potential. It should be noted, however, that even at +100 mV the DIDS-modified channel still has a very high P_{oE} (0.88 ± 0.02; $n = 4$). There are two factors to be considered in explaining how this dependence on holding potential arises: 1) the frequency of closing events, and 2) the duration of closing events. Fig. 8 *A* shows the voltage-dependence of the first of these factors, the frequency of closing events. The figure shows that between holding potentials of −80 mV and +40 mV, there is little change in the frequency of closings. Increasing positive holding potential above +40 mV causes a sharp increase in the frequency of events from 0.53 ± 0.17 events/s at +40 mV to 4.05 ± 0.69 events/s (SEM; $n = 4$) at +100 mV. A Boltzmann distribution (*solid line*) through the points gives a *z* value of 2.84 for the voltage-dependence of closing event frequency. Fig. 8 *B* illustrates how the second of these factors, duration of closing events, is affected by holding potential. Mean closed event duration increases with both increasing positive and negative holding potentials. However, a greater lengthening of the closed state is observed with increasing negative holding potential (mean event duration was 39.9 ± 14.5 ms at −80 mV compared with 16.9 ± 1.5 ms at +80 mV (SEM; $n = 4$)). The combination of the effects of these two factors leads to the greater decrease in P_{oE} observed at positive holding potentials (Fig. 7) compared with negative holding potentials. As expected, the increased frequency of closing at potentials more positive to +20 mV results from the reduction in the duration of open lifetimes at these potentials, as demonstrated in Fig. 8 *C*.

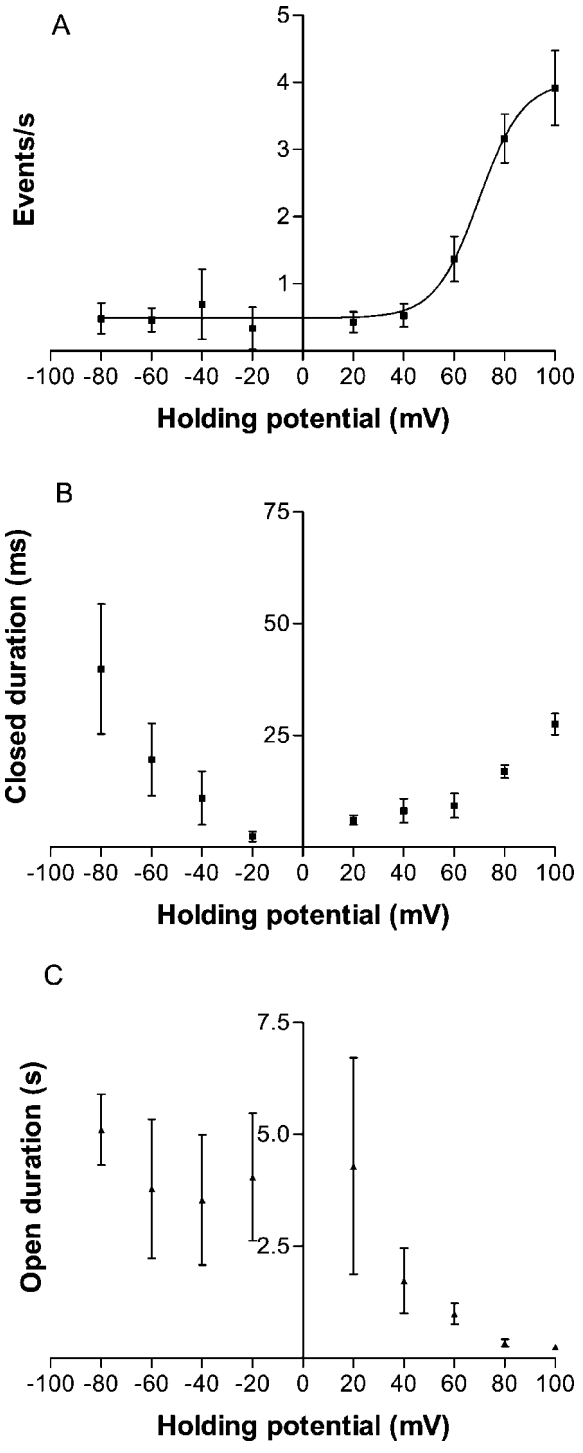


FIGURE 8 Voltage-dependence of the frequency (*A*), mean dwell times of the brief closing events (*B*), and the mean dwell times of the open events (*C*) of DIDS-modified channels. The solid line in (*A*) represents the Boltzmann distribution (fitted using GraphPad Prism (GraphPad Software Inc., San Diego, CA)) according to the equation $y = (A_1 - A_2)/\{1 + \exp[(x - x_0)/dx]\} + A_2$, where A_1 and A_2 are the initial and final values respectively, x_0 is the voltage at which half the maximum effect was observed, and dx is the slope. dx is equivalent to RT/zF, where R is the universal gas constant, T is the temperature in K, F is the Faraday constant and z is the valence of the particle moving across the voltage drop. At 23°C $RT/F = 25.5$; z is therefore $25.6/dx$. This yielded a z value of 2.84. The mean values and SEM ($n = 4$) are shown.

