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Modification of the Conductance and Gating Properties of Ryanodine Receptors by Suramin

R. Sitsapesan, A.J. Williams

Cardiac Medicine, The National Heart & Lung Institute, Imperial College, Dovehouse Street, London SW3 6LY

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Abstract. Suramin, a polysulfonated napthylurea, increases the open probability and the single-channel conductance of rabbit skeletal and sheep cardiac ryanodine receptor channels. The main mechanism for the increase in P_o is an increase in the duration of open lifetimes. The effects on conduction and gating are completely reversible and involve an interaction with the cytosolic side of the channel. 10 mM dithiothreitol had no effect on the suramin-induced increase in conductance or P_o. Therefore oxidation of sulfhydryl groups on the channels does not appear to be involved. Suramin has been used as an antagonist of ATP at P_2 purinoceptors, however, we find that suramin does not antagonize the effect of ATP at skeletal or cardiac ryanodine receptor channels. The unusual gating kinetics induced by suramin suggest that it does not interact with the adenine nucleotide binding site on the ryanodine receptor but rather binds at a novel site(s). The suramin-induced changes to channel gating and conduction do not prevent the characteristic modification of single-channel properties by micromolar ryanodine.

Key words: Suramin — Ryanodine receptor — Sarcoplasmic reticulum — Cardiac Ca^{2+}-release channel — Skeletal muscle — ATP

Introduction

The sarcoplasmic reticulum (SR) Ca^{2+}-release/ryanodine receptor channel plays a key role in the regulation of muscle contraction. In cardiac muscle the mechanism for excitation-contraction (EC) coupling is Ca^{2+}-induced Ca^{2+}release [4, 5, 10, 23]. Influx of Ca^{2+} through sarcolemmal dihydropyridine (DHP) receptors triggers the opening of ryanodine receptors allowing a much greater flux of Ca^{2+} from the terminal cisternae of the SR to the cytosol. In skeletal muscle, Ca^{2+} does not act as the trigger for SR Ca^{2+}-release, rather the DHP receptors act as voltage sensors which activate the ryanodine receptors [25, 26]. Despite such fundamental differences in trigger mechanisms, the gating of both cardiac and skeletal ryanodine receptors, once activated, depend on other regulatory factors. In addition to changes in local [Ca^{2+}], such factors may include levels of secondary modulatory ligands (for example ATP, Mg^{2+} and calmodulin [20–22, 38, 41]), the phosphorylation state of the channels [11, 12, 15], possible changes in membrane potential across the SR [16, 24, 31] and fluctuations in luminal [Ca^{2+}] [33, 35].

Based on planar lipid bilayer experiments, Ca^{2+} is thought of as 'the primary' ligand for both cardiac and skeletal ryanodine receptors. This is because Ca^{2+} can act as the sole activating ligand whereas many other ligands have either no or very little effect in the absence of cytosolic Ca^{2+}. However, in the absence of secondary ligands, Ca^{2+} itself cannot fully activate the sheep cardiac or the sheep and rabbit skeletal ryanodine receptor channels [3, 35, 38]. Maximum P_o values are usually less than 0.5. ATP is present in muscle cells in high (millimolar) concentrations and such levels of ATP can fully activate ryanodine receptors incorporated into bilayers in the presence of activating levels of cytosolic Ca^{2+}. Therefore, the [ATP] at the cytosolic ryanodine receptor channel face is likely to serve as an important mechanism for amplification of SR Ca^{2+}release by potentiation of Ca^{2+}-induced channel openings.

There are other agents present intracellularly in cardiac and skeletal muscle cells that are also thought to bind to the adenine nucleotide site on the ryanodine receptor channel. These agents include adenosine, ADP, cyclic ADP-ribose (cADPR), βNAD^+ and ADP-ribose [14, 18, 29, 36]. Other structurally related compounds,

Correspondence to: R. Sitsapesan

the effects of which have not been investigated at the single-channel level, have been shown to elicit rapid release of $^{45}Ca^{2+}$ from isolated SR vesicles [20–22]. The levels of many of these agents are thought to change under conditions of increased work, fatigue and ischemia [2], which may result in changes in intracellular Ca^{2+} homeostasis. For example, significant changes in the level of an agent which can bind with high affinity but possesses little efficacy at the adenine nucleotide site would be expected to result in impairment of SR Ca^{2+}release.

The pharmacological profile of the adenine nucleotide binding site on ryanodine receptors is different from that of any cell surface purinoceptor, however quantitative pharmacological classification of the site has been hindered by the lack of a specific antagonist. Suramin is a polysulfonated napthylurea which has been shown to act as a P_2-purinoceptor antagonist [8, 13]. Suramin has been reported to have many varied pharmacological effects [42], but in particular it has been reported to affect Ca^{2+}-uptake and efflux from skeletal muscle SR vesicles [9]. We therefore examined the possibility that suramin may bind to the adenine nucleotide site on the sheep cardiac and rabbit skeletal ryanodine receptors and modulate single-channel gating. Our results demonstrate that suramin invokes novel changes in the gating and conductance properties of cardiac and skeletal ryanodine receptors. Suramin does not appear to exert these effects via the adenine nucleotide site and we propose that suramin acts via a separate and possibly novel binding site. A preliminary report of these results has been published in abstract form [37].

Materials and Methods

Preparation of SR Membrane Vesicles and Planar Lipid Bilayer Methods

SR membrane vesicles were prepared from rabbit skeletal and sheep cardiac muscle as previously described for cardiac SR vesicles by Sitsapesan et al. [30]. Heavy SR membrane vesicles were frozen rapidly in liquid nitrogen and stored at –80°C. Vesicles were fused with planar phosphatidylethanolamine lipid bilayers as previously described [30]. The vesicles fused 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 held at potentials relative to ground. Following fusion, the *cis* chamber was perfused with 250 mM HEPES, 125 mM TRIS, 10 μM free Ca^{2+}, pH 7.4, and the *trans* chamber was perfused with a solution containing 250 mM glutamic acid, 10 mM HEPES, pH 7.4 with $Ca(OH)_2$ (free [Ca^{2+}] approximately 50 mM). The free [Ca^{2+}] and pH of the solutions were determined at 23°C using a calcium electrode (Orion 93–20) and Ross-type pH electrode (Orion 81–55) as described previously [30]. Additions of ATP, dithiothreitol (DTT) and ryanodine were made to the *cis* chamber only. Suramin was added to the *cis* chamber unless stated otherwise. Subnanomolar Ca^{2+} concentrations were obtained by additions of EGTA (12 mM) and the free [Ca^{2+}] calculated using the computer program EQCAL (Biosoft, Cambridge, UK). Suramin was obtained from Calbiochem (Nottingham, UK), ATP from Sigma (Poole, UK), DTT from BDH (Poole, UK) and ryanodine from Agri Systems (Wind Gap, PA).

