Functional Regulation of the Cardiac Ryanodine Receptor by Suramin and Calmodulin Involves Multiple Binding Sites

Adam Parker Hill, Olivia Kingston, and Rebecca Sitsapesan

University of Bristol, Department of Pharmacology, School of Medical Sciences, Bristol United Kingdom (O.K., R.S.); and Imperial College School of Medicine, National Heart & Lung Institute, London, United Kingdom (A.P.H.)

ABSTRACT

Suramin and structurally related compounds increase not only the open probability ($P_o$) of ryanodine receptor (RyR) channels but also the single-channel conductance in a unique characteristic manner. In this report, we examine the mechanisms underlying the complex changes to cardiac RyR channel function caused by suramin and the evidence that these changes result from an interaction with calmodulin (CaM) binding sites. In the presence of 100 μM cytosolic Ca$^{2+}$, we demonstrate that suramin exerts a triphasic effect on $P_o$, indicating the presence of high-, intermediate-, and low-affinity suramin binding sites. The effects of suramin binding to high-affinity sites are Ca$^{2+}$-dependent; $P_o$ is decreased and seems to result from a reduction in the sensitivity of the channel to cytosolic Ca$^{2+}$. We suggest that this site is the CaM inhibition site. Suramin also binds to intermediate-affinity sites that mediate an increase in $P_o$ and an increase in conductance. Cytosolic Ca$^{2+}$ is not an absolute requirement for the effects mediated via intermediate-affinity suramin sites. The suramin-induced increase in $P_o$ and conductance are both concentration-dependent. The correlation between the increase in $P_o$ and increase in conductance indicates that the binding events which produce an increase in $P_o$ also lead to an increase in conductance and, because the effect is concentration-dependent, multiple suramin molecules must bind to produce the maximum effect. The low-affinity suramin binding sites are inhibition sites and mediate a reduction in $P_o$ caused by changes to both open and closed lifetimes.

There has recently been much interest in the effects of suramin on RyR channels because biochemical evidence indicates that suramin and calmodulin compete for common binding sites on the skeletal isoform of RyR (Klinger et al., 1999; Papineni et al., 2002). Single-channel experiments, however, indicate that suramin causes very unusual changes to RyR channel function. We have demonstrated previously that suramin produces an increase in the $P_o$ of single RyR channels that is associated with an increase in single-channel conductance (Sitsapesan and Williams, 1996). This distinctive, simultaneous modification to the gating and conduction properties of RyR channels is shared by other structurally related ligands, including 4,4'-disothiocyanatostilbene-2,2'-disulphonic acid and 4,4'-dibenzamidostilbene-2,2'-disulphonic acid (Sitsapesan, 1999; Hill and Sitsapesan, 2002). Another anomalous effect of suramin is that in the presence of 10 μM cytosolic Ca$^{2+}$, the increase in $P_o$ of the cardiac RyR is caused by an increase in the duration of open times, with no detectable increase in the frequency of the openings (Sitsapesan and Williams, 1996). No other activators of RyR channels increase $P_o$ by this mechanism. All known agonists of RyR, including ligands such as ATP, caffeine, and Ca$^{2+}$, increase $P_o$ by increasing the frequency of channel openings and may or may not also increase the duration of open states. These unusual effects of suramin and related compounds do not seem to be shared by CaM (Smith et al., 1989; Tripathy et al., 1995; Fruen et al., 2000). The question therefore arises as to what functional effects occur as a consequence of binding to sites on RyR that are common to CaM and suramin. Because binding studies provide no information about whether common binding sites mediate the functional effects of CaM and suramin, we investigated the mechanisms underlying the single-channel effects of suramin on cardiac RyR (RyR2) channels and examined the evidence that the functional effects of suramin are mediated via calmodulin binding sites. We chose to activate the channels with 100 μM Ca$^{2+}$ because this is a concentration of cytosolic Ca$^{2+}$ at which CaM has been demonstrated to be most effective as an inhibitor of channel activity (Tripathy et al., 1995; Zhang et al., 1999; Fruen et al., 2000; Rodney et al., 2000). If suramin and CaM bind to common sites on RyR channels, it is possible that both ligands require the same conditions for maximum efficacy. We show that by raising the cytosolic [Ca$^{2+}$], the complex actions of suramin are exposed, revealing that suramin interactions with RyR channels involve multiple binding sites.