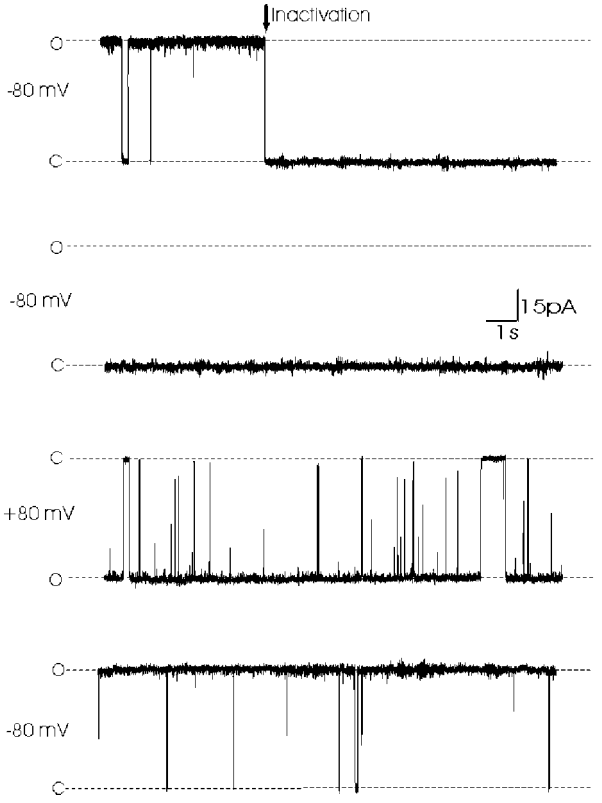


FIGURE 9 A representative example of the voltage-dependent inactivation of DIDS-modified RyR. Dotted lines labeled "O" and "C" indicate the fully open and fully closed levels, respectively. In the top trace the holding potential is −80 mV and brief closings can be observed. The channel inactivates at the arrow and does not open again (*second trace*) until the holding potential is switched to a positive holding potential (*third trace*). At the positive holding potential, the brief closing events are observed but the channels never inactivate. When a negative holding potential is applied again (*bottom trace*), the brief closing events are restored until the channel inactivates again, 20 s after the change in holding potential.