The mean value ± standard error of the mean (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.

Data Acquisition and Analysis

Single-channel recordings were displayed on an oscilloscope and recorded on Digital Audio Tape (DAT). All steady-state recordings were carried out at 0 mV. Current recordings were filtered at 1 kHz and digitized at 2 kHz. Channel open probability (P_o) and the lifetimes of open and closed events were monitored by 50% threshold analysis. Channel P_o values were obtained from 3 min of steady-state recording. Lifetime analysis was carried out only when a single channel incorporated into the bilayer. Events <1 msec in duration were not fully resolved and were excluded from lifetime analysis. Lifetimes accumulated from approximately 3 min steady-state recordings were stored in sequential files and displayed in noncumulative histograms. Individual lifetimes were fitted to a probability density function (pdf) by the method of maximum likelihood [7] according to the equation: $f(t) = a_1(1/T_1)\exp(-t/T_1) + \ldots\ldots + a_n(1/T_n)\exp(-t/T_n)$ with areas a and time constants T. A missed events correction was applied as described by Colquhoun & Sigworth [7]. A likelihood ratio test [6] 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.

Results

Effects of Suramin on Single-Channel Conductance

Figure 1*a* demonstrates the effect of 100 μM suramin on current amplitude at the holding potential of 0 mV in a representative cardiac channel. This is the potential at which all steady-state current fluctuation measurements were made. Clearly, a 20–25% increase in current amplitude occurs. Figure 1*b* demonstrates the current-voltage relationship of the channel activated by 10 μM cytosolic Ca^{2+} alone and by Ca^{2+} plus 100 μM suramin. The current-voltage relationship of the ryanodine receptor is not linear over the full voltage range under the experimental conditions of this study [40]. Therefore conductance was measured by linear regression only over the range –40 to +20 mV where the relationship is essentially linear. Suramin increased the conductance of the channel from 103.4 ± 3.4 pS (SD; $n = 5$) to 124 ± 4.6 pS (SD; $n = 3$). The extrapolated, apparent reversal potential was not altered (41.1 ± 5.2 mV (SD; $n = 5$) before and 43.6 ± 0.97 mV (SD; $n = 3$) after 100 μM suramin). Qualitatively similar results were observed in the rabbit skeletal channel as can be observed in Figs. 3 and 7.

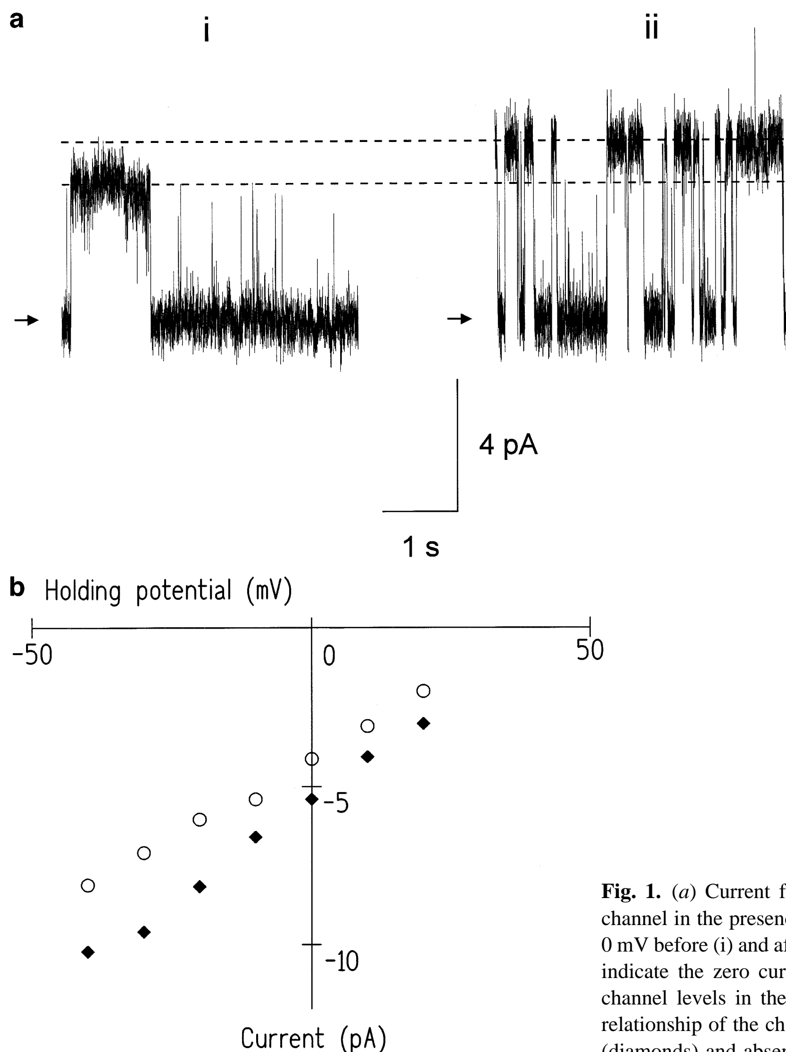

Fig. 1. (*a*) Current fluctuations through a sheep cardiac ryanodine receptor channel in the presence of 10 μM cytosolic Ca^{2+} with the holding potential of 0 mV before (i) and after (ii) addition of 100 μM cytosolic suramin. The arrows indicate the zero current level and the broken lines indicate the fully open channel levels in the presence and absence of suramin. (*b*) Current-voltage relationship of the channel activated by 10 μM cytosolic Ca^{2+} in the presence (diamonds) and absence (circles) of 100 μM cytosolic suramin.

Effects of Suramin on Open Probability

Cytosolic suramin increased the P_o of cardiac and skeletal ryanodine receptors in a concentration dependent manner. In the presence of 10 μM cytosolic Ca^{2+}, suramin increased the P_o of sheep cardiac ryanodine receptors from 0.037 ± 0.018 (SEM; n = 10) to 0.254 ± 0.026 (n = 6) at 10 μM and 0.809 ± 0.040 (n = 5) at 100 μM. At 1 mM suramin P_o was 0.996 (n = 3). Figure 2 illustrates the effect of suramin on the gating of sheep cardiac ryanodine receptors. The relationship between suramin concentration and P_o is shown in Fig. 3. The EC_{50} value was 22.4 μM and the Hill slope was 1.39 (n = 7) suggesting that more than one molecule of suramin must bind to the channel for maximal activation.