ABBREVIATIONS: RyR, ryanodine receptor; CaM, calmodulin; PIPES, 1,4-piperazinediethanesulfonic acid; SR, sarcoplasmic reticulum.
Materials and Methods

Preparation of SR Membrane Vesicles and Planar Lipid Bilayer Methods. Heavy SR membrane vesicles were prepared from sheep hearts as described previously (Sitsapesan et al., 1991) and were rapidly frozen and stored in liquid nitrogen. Vesicles were fused with planar phosphatidylethanolamine lipid bilayers as described previously (Sitsapesan et al., 1991). SR 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. After fusion, the cis chamber was perfused with 250 mM HEPES, 125 mM Tris, and 10 mM free [Ca$^{2+}$], pH 7.2. The free [Ca$^{2+}$] in the cis chamber was adjusted by the addition of CaCl$_2$ or EGTA. The trans chamber was perfused with 250 mM glutamic acid and 10 mM HEPES, pH 7.2 to 7.2 with Ca(OH)$_2$ (free [Ca$^{2+}$]), approximately 50 mM. Experiments were performed at room temperature (22 ± 2°C). The free [Ca$^{2+}$] and pH of the solutions were determined at 22°C using a Ca$^{2+}$ electrode (Orion 93-20; Thermo Electron, Franklin, MA) and Ross-type pH electrode (Orion 81-55; Thermo Electron) as described previously (Sitsapesan et al., 1991). Additions of suramin and caffeine and CaM were made to the cis chamber and, at the cytosolic free [Ca$^{2+}$] used, caused no change to the pH or free [Ca$^{2+}$] of the solutions. The free [Ca$^{2+}$] and pH of the solutions were measured using a Ca$^{2+}$ electrode (Orion 93-20) and Ross-type pH electrode (Orion 81-55) as described previously (Sitsapesan et al., 1991).

Data Acquisition and Analysis. Single-channel recordings were displayed on an oscilloscope and recorded on digital video tape. Steady-state recordings were carried out at 0 mV. At this holding potential, Ca$^{2+}$ currents flow in the luminal to cytosolic direction. The current recordings were filtered at 0.5 kHz (~3 dB) and digitized at 2 kHz using the single-channel analysis program Satori (Intracel, Cambridge, MA). P$_o$ and the lifetimes of the open and closed single-channel events were determined over 3 min of recording using 50% threshold analysis (Colquhoun and Sigworth, 1983). When more than one channel was incorporated into the bilayer, average P$_o$ was calculated according to the formula Average P$_o$ = (T$_{open1}$ + 2T$_{open2}$ + 3T$_{open3}$... + nT$_{open n}$)/nNT$_{total}$, where T$_{open1}$, T$_{open2}$ and T$_{open3}$ are the times in the first, second, and third open channel levels, respectively. T$_{total}$ is the total recording time, and N is the number of channels in the bilayer. Lifetime analysis was carried out only when a single channel incorporated into the bilayer. Events <1 ms in duration were not fully resolved and were excluded from lifetime analysis. Lifetimes accumulated from 3-min steady-state recordings were stored in sequential files and displayed in noncumulative histograms. Individual lifetimes were fitted to a probability density function by the method of maximum likelihood (Colquhoun and Sigworth, 1983) according to the equation f(t) = a$_1$(1/τ$_{1}$)exp(−t/τ$_{1}$) + ... + a$_n$(1/τ$_{n}$)exp(−t/τ$_{n}$), with areas a and time constants τ. A missed events correction was applied as described by Colquhoun and Sigworth (1983). A likelihood ratio test (Blatz and Magleby, 1986) was used to compare fits to up to four exponentials by testing twice the difference in log$_2$ (likelihood) against the χ$^2$ distribution at the 1% level. Single-channel current amplitudes were measured from digitized data using manually controlled cursors. Amplitude histograms were also used to confirm measurements.

[3H]Ryanodine Binding. Known amounts of heavy SR membrane vesicles were incubated for 90 min at 37°C in the presence of 5 nM [3H]Ryanodine in 500 μl of binding buffer containing 250 mM KCl, 25 mM PIPES, pH 7.4, and varying concentrations of CaM or suramin. Free Ca$^{2+}$ in the binding solutions was adjusted by addition of 1 mM EGTA and titration of free Ca$^{2+}$ to the desired concentration (determined by measurement with a Ca$^{2+}$-sensitive electrode) with CaCl$_2$. Each experiment was performed in triplicate. Nonspecific (background) binding was quantified by performing the incubation in the presence of 5 μM (a thousand-fold excess) unlabeled ryanodine. Binding reactions were terminated by addition of 5 ml of ice-cold binding buffer. The samples were then filtered through Whatman GF/B filters (Whatman, Clifton, NJ) that had been pre-soaked for 1 h in binding buffer, followed by two 5-ml washes of ice-cold binding buffer to wash through unbound [3H]Ryanodine. Filters were then placed in 10 ml of aqueous scintillant and counted the following day using a liquid scintillation counter.