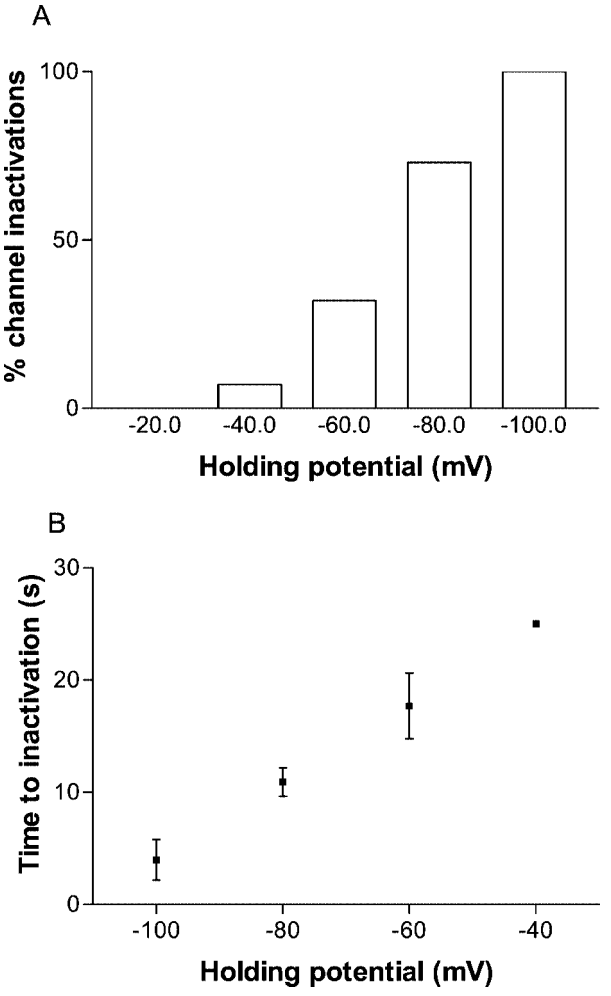


FIGURE 10 Voltage-dependent inactivation of the DIDS-modified channel. The polarity of the holding potential was switched every 30 s. Fourteen to 19 sweeps from four separate channels at each holding potential were obtained. At −20 mV and at more positive potentials, channel inactivation never occurred. (*A*) Percentage of switches (30-s data sweeps) to a negative holding potential that resulted in inactivation. (*B*) Time to the inactivation at −40, −60, −80, and −100 mV of those channels that inactivated during the 30 s following the switch to a negative holding potential.

Voltage-dependent inactivation

In addition to the brief closing events described above we also observed closings to an inactivated state. Inactivation occurred in 100% of the channels ($n = 29$). Inactivation occurred to the fully closed channel level and was only observed at negative holding potentials. Once inactivated (during the 30 s of measurement at a negative potential), channels could only be re-opened by switching to positive holding potentials. Fig. 9 shows a typical inactivation of the channel at −80 mV, and the subsequent re-opening upon switching to +80 mV. The relationship between the negative holding potential and channel inactivation can be seen in Fig. 10. In these experiments, the polarity of the holding potential was reversed every 30 s. Fig. 10 *A* shows the percentage of switches to a negative holding potential resulting in channel inactivation within 30 s. A clear voltage-dependence is evident with only 2 from 28 switches (7%) (8 channels) resulting in inactivation at −40 mV, while at −80 mV, 22 of 30 switches (73%) (8 channels) resulted in channel inactivation. At −100 mV, all channels inactivated with every switch (4 channels). Fig. 10 *B* shows the time to inactivation for the channels that inactivated during the 30-s switch to a negative holding potential. The strong tendency of the channels to inactivate at high negative voltages leads to the overall P_{oI}-voltage relationship shown in Fig. 5, in which the channels are closed after the first few seconds following a switch to a high negative voltages.

Irreversible effects of DIDS

To demonstrate that the effects of DIDS reported here are irreversible, and that all our single channels are irreversibly

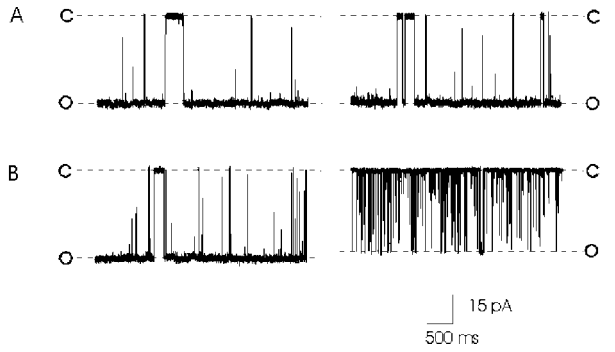


FIGURE 11 Irreversible effects of DIDS and reversible effects of DBDS. (*A*) and (*B*) are different experiments in which a single cardiac RyR channel has incorporated into a bilayer. The left panel illustrates the typical effects of (*A*) 1 mM DIDS and (*B*) 200 μM DBDS at the holding potential of +60 mV in symmetrical 210 mM KCl solutions. The right panel demonstrates the effects of removing the DIDS (*A*) and the DBDS (*B*) from the *cis* chamber by perfusion with the 210 mM KCl recording solution. The *cis* chamber was perfused for a period of 2.5 min, after which current recordings were immediately acquired. For channels activated by DIDS the high P_o, increased current amplitude, and brief closing events remain after perfusion, whereas the gating and conductance of channels activated by DBDS return to the control values observed when the channels are activated by 10 μM Ca^{2+} alone. Dotted lines labeled "O" and "C" indicate the open and closed channel levels, respectively.