In contrast, suramin was not as potent at increasing the P_o of rabbit skeletal ryanodine receptor channels. In the presence of 10 μM cytosolic Ca^{2+}, 10 μM and 100 μM suramin increased P_o from 0.011 ± 0.006 (SEM; n = 10) to 0.032 ± 0.019 (n = 4) and 0.285 ± 0.076 (n = 8) respectively. Even 1 mM suramin did not fully activate the rabbit skeletal ryanodine receptor channels (P_o = 0.628, n = 3). The EC_{50} was 250 μM (n = 4). Figure 4 demonstrates the effect of suramin on the skeletal channels. A comparison of Figs. 2 and 4 illustrates one very obvious difference in the mechanism of suramin activation of the cardiac and skeletal channels. In the cardiac channel, the duration of the open lifetimes was very markedly increased. The mean open lifetimes increased from 0.76 ± 0.12 msec in the presence of 10 μM cytosolic Ca^{2+}, to 22.46 ± 11.23 msec (n = 4) after the addition of 10 μM suramin. In the skeletal channel the increase in P_o was not accompanied by such a large measurable increase in the duration of open lifetimes. The mean open lifetime was 0.74 ± 0.13 msec (n = 6) before and 1.35 ± 0.28 msec (n = 4) after the addition of 10 μM suramin. Even 100 μM suramin only increased the mean open lifetime to 3.87 ± 1.31 msec (n = 6).

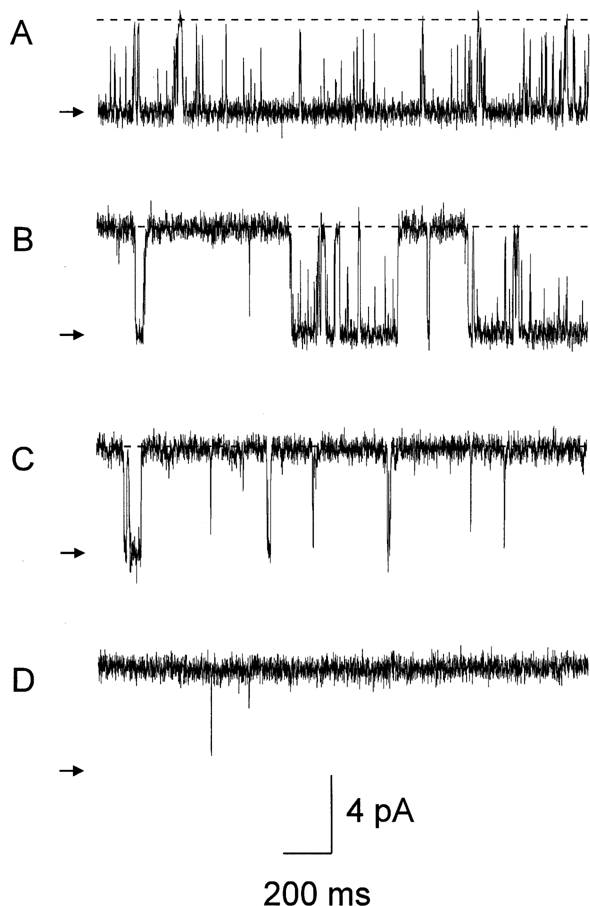

Fig. 2. Activation of a representative single cardiac ryanodine receptor channel by suramin. The arrows indicate the zero current level and the broken lines indicate the fully open channel level. In (*A*) the channel is activated solely by 10 μM Ca^{2+} and very brief openings to the usual conductance level occur. P_o (recorded over 3 min) = 0.048. Subsequent additions of 10 μM (*B*), 100 μM (*C*) and 1 mM suramin (*D*) to the cytosolic (*cis*) channel side resulted in increases in P_o to 0.213, 0.777 and 0.991. Note that the full current amplitude is also increased in the presence of suramin.

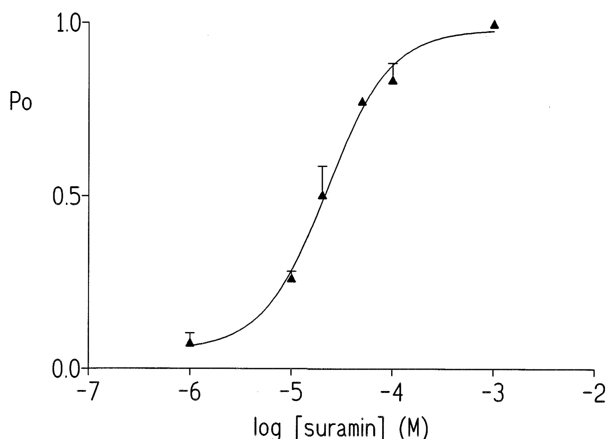

Fig. 3. Effect of cytosolic suramin on the P_o of the sheep cardiac channel. The experiments were carried out in the presence of 10 μM cytosolic Ca^{2+}. The data points shown are the mean of 3–7 observations. Standard error bars are shown where $n > 3$. The EC_{50} value and Hill coefficient were 22.4 μM and 1.39 respectively.

Figure 5*a* demonstrates the changes in mean open and closed lifetimes that underly the suramin-induced increase in P_o in a typical cardiac ryanodine receptor channel. Clearly the increase in P_o results predominantly from an increase in the open lifetime duration. Very little decrease in closed lifetime duration appears to occur until high P_o values (>0.75) are reached. Figure 5*b* illustrates the open and closed lifetime distributions from the same channel before and after 50 μM suramin. In the presence of 10 μM cytosolic Ca^{2+}, the best fits to the open and closed lifetime distributions are given by two and three exponentials respectively indicating at least two open and three closed states. The open lifetimes are very brief with more than 90% of events occurring to the shortest time constant. After adding 50 μM suramin to the cytosolic channel face the P_o was elevated from 0.119 to 0.877. The open lifetime distribution was distinctly altered with an increased number of very long events. Only 4% of the events occurred to the shortest time constant and a third open state could be detected with a time constant of 100 msec. In comparison, suramin has very little effect on closed lifetime durations.

Reversibility and Effects of DTT

The effect of suramin was completely reversible on both skeletal and cardiac channels. This is demonstrated for the cardiac channel in Fig. 6.