Results

We have previously demonstrated that in the presence of 10 μM cytosolic [Ca$^{2+}$], the threshold for channel activation by suramin was approximately 1 μM, the EC$_{50}$ was 22.4 μM, and the Hill slope was 1.39 (Sitsapesan and Williams, 1996). Figure 1 demonstrates that if P$_o$ is first raised by increasing cytosolic [Ca$^{2+}$] to 100 μM, then low concentrations of suramin actually cause a reduction in P$_o$. In the control trace in the presence of 100 μM cytosolic Ca$^{2+}$, P$_o$ was 0.210 ± 0.008 (S.E.M.; n = 6). The second trace demonstrates the reduction in P$_o$ and the development of long closed states caused by 500 nM suramin (P$_o$ = 0.100 ± 0.06; n = 4; p < 0.05). Increases in suramin concentration to micromolar levels and greater reversed the inhibitory effect of suramin and caused long open events and high P$_o$ (traces 3 and 4). For example, in the presence of 500 μM suramin, P$_o$ was 0.987 ± 0.02 (n = 4). The bottom trace demonstrates that supraoptimal concentrations of suramin produce a decrease in P$_o$ and the appearance of shorter open states. P$_o$ in the presence of 2 mM suramin was reduced to 0.665 ± 0.103 (n = 4). The overall relationship between P$_o$ and [suramin] can be observed in Fig. 2 and demonstrates the triphasic nature of the actions of suramin. Concentrations of suramin between 1 mM and 1 μM reduce P$_o$ to lower than the control values that occur in the presence of 100 μM cytosolic Ca$^{2+}$. Higher concentrations produce activation, with an EC$_{50}$ of 2.5 μM and Hill slope of 1.2. Supraoptimal levels of suramin (>1 mM) lead to channel inactivation. Figure 3 demonstrates a similar relationship between [3H]Ryanodine binding to heavy SR and suramin concentration in the presence of 100 μM Ca$^{2+}$ and...
shows that at 10 μM Ca²⁺, the high-affinity inhibition is undetectable.

How are the concentration-dependent changes in $P_o$ brought about? Information about the underlying mechanisms can be obtained by examining the concentration-dependent changes in event duration. Figure 4 illustrates how mean open and closed times change with increasing suramin concentration in the presence of 100 μM Ca²⁺. Low concentrations of suramin (500 nM) produced a 5-fold increase in mean closed time [6.0 ± 1.5 ms in 100 μM cytosolic Ca²⁺; 34.8 ± 28.3 ms (S.E.M.; n = 4) after the addition of 500 nM suramin] but no reduction in mean open time [1.66 ± 0.84 ms in 100 μM cytosolic Ca²⁺; 2.54 ± 0.41 ms (S.E.M.; n = 4) after the addition of 500 nM suramin]. The reduction in $P_o$ observed at these concentrations must therefore be caused by the lengthening of closed lifetimes. As the concentration of suramin is increased higher than 500 μM, mean closed times

Fig. 1. The effects of suramin in the presence of 100 μM cytosolic Ca²⁺ on the function of a typical single sheep cardiac RyR channel. The holding potential was 0 mV. The dotted lines labeled “O” and “C” indicate the open and closed channel levels, respectively. The $P_o$ values to the right of each trace were obtained from 3 min of consecutive recordings. The top trace shows the control channel activated by 100 μM cytosolic Ca²⁺. Subsequent traces show the effects of increasing concentrations of suramin added to the cytosolic channel side.

Fig. 2. Relationship between $P_o$ and [suramin] in the presence of 100 μM cytosolic Ca²⁺. Values are mean ± S.E.M. for $n = 4$. Where error bars are not shown, they are within the symbol. The broken line indicates the mean $P_o$ of channels activated by 100 μM cytosolic Ca²⁺ alone.

Fig. 3. Effect of suramin on [³H]ryanodine binding to cardiac heavy SR in the presence of 10 μM Ca²⁺ (A) and 100 μM Ca²⁺ (B). The mean values ± S.E.M. are shown for $n = 4$. At 10 μM Ca²⁺ the EC₅₀ and Hill slope were 64.5 μM and 2.0, respectively; supraoptimal concentrations reduced binding with an IC₅₀ of 912 μM. At 100 μM Ca²⁺ the EC₅₀ and Hill slope were 2.9 μM and 0.62, respectively. The IC₅₀ and Hill coefficient for inactivation at high concentrations were 1.58 mM and 1.35, respectively. The broken lines illustrate the control level of binding obtained in the absence of suramin. Control binding was 0.173 ± 0.2 and 1.56 ± 0.2 pmol[³H]/mg of protein in the presence of 10 μM Ca²⁺ and 100 μM Ca²⁺, respectively.
tend to decline until supraoptimum levels of suramin are obtained, and closed lifetimes abruptly increase. In contrast, mean open times increase steeply at concentrations greater than 500 nM suramin and plateau at approximately 10 μM suramin. For example, in the presence of 5 μM suramin, a several hundred-fold increase in mean open time above control values is observed (836 ± 1490 ms). Note the log scale required to plot mean open times over the range of suramin concentrations. Mean open times remain very high until supraoptimal doses are reached, and mean open times fall below 100 ms. Lifetime analysis examines these effects of suramin in more detail, and an example of how various concentrations of suramin alter the distribution of open and closed events can be observed in Fig. 5. At 500 nM suramin, no significant change in open lifetime distributions