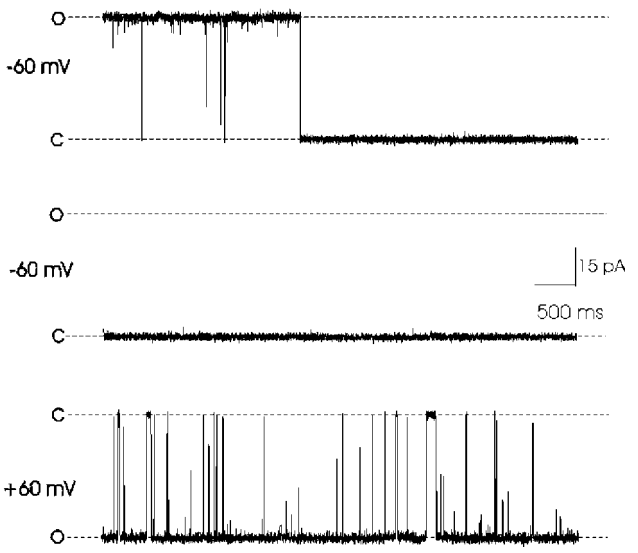


FIGURE 12 The effects of DBDS (200 μM) on the closing events of a representative single RyR held at ±60 mV. Channels activated by DBDS exhibited both the brief closing events and the voltage-dependant inactivation events. Dotted lines labeled "O" and "C" indicate the open and closed channel levels, respectively. In the top trace, at −60 mV, the DBDS-activated channel gates with a high P_o and the channel abruptly inactivates. The channel remains inactivated (*middle trace*) until the polarity of the holding potential is reversed (*bottom trace*).

modified, we removed DIDS from the *cis* chamber by perfusion with the 210 mM K^+ solution. Fig. 11 *A* (*left panel*) shows a typical DIDS-modified channel held at +60 mV. The right panel shows the effect of removing DIDS from the *cis* chamber by perfusion and demonstrates that the increase in P_o was irreversible; P_o remained close to unity. The DIDS-induced increase in conductance was also irreversible. Single-channel current amplitude before (46.7 ± 0.2 pA) and after (47.02 ± 0.2 pA) perfusion of DIDS from the *cis* chamber was not significantly different ($p < 0.05$; SEM; $n = 4$). The effects of DIDS were irreversible in all experiments ($n = 29$).

Reversible effects of DBDS

It has previously been shown that DIDS and DBDS have very similar effects on the gating and conductance of native cardiac RyR when Ca^{2+} is the permeant ion (Sitsapesan, 1999) with the exception that the effects of DBDS are completely reversible. Although it has not been proved unequivocally, the results indicate that DIDS and DBDS may bind to the same sites on RyR (Sitsapesan, 1999). To investigate whether structural analogs of DIDS without the isothiocyanate groups can also produce similar effects on conduction and voltage-dependence of gating when K^+ is the permeant ion, the effects of DBDS were examined. Fig. 11 *B* illustrates that, as for DIDS, DBDS was able to increase both P_o and conductance but, unlike DIDS, the effects of DBDS were completely reversible. The left panel in Fig. 11 *B* shows a channel activated to a high P_o with DBDS (200 μM). After perfusing away the DBDS from the *cis* chamber, both P_o and conductance revert to control levels. The trace shown after DBDS perfusion is not representative of channel P_o, but has been chosen to include a long opening so that the change in current amplitude can be observed. P_o values before and after addition of 200 μM DBDS were 0.086 and 0.95, respectively. Following washout of DBDS, P_o decreased to 0.093. Channels activated by DBDS also exhibited inactivation at negative potentials, but not at positive potentials (Fig. 12), as was observed with DIDS. Following inactivation at negative potentials, switching to a positive potential reversed the inactivation (Fig. 12, *bottom trace*).

DISCUSSION

DIDS-modification of conductance

We have demonstrated that the effects of DIDS on the single channel-conductance of RyR2 are dependent on the permeant ion (compare Figs. 1 and 2). With Ca^{2+} as the permeant ion we see a 20% increase in conductance in comparison with the 8% increase that we observe with K^+. Although we have not investigated the effects of DIDS using Cs^+ as the permeant ion, in a previous paper using Cs^+ we were not able to detect any increase in conductance with suramin. As there is good evidence that suramin and DIDS act at the same sites on RyR (Sitsapesan, 1999) and as suramin and DIDS increase conductance to the same

amount with Ca^{2+} as the permeant ion, we might expect them to have similar effects with monovalent cations as the permeant species. It is therefore clear that while DIDS, suramin, and related agents only cause small or undetectable changes in conductance with monovalent cations as permeant ions, they produce far greater effects when a divalent cation is the permeant species. A small increase in conductance may go undetected, and therefore these results explain why authors using Cs^+ or K^+ as the permeant ion do not observe any increase in conductance after cytosolic addition of DIDS or suramin (Zahradníková and Zahradník, 1993; Oba et al., 1996; Hohenegger et al., 1996; Xu et al., 1998). It is possible that the changes in conduction reflect differential decreases in the affinity of the permeant ions within the conduction pathway or changes to Ca^{2+}/K^{2+} permeability ratio. The conformational changes to the conduction pathway that result when a ligand such as DIDS or suramin binds to RyR may produce changes in ionic selectivity that could lead to alterations in Ca^{2+} flux through the RyR under the physiological ionic conditions of the cell. The DIDS-induced changes in ion conduction in RyR should be investigated in more detail, as it is possible that there are physiological regulators of RyR that exert similar effects on RyR.