Abramson et al. [1] have shown that certain polysulfonated dyes (suramin was not examined) cause Ca^{2+}-release from skeletal SR vesicles; an effect which can be abolished by the presence of DTT. Thus it was suggested that the mechanism for the dye-induced Ca^{2+}-release was oxidation of sulfhydryl groups on the ryanodine receptor channels. To determine whether the suramin-induced increase in P_o or single-channel conductance could be explained by oxidation of sulfhydryl groups, 10 mM DTT was added to the cytosolic channel side 5 min before addition of suramin. No significant effect of DTT was observed. After pretreatment of the channel with 10 mM cytosolic DTT, 10 μM suramin increased P_o to 0.252 ± 0.081 (SD;$n = 3$) in comparison with a P_o of 0.256 ± 0.059 (SD; $n = 3$) in the absence of DTT. The effect of suramin in the presence of DTT is shown in Fig. 7. Note that both current amplitude and P_o are increased.

Effects of Luminal Suramin

No change in single-channel conductance was observed with luminal applications of suramin (100 μM). How-

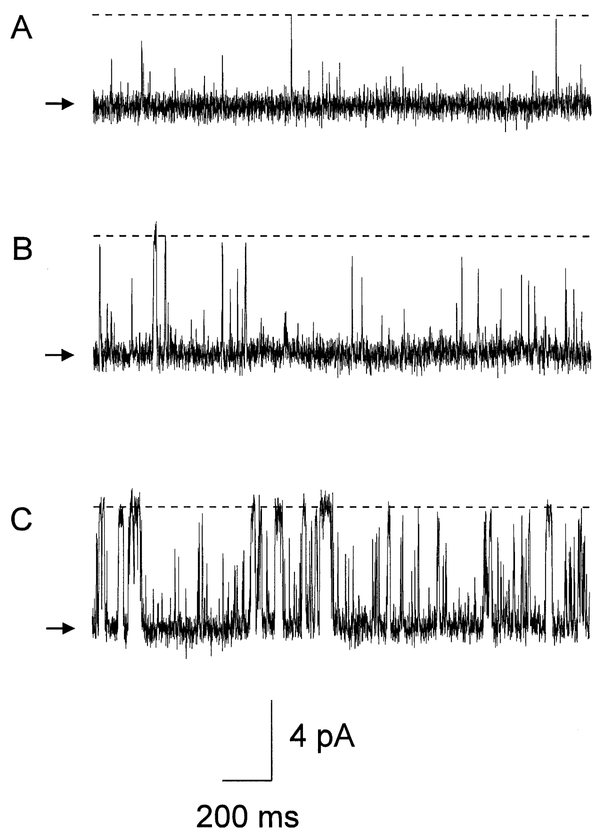

Fig. 4. Activation of a typical single skeletal ryanodine receptor channel by suramin. The arrows indicate the zero current level and the broken lines indicate the fully open channel level. The single channel current fluctuations resulting from activation by 10 μM cytosolic Ca^{2+} alone are shown in (*A*) (P_o = 0.002). 10 μM and 100 μM suramin were added to the cytosolic (*cis*) channel side in (*B*) (P_o = 0.075) and (*C*) (P_o = 0.113) respectively. As in Fig. 2, note the increase in current amplitude after addition of suramin.

ever, 100 μM suramin at the luminal side of either skeletal or cardiac channels appeared to cause an immediate slight increase in P_o resulting from an increase in the frequency of channel opening. For example in the skeletal channels, P_o was 0.023 ± 0.008 before and 0.062 ± 0.017 (SEM; n = 6) after the addition of 100 μM suramin. However this effect was not statistically significant ($P >$ 0.05). No change in open lifetime duration occurred (mean open lifetime before and after 100 μM suramin was 0.88 ± 0.153 and 1.09 ± 0.196 msec (SEM; n = 6) respectively for skeletal channels). Similarly, no significant change in P_o or mean open lifetime was observed in cardiac channels after luminal addition of suramin (*data not shown*). Therefore, any trend towards an increase in P_o must result solely from an increase in frequency of channel opening. The effect of luminal suramin did not increase with time and even up to 10 min after addition the large increase in P_o and mean open time characteristic of the cytosolic action of this compound were not observed. Trace *B* in Fig. 8 illustrates the typical gating behavior of the rabbit skeletal channel in the presence of 100 μM luminal suramin. Subsequent addition of 100 μM suramin to the cytosolic channel face resulted in a large increase in P_o and open lifetime duration coupled with an increase in current amplitude (Fig. 8, trace C).

Site of Action of Suramin

Suramin has been used as an antagonist of ATP at cell surface P_2 purinoceptors [8, 13] and therefore the obvious site of action for suramin on ryanodine receptor channels would appear to be the adenine nucleotide binding site. However, the unique gating kinetics induced by suramin suggest that this is probably not the case. Trace A in Fig. 9 demonstrates the typical gating pattern of cardiac ryanodine receptors activated by 100 μM ATP in the presence of 10 μM cytosolic Ca^{2+}. Low concentrations of ATP activate ryanodine receptor channels by causing an increase in the frequency of very short events. The figure clearly demonstrates how these short, and therefore poorly resolved events lead to a flickery appearance of channel gating. However, as shown in Figs. 2, 4 and 5, the primary mechanism for suramin-induced increases in P_o is an increase in the duration of open lifetimes, even at low concentrations of suramin. This is strong evidence that suramin and ATP bind to different sites on the channel. In trace B, Fig. 9, a low concentration of suramin (10 μM) has been added to the cytosolic channel side in the presence of 100 μM ATP. There is no evidence of a decrease in P_o as would be expected if suramin was acting as an antagonist of ATP; in fact, suramin causes a further increase in P_o. The figure shows that both long, resolvable openings and brief short events now occur. After the subsequent increase in [suramin] to 50 μM (trace C), very long openings and brief closings occur as P_o approaches 1. The mean P_o in the presence of 100 μM ATP was 0.189 ± 0.014 (SEM, n = 4) before and 0.544 ± 0.147 after the addition of 10 μM suramin. Thus at these concentrations, the effect of ATP and suramin together appears to be greater than the sum of their individual effects. This is further evidence that suramin and ATP bind to distinct sites on the channel although for accuracy this should really be tested over a wider concentration range. These results suggest that suramin is not an antagonist of ATP at ryanodine receptor channels but is a full agonist at a separate and perhaps novel site.