DIDS modification of channel gating

When Ca^{2+} is the permeant ion we observe no closing events after irreversible modification (Fig. 1), whereas when K^+ is the permeant ion we observe distinct closing and inactivation events, indicating that the effects of DIDS on channel gating may depend on the permeant ion. We have shown that the P_o of RyR, activated solely by 10 μM cytosolic Ca^{2+}, increases with increasing positive or negative transmembrane potential (Fig. 4). This voltage-dependence, however, is dramatically altered once the DIDS molecule is bound to the channel. The P_o of the DIDS-modified channel is close to unity at voltages between ± 40 mV and we see two types of closing event, namely brief closing events and closings to an inactivated state. Both types of closing are sensitive to changes in holding potential, yet display their own unique voltage dependence. While we only see inactivation of the DIDS-modified channel at negative holding potentials, the frequency of brief closing events is primarily increased as the holding potential becomes more positive. Neither of the voltage-dependent events that we see, the brief closings or closings to the inactivated state, are eliminated upon perfusion of DIDS from the *cis* chamber (Fig. 11). They are, therefore, intrinsic voltage-dependent gating properties of RyR bound to DIDS as opposed to events corresponding to the binding and unbinding of the ligand. The irreversible binding of the DIDS molecule to the channel protein must therefore stabilize conformational states that give rise to the high conductance, high P_o mode and lead to the appearance of a unique voltage-dependence of inactivation not observed with ligands such as ATP or caffeine.

Previous publications give a varied account of the effects of DIDS on the voltage-dependence of RyR channels (Zahradníková and Zahradník, 1993; Oba et al., 1996; Kawasaki and Kasai, 1989). All the effects of DIDS reported in the present study are those that can be observed *after* irreversible DIDS-modification of RyR to a high P_o and increased conductance. We prove this by using only bilayers with a single channel incorporated and by washing away the DIDS. This is not the case with other studies of DIDS in the literature and explains the diversity of the reported effects of DIDS. The results of Oba et al. (1996) correspond very well with the effects of DIDS that we observe *before* irreversible modification, as shown in Fig. 1 (*top right trace*), and described in more detail in a previous publication (Sitsapesan, 1999). At the concentrations of DIDS used by Oba et al. (1996) the on-rate for DIDS modification is low (Sitsapesan, 1999) and the P_o values were low. As Oba et al. (1996) were monitoring the activating effects of DIDS *before* irreversible modification, with multiple channels incorporated and with low P_o values, they may not have observed any significant voltage-dependent channel gating. Zahradníková and Zahradník (1993) and Kawasaki and Kasai (1989) also incorporated multiple channels into the bilayers, but appeared to be monitoring the current fluctuations through a mixture of reversibly and irreversibly modified channels. Although it was not possible to distinguish between reversible and irreversible effects of DIDS in these experiments (because of the multiple channels and because they did not perfuse away the DIDS), it was possible to show some voltage dependence in that P_o was lower at -50 mV than at 0 mV (Zahradníková and Zahradník, 1993). Our results now indicate that the reduced P_o at -50 mV observed by the above authors was likely to have been due to voltage-dependent inactivation of some of the multiple channels in the bilayer.

Voltage-dependent gating

The cardiac RyR channel clearly displays voltage-dependence of gating both before and after modification by DIDS, but a marked change in voltage-dependence is caused by DIDS. In fact, the U-shaped P_o-voltage relationship for the control channels activated solely by 10 μM cytosolic Ca^{2+} has not previously been reported. Our earlier experiments with the native cardiac RyR using Cs^+ as the permeant ion (Sitsapesan and Williams, 1994a) or the purified channel using K^+ as the permeant ion (Sitsapesan and Williams, 1994b) indicated that P_o increased in a roughly linear fashion as the holding potential was increased from -50 to $+50$ mV. By examining a greater range of holding potentials we now demonstrate the U-shaped dependence of P_o on voltage.