Suramin Can Activate Ryanodine Receptors at Subactivating [Ca^{2+}]

All the above experiments have been carried out in the presence of 10 μM cytosolic-free Ca^{2+}. In the absence of activating levels of Ca^{2+} (picomolar), suramin can still activate both skeletal and cardiac ryanodine receptor

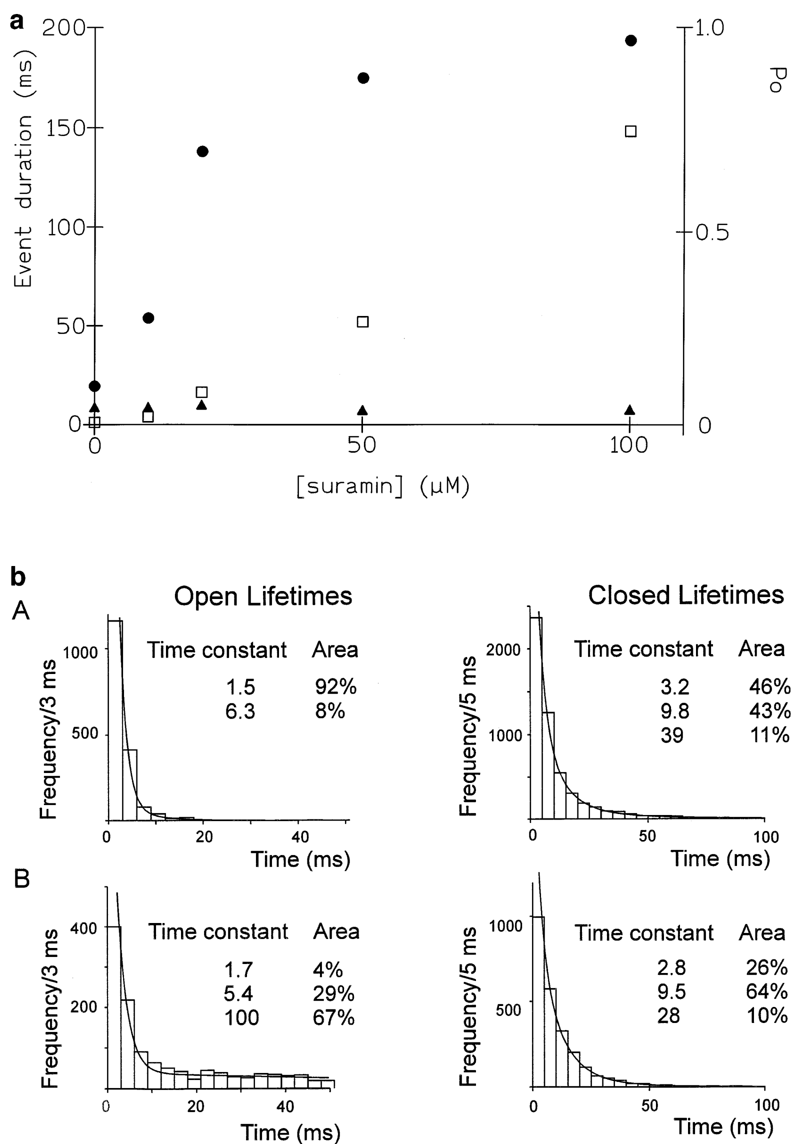

Fig. 5. (*a*) The effect of suramin on mean open lifetimes (squares), mean closed lifetimes (triangles), and P_o (circles) from a representative cardiac ryanodine receptor channel. (*b*) The open and closed lifetime distributions and probability density functions (pdf) from the same channel activated by 10 μM cytosolic Ca^{2+} alone (*A*) (P_o = 0.119) and after addition of 50 μM cytosolic suramin (*B*) (P_o = 0.877). The best fit to each lifetime distribution was obtained by the method of maximum likelihood (described in Materials and Methods) and the resulting time constants and percentage areas are shown in the figure.

channels however the open and closed events become exceptionally long. For both skeletal and cardiac channels the increase in conductance was unchanged by reducing the free cytosolic [Ca^{2+}]. Figure 10 shows a representative cardiac channel. Note the change in the time scale of the figure necessary to illustrate the durations of the events. Whereas in the presence of Ca^{2+}, suramin caused openings in the order of tens of milliseconds in duration, at subactivating [Ca^{2+}], suramin elicited openings in the order of seconds. Since often only two or three openings a minute were observed, recordings of more than an hour are required to obtain sufficient events for lifetime analysis. Thus suramin can activate ryanodine receptor channels by at least two mechanisms; a Ca^{2+}-dependent and a Ca^{2+}-independent action.

Ryanodine Modification of the Suramin-Activated Channel

As suramin appeared to completely alter the gating of ryanodine receptor channels and also produced an increase in single-channel conductance it seemed possible that suramin might be activating a channel other than the ryanodine receptor or that ryanodine-induced modification of conductance and gating might be altered. Figure 11 demonstrates that suramin-activated channels are ryanodine sensitive and are modified by ryanodine to a long-duration open state at approximately 40% of the full conductance. This is similar to the percentage reduction in conductance observed in channels modified by ryanodine in the absence of suramin [44].

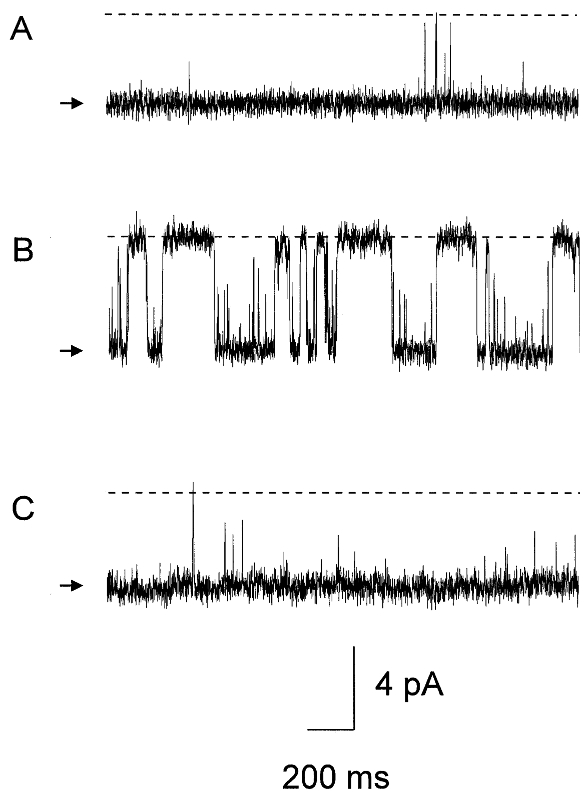

Fig. 6. Reversibility of suramin. In (*A*) a cardiac ryanodine receptor channel is activated by 10 μM cytosolic Ca^{2+}. The broken line indicates the open channel level and the arrow indicates the closed channel level. In (*B*), the channel has been further activated by the addition of 10 μM cytosolic suramin. In (*C*) the *cis* chamber has been perfused to remove the suramin and P_o and current amplitude have returned to control levels.

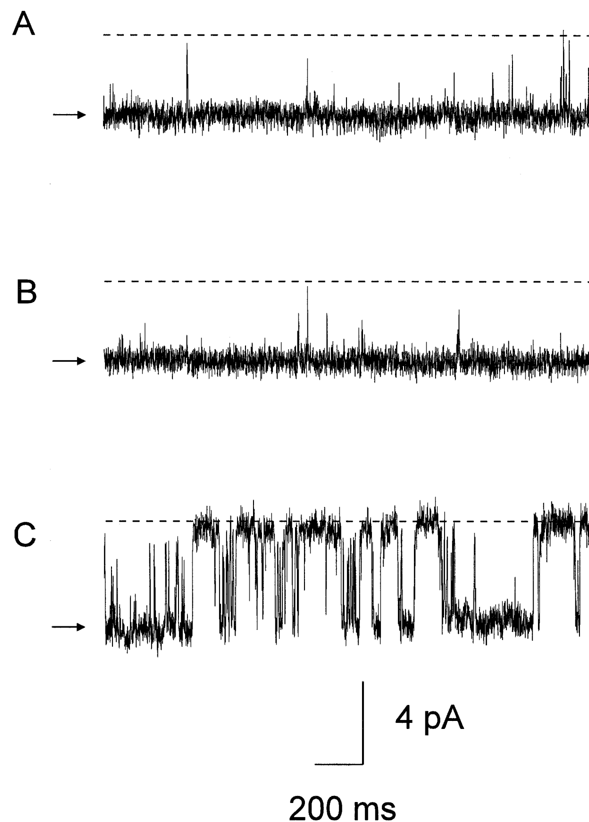

Fig. 7. Effects of suramin on the cardiac ryanodine receptor channel in the presence of DTT. The broken lines indicate the fully open channel level; the arrows indicate the zero current level. In (*A*) a representative channel has incorporated into the bilayer and is activated solely by 10 μM cytosolic Ca^{2+}. In (*B*) 10 mM DTT has been added to the *cis* chamber. Subsequent addition of suramin (10 mM) results in the usual increases in open lifetime duration and current amplitude (*C*).

Discussion

We have demonstrated that cytosolic additions of suramin increase both the P_o and the single-channel conductance of rabbit skeletal and sheep cardiac ryanodine receptor channels. The effect on P_o is concentration dependent but it is not yet clear if the same is true of the effect on conduction. This would require improved resolution of the single-channel events. The effects of suramin are completely reversible and do not appear to result from oxidation of sulfhydryl groups on the channels. Protection of sulfhydryl groups with high levels of DTT (10 mM) in the cytosolic solution does not prevent the suramin-induced increase in conductance or P_o. This observation is in agreement with previous work examining the effects of suramin on efflux of Ca^{2+} from rabbit skeletal SR vesicles [9].

In the cardiac channel, in the presence of activating cytosolic Ca^{2+}, suramin increases the frequency of channel openings although the predominant mechanism for the increase in P_o is an increase in the duration of open lifetimes. To cause an increase in the frequency of channel opening, suramin must bind to the closed conformation of the channel. However, increasing the suramin concentration increases the duration of the open lifetimes and therefore suramin must also bind to the open channel state(s) to prolong channel opening. In comparison, the effect of cytosolic Ca^{2+} alone is to increase the frequency of channel opening without a significant change in the duration of the open events [3, 34, 35]. P_o values greater than 0.5 are rarely achieved when Ca^{2+} is the sole ligand. It is clear that the P_o in the presence of Ca^{2+} and suramin together is greater than the sum of the effects of Ca^{2+} alone and suramin alone. Therefore, suramin and Ca^{2+} must act synergistically to produce a second, different gating scheme whereby long open events occur and the channels can be fully opened.

In the absence of activating cytosolic Ca^{2+} suramin can still induce channel openings. However, the open and closed events under these conditions are at least

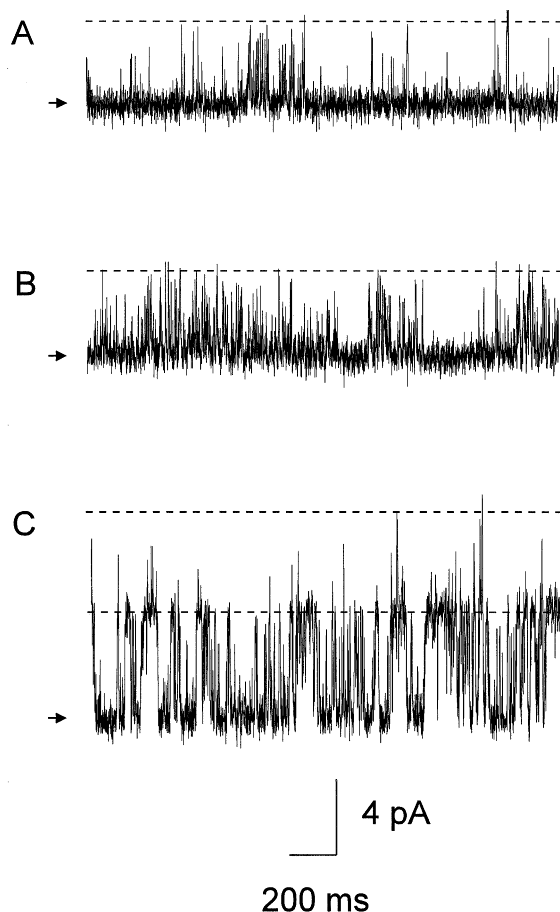

Fig. 8. The effects of luminal addition of suramin. In (*A*) two skeletal ryanodine receptor channels have incorporated into the bilayer and are activated solely by 10 μM cytosolic Ca^{2+}. The broken lines indicate the open channel levels and the arrows indicate the zero current level. In (*B*), 100 μM suramin was added to the luminal (*trans*) side of the bilayer. There appears to be a slight increase in the frequency of channel opening but no increase in open lifetime duration or in current amplitude is observed. After addition of 100 μM suramin to the *cis* chamber, a marked increase in open lifetimes coupled with an increase in current amplitude is observed (*C*).