Modification of voltage-dependent inactivation by DIDS

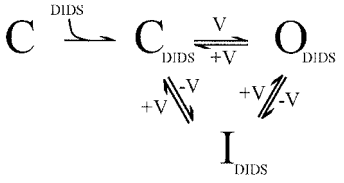
In the present study, when the channels were activated by 10 μM cytosolic Ca^{2+} only, no inactivation was observed. These results are in agreement with our earlier experiments (Sitsapesan et al., 1995a,b; Sitsapesan and Williams, 1994b) indicating the need for a higher [Ca^{2+}] or the presence of a second ligand before inactivation is manifest. After modification of RyR channel function by DIDS, voltage-dependent inactivation was observed at negative voltages only, and the rate of inactivation was increased as the polarity increased. Previous reports of voltage-dependent inactivation of RyR indicated that this was not a property of all RyR, but only a subgroup of channels (Sitsapesan et al., 1995b; Ma, 1995; Laver and Lamb, 1998). Additionally, it was suggested that inactivation was only observed at positive potentials (Sitsapesan et al., 1995b; Chen et al., 1994; Percival et al., 1994), negative potentials (Ma, 1995), or both negative and positive potentials (Laver and Lamb, 1998). However, we find that 100% of the DIDS-modified channels exhibited voltage-dependent inactivation at negative holding potentials ($n = 29$). Importantly, voltage-dependent inactivation was never observed at positive holding potentials ($n = 29$).

It has also been proposed that inactivation of RyR channels is correlated with long open time duration and high P_o values (Sitsapesan et al., 1995b; Laver and Lamb, 1998). However, evidence is emerging to suggest that our original explanation was too simplistic. After DIDS modification, P_o is always >0.8 (consistently higher than in any other investigation of inactivation) with long open times over the entire range of holding potentials. Open times do decrease as the holding potential is increased from +20 mV to +100 mV (see Fig. 8 *C*), but even the shortest open times are of the order of 100-1000 ms compared with the 1–10 ms that Laver and Lamb (1998) indicate may be correlated with inactivation, and yet we still observe no inactivation at positive potentials. A recent study by Bannister et al. (2000) in which cardiac RyR were activated with the caffeine analog EMD 41000 also demonstrates very clearly that long open events and high P_o values alone are not enough to trigger inactivation. In fact, a reduced probability of inactivation was observed under conditions with the highest P_o and the longest open times (Bannister et al., 2000). Examination of Fig. 8 indicates that inactivation is favored when the channel is gating with the longest open states *and* the longest closed states. After DIDS activation, it is possible that the channel must dwell in a particular long open state *or* a particular long closed state (or both) before inactivation can occur. All studies indicate that P_o values above 0.3–0.5 are required before inactivation is observed, but our results suggest that once this "threshold" level has been reached there is no correlation between P_o (or open time duration) and inactivation. Rather than long open times per se being important, it is more likely that the particular ligand activating the channel changes the voltage-dependence of the channel in a characteristic manner and sets the voltage at which the channel will inactivate. For example, structural analogs of DIDS will induce inactivation at negative holding potentials only where ligands that bind to the caffeine sites are more likely to induce inactivation at positive holding potentials (Bannister et al., 2000). We also observed voltage-dependent inactivation with the reversible ligand DBDS (Fig. 12) and, as for DIDS, the inactivation occurred only at negative holding potentials. Thus, the voltage-dependent inactivation reported in this study is likely to be an effect that this class of activator confers on RyR and is not simply the result of covalent modification produced by the isothiocyanate groups.

Laver and Lamb (1998) detected inactivation at both polarities. They suggested that either two different inactivated states could be induced, one at positive potentials and one at negative potentials, or that the sign of the transmembrane potential was unimportant and that dielectric forces (or electrostriction) led to the plugging of the channel in response to the change in the magnitude of the transmembrane potential. Our results with DIDS demonstrate a very distinct inactivation pattern that is observed only at negative holding potentials. The results suggest that voltage-dependent inactivation of DIDS-modified channels, and probably that of RyR channels in general, is not due to electrostriction but to the movement of charged, dipolar amino acid residues of the RyR channel complex by the electric field. DIDS presumably causes a conformational change that alters the movement of the charged particles under the influence of voltage, leading to either direct block of the channel or conformational changes that lead to channel closure. As inactivation in RyR channels activated by caffeine analogs shows a distinctly different voltage dependence (Bannister et al., 2000) this suggests that the conformational changes produced by different ligands allow the movement of different voltage sensors and/or allow different degrees of movement of the same charged particles.