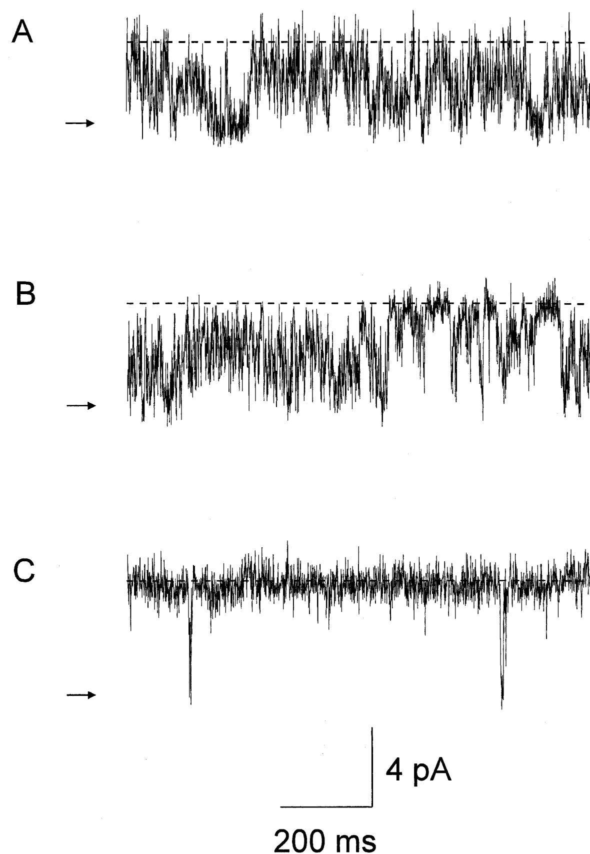

Fig. 9. Effects of suramin on the cardiac ryanodine receptor channel in the presence of ATP. In (*A*) the channel has been activated by 100 μM cytosolic ATP in the presence of 10 μM cytosolic Ca^{2+}. Note the rapid flickering gating which results from this concentration of ATP; P_o = 0.190. In (*B*), 10 μM suramin has been added to the *cis* chamber. In addition to the rapid flickery gating, longer opening events of increased current amplitude now occur; P_o = 0.525. After increasing [suramin] to 50 μM (*C*), P_o increases to 0.968.

10–100 times longer than the events which occur when the channels are activated by suramin plus cytosolic Ca^{2+} (Fig. 10). (It is difficult to give an accurate value since the events are so long in the absence of Ca^{2+} and therefore very long recordings would be required to give meaningful mean lifetime values.) The results indicate however, that in the absence of activating cytosolic Ca^{2+}, suramin binds to the channel inducing a conformation change in the channel protein which results in a third, very slow mode of channel gating, different to that observed when the channels are activated solely by cytosolic Ca^{2+} or by Ca^{2+} plus suramin. Thus, as has been observed for certain ligands which bind to the caffeine site [19, 32, 43] or to the adenine nucleotide site [3, 14] on ryanodine receptor channels, suramin induces two distinct gating schemes: a Ca^{2+}-dependent and a Ca^{2+}-independent mode of gating.

Suramin is ten times more effective at increasing P_o in the cardiac than in the skeletal channel. This appears to be related to the observation that suramin causes a far greater increase in open lifetime duration in the cardiac than in the skeletal channel (*see* Figs. 2 and 4). The differences could be explained on the basis that the binding sites for suramin on the two channels are different. Another explanation could be that skeletal channels possess a slightly different intrinsic gating mechanism ensuring that the open lifetimes are always shorter than those of the cardiac channel given the same conditions. The literature supports this idea if the lifetimes of channels activated solely by cytosolic Ca^{2+} are compared. The open lifetimes of both the cardiac and skeletal channels are very brief [34, 35] and under the experimental conditions of the present study the mean open lifetimes

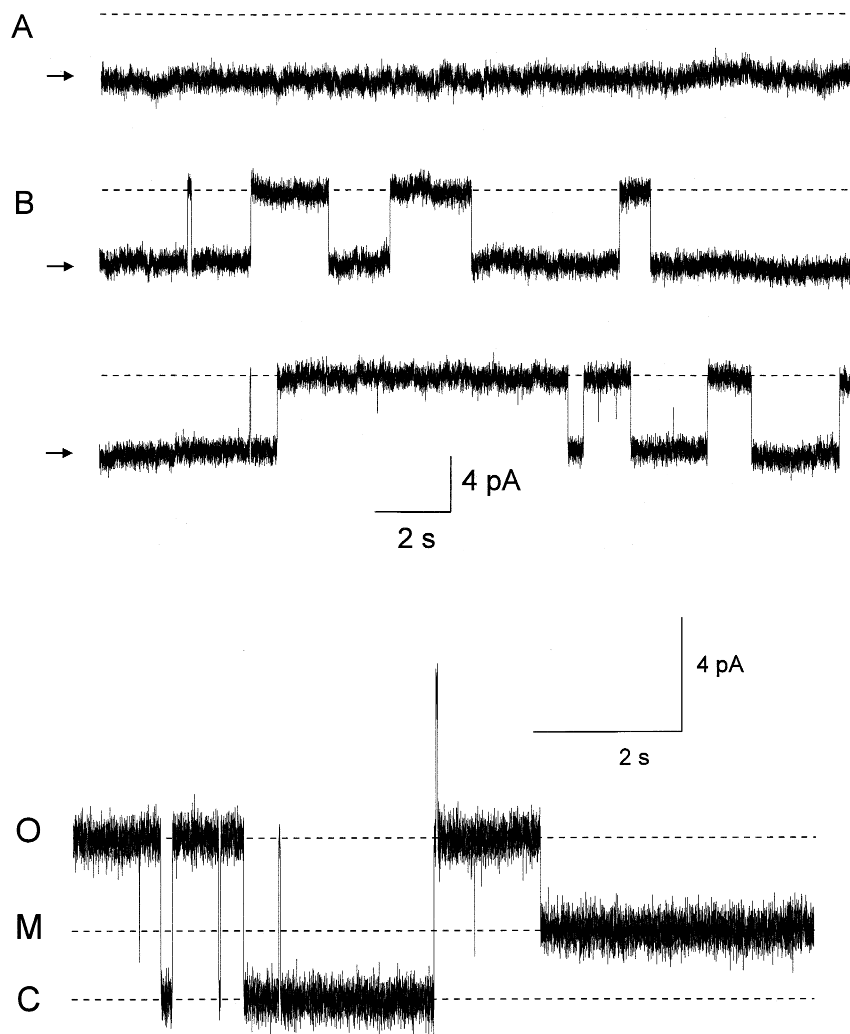

Fig. 10. Suramin can activate ryanodine receptor channels by a Ca^{2+}-independent mechanism. (*A*) A single cardiac channel has incorporated into the bilayer and the cytosolic-free [Ca^{2+}] has been lowered to picomolar levels by the addition of 12 mM EGTA. Under these conditions there are no channel openings. The broken line indicates the fully open channel level and the arrow indicates the closed channel level. (*B*) 100 μM suramin has been added to the *cis* chamber and very long but relatively infrequent openings occur. Forty seconds of consecutive single-channel fluctuations in the presence of suramin is shown to illustrate the exceptionally long events.