The rate of the inactivation actually increases as the holding potential becomes more negative, although open times remain almost constant. In fact, rather than seeing a correlation between longer open times and inactivation we see closed times increasing as the rate of inactivation increases (Fig. 8). If we consider how closed times change with holding potential (Fig. 8 *B*) the relationship is not completely symmetrical. Closed lifetimes tend to increase more with increases in negative holding potential than with the corresponding increase in positive potential. The increased probability of dwelling in longer closed states may be linked to the inactivation we observe. Unfortunately, we have no evidence to suggest whether inactivation occurs directly from an open state or whether transitions from the open state to another closed state precede inactivation. As applying increasingly negative holding potentials is corre-

lated with an increase in the duration of the brief closing events and an increase in the probability of inactivation, it may be that transitions to the inactivated state occur from a particular long closed state. Lifetime analysis of open and closed dwell times at negative holding potentials would shed light on this issue; however, it is not possible to collect enough events at negative potentials because the channels inactivate too rapidly. In the absence of such information we propose the following simple kinetic scheme that can allow for the possibility that the channel could inactivate from an open or a closed state.



where C, O, and I represent open, closed and inactivated states. The voltage-dependent transitions are indicated by V.

DIDS binding sites

DIDS is a negatively charged, membrane-impermeant compound that modifies RyR function by binding from the cytosolic side of the bilayer. There is, however, question as to whether the molecule binds to the channel protein itself, or to a smaller associated protein of around 30 kDa (Yamaguchi et al., 1995). Our experiments with Ca^{2+} as the permeant ion have demonstrated that native and purified channels are both modified by DIDS in an identical manner, causing irreversible changes to both gating and conductance. As we detected no difference in the effects of DIDS after the purification procedure, we suggest that the most likely explanation is that DIDS acts upon the RyR protein itself to cause the changes we report here. We have evidence to suggest that the effects of DIDS and DBDS occur by binding to suramin sites on the cardiac RyR (Sitsapesan, 1999). There are increasing reports that calmodulin and suramin compete for the same binding sites on skeletal RyR channels (Klinger et al., 1999; Fruen et al., 2002) and therefore it is interesting to speculate that calmodulin's ability to reduce P_o at high cytosolic [Ca^{2+}] may be related to the general ability of ligands acting at this site to change the voltage-dependence of inactivation. Such an effect would be important physiologically as a mechanism that could lead to termination of SR Ca^{2+} release.

CONCLUSIONS

We have described the effects of DIDS on the purified cardiac RyR channel with Ca^{2+} and K^+ as the permeant ions. We have established that DIDS causes multiple effects on the conductance, gating, and the intrinsic voltage-dependence of the channel. The results lead us to conclude that inactivation of RyR channels depends heavily on the ligand activating the channel. We suggest that different ligands cause distinct alterations to the intrinsic voltage-dependence of inactivation of RyR. In addition, our results demonstrate that the effects of DIDS on conduction and gating are dependent on the permeant ion. This may also be true for other ligands that act at other sites on RyR. Therefore, not only may the Ca^{2+}-flux through RyR be altered by the ligands activating the channel, but the ions diffusing through RyR may influence how the various ligands modify gating.

APPENDIX

In a previous report we described a DIDS-induced subconductance state (Hill and Sitsapesan, 2000). However, the results were obtained using a single (new) batch of DIDS from CN Biosciences (Beeston, UK). All subsequent batches from different suppliers (CN Biosciences (Beeston, UK) and Sigma-Aldrich (Poole, UK)) did not induce any subconductance states. We therefore concluded that the subconductance state was caused by contamination of the original batch of DIDS.

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REFERENCES

Bannister, M. L., A. J. Williams, and R. Sitsapesan. 2000. Voltage-dependent gating in sheep cardiac ryanodine receptor channels. *J. Physiol.* 527:83*a* (Abstr.).

Chen, S. R. W., L. Zhang, and D. H. MacLennan. 1994. Asymmetrical blockade of the Ca^{2+} release channel (ryanodine receptor) by 12-kDa FK506 binding protein. *Proc. Natl. Acad. Sci. U.S.A.* 91:11953–11957.

Colquhoun, D., and F. J. Sigworth. 1983. Fitting and statistical analysis of single-channel recording. *In* Single-Channel Recording. B. Sakmann and E. Neher, editors. Plenum Publishing Corp., New York and London. 191–263.

Fruen, B. R., J. M. Bardy, T. M. Byrem, G. M. Strasburg, and C. F. Louis. 2002. Differential Ca^{2+} sensitivity of skeletal and cardiac muscle ryanodine receptors in the presence of calmodulin. *Am. J. Physiol. Cell Physiol.* 279:C724–C733.