Fig. 11. Ryanodine modification of suramin-activated channels. More than one cardiac channel is present in the bilayer. The broken lines indicate the closed (*C*), ryanodine modified (M) and open (O) channel levels. The channels are activated by 100 μM suramin at subactivating (picomolar) cytosolic [Ca^{2+}]. The characteristic modification of one of the channels by ryanodine to a reduced-conductance long-lived open state (M) is shown.

appear to be identical (approximately 0.75 msec; *see* results section). However, when a monovalent cation is used as the permeant ion (to maximize the resolution of the single channel events) the open lifetime duration of skeletal channels appears to be shorter than that of cardiac channels [35]. Open lifetime distributions suggest that the cardiac channel has at least three open states whereas the skeletal channel has only two resolvable open states. The sheep skeletal channel does not appear to open to a state corresponding to the long third open state of the cardiac channel when activated solely by Ca^{2+} [35]. The absence of a third long open state for the skeletal channel may of course only be relevant for channels activated by Ca^{2+} as the sole ligand however, it is possible that there exists a gating pattern intrinsic to each isoform of the channel which is merely modified by other agents including suramin.

Luminal addition of suramin appeared to slightly increase the P_o of the channels by causing an increase in the frequency of channel opening however this effect was not statistically significant. Luminal addition of suramin did not increase the conductance of the skeletal or cardiac channels or induce long open events characteristic of the cytosolic action of suramin. Thus it appears that suramin does not cross the membrane (at least over the time course of these experiments $\leq$10 min) and that the effects observed when suramin is added to the cytosolic channel side result from an interaction of suramin with the cytosolic portion of the ryanodine receptor channel.

Based on Ca^{2+}-flux and ryanodine binding studies it has been suggested that suramin may bind to the adenine nucleotide site on the skeletal channel [9]. By investigating the effect of suramin on single channels we have been able to extract more information about the mechanisms underlying the effects of suramin. Agents which increase P_o by binding to the adenine nucleotide site are characterized by the very rapid gating kinetics that they induce. In the presence of activating levels of cytosolic Ca^{2+} and at suboptimal levels of the adenine compound,

very short open and closed events are observed [29]. Thus the predominant mechanism for an increase in P_o at suboptimal agonist concentrations is an increase in frequency of channel opening. At high P_o values, increases in open lifetime duration also occur. This effect was first observed in skeletal channels by Smith, Coronado & Meissner [38] but has subsequently been shown to occur in cardiac channels [27]. In contrast, suboptimal concentrations of suramin produce a marked increase in open lifetime duration with only a slight increase in frequency of opening (Figs. 2 and 5). In addition, suramin causes an increase in conductance; an effect never observed with any agent thought to act at the adenine nucleotide binding site [18, 27, 38]. The differences in ATP and suramin induced gating behavior can be observed in Fig. 9. Moreover, the figure illustrates that suramin does not antagonize the effect of ATP but that the combined effects of the two agents are more than additive. Thus there is strong evidence to suggest that suramin does not interact with the adenine nucleotide binding site but binds at distinct site(s) on the channel. In fact the effects of suramin on gating and conduction in the ryanodine receptor are incompatible with suramin acting at any other known sites on the channel (e.g., Ca^{2+}, caffeine [32], digoxin [17], calmodulin [39, 41], ryanodine [28]). We therefore propose that suramin binds to previously uncharacterized sites on ryanodine receptor channels to modulate not only the gating but also the conduction of ions (at least of Ca^{2+}) through the channel.

How does suramin interact with the channel to alter conduction? It could be argued that the open events of ryanodine receptors activated solely by cytosolic Ca^{2+} are too brief to measure accurately and that suramin, by increasing the duration of the events, merely allows the full current amplitude to be observed. However, even with Ca^{2+} as the sole agonist, very occasional long open events do occur as shown in Fig. 1*b*. A comparison of such events with those occurring in the presence of suramin demonstrates quite clearly that there is a 20–25% increase in conductance. The fact that caffeine and ATP also increase the duration of open lifetimes at higher concentrations, but do not increase conductance [32, 38] also argues against this idea. We are uncertain as to whether the suramin-induced effects on P_o and conductance result from the binding of suramin to the same or to distinct sites. It is possible that the binding of suramin to one site on the channel leads to a conformational change which results both in an altered conduction pathway and modification to channel gating kinetics. Alternatively, the effects on gating and conduction may result from the interaction of suramin with two different binding sites. One way of testing this would be to investigate if the effects of suramin on conductance are concentration dependent and if the concentration ranges for P_o and conduction changes can be differentiated. Other lines of investigation include examining the effects of structurally related compounds to determine if changes in conduction and P_o are always linked. It is also not known which parameters determining conduction and ion discrimination have been altered by suramin. For example, does the suramin-induced increase in conductance reflect a decrease in the affinity of the divalent cation binding site within the conduction pathway for Ca^{2+}? Does suramin also increase the conductance of monovalent cation current through the channel? Is the relative permeability of monovalent and divalent cations altered? These questions are currently under investigation.

In summary, we report that suramin increases the conductance and P_o of rabbit skeletal and sheep cardiac ryanodine receptor channels. Both effects would be expected to contribute to the increased Ca^{2+} efflux from skeletal SR vesicles reported by Emmick et al. [9]. Suramin increases conductance in both skeletal and cardiac channels by approximately 20–30%. Suramin is more effective at increasing P_o in cardiac than skeletal channels. This may reflect the greater increase in open lifetime duration observed with the cardiac channel. Finally, suramin may be acting at a unique binding site on the cytosolic face of ryanodine receptor channels. Certainly, it appears unlikely that suramin is interacting with the adenine nucleotide site.

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