Hill, A. P., and R. Sitsapesan. 2000. DIDS increases open probability and conductance and induces a subconductance state in RyR2. *Biophys. J.* 78:191*a* (Abstr.).

Hohenegger, M., M. Matyash, K. Poussu, A. Herrmann-Frank, S. Sarközi, F. Lehmann-Horn, and M. Freissmuth. 1996. Activation of the skeletal muscle ryanodine receptor by suramin and suramin analogs. *Mol. Pharmacol.* 50:1443–1453.

Kawasaki, T., and M. Kasai. 1989. Disulfonic stilbene derivatives open the Ca release channel of sarcoplasmic reticulum. *J. Biochem. (Tokyo).* 106:401–405.

Klinger, M., M. Freissmuth, P. Nickel, M. Stäbler-Schwarzbart, M. Kassack, J. Suko, and M. Hohenegger. 1999. Suramin and suramin analogs activate skeletal muscle ryanodine receptor via a calmodulin binding site. *Mol. Pharmacol.* 55:462–472.

Laver, D. R., and G. D. Lamb. 1998. Inactivation of Ca^{2+} release channels (ryanodine receptors RyR1 and RyR2) with rapid steps in [Ca^{2+}] and voltage. *Biophys. J.* 74:2352–2364.

Lindsay, A. R. G., and A. J. Williams. 1991. Functional characterisation of the ryanodine receptor purified from sheep cardiac muscle sarcoplasmic reticulum. *Biochim. Biophys. Acta.* 1064:89–102.

Ma, J. 1995. Desensitization of the skeletal muscle ryanodine receptor: evidence for heterogeneity of calcium release channels. *Biophys. J.* 68:893–899.

Oba, T., M. Koshita, and D. F. Van Helden. 1996. Modulation of frog skeletal muscle Ca^{2+} release channel gating by anion channel blockers. *Am. J. Physiol. Cell Physiol.* 271:C819–C824.

Percival, A. L., A. J. Williams, J. L. Kenyon, M. M. Grinsell, J. A. Airey, and J. L. Sutko. 1994. Chicken skeletal muscle ryanodine receptor isoforms: ion channel properties. *Biophys. J.* 67:1834–1850.

Sitsapesan, R. 1999. Similarities in the effects of DIDS, DBDS and suramin on cardiac ryanodine receptor function. *J. Membr. Biol.* 168: 159–168.

Sitsapesan, R., R. A. P. Montgomery, K. T. MacLeod, and A. J. Williams. 1991. Sheep cardiac sarcoplasmic reticulum calcium release channels: modification of conductance and gating by temperature. *J. Physiol.* 434:469–488.

Sitsapesan, R., R. A. P. Montgomery, and A. J. Williams. 1995a. A novel method for incorporation of ion channels into a planar phospholipid bilayer which allows solution changes on a millisecond timescale. *Pflugers Arch.* 430:584–589.

Sitsapesan, R., R. A. P. Montgomery, and A. J. Williams. 1995b. New insights into the gating mechanisms of cardiac ryanodine receptors revealed by rapid changes in ligand concentration. *Circ. Res.* 77: 765–772.

Sitsapesan, R., and A. J. Williams. 1994a. Regulation of the gating of the sheep cardiac sarcoplasmic reticulum Ca^{2+}-release channel by luminal Ca^{2+}. *J. Membr. Biol.* 137:215–226.

Sitsapesan, R., and A. J. Williams. 1994b. Gating of the native and purified cardiac SR Ca^{2+}-release channel with monovalent cations as permeant species. *Biophys. J.* 67:1484–1494.

Sitsapesan, R., and A. J. Williams. 1996. Modification of the conductance and gating properties of ryanodine receptors by suramin. *J. Membr. Biol.* 153:93–103.

Xu, L., A. Tripathy, D. A. Pasek, and G. Meissner. 1998. Potential for pharmacology of ryanodine receptor calcium release channels. *Ann. NY. Acad. Sci.* 853:130–148.

Yamaguchi, N., T. Kawasaki, and M. Kasai. 1995. DIDS binding 30-kDa protein regulates the calcium release channel in the sarcoplasmic reticulum. *Biochem. Biophys. Res. Commun.* 210:648–653.

Zahradníková, A., and I. Zahradník. 1993. Modification of cardiac Ca^{2+} release channel gating by DIDS. *Pflugers Arch.* 425:555–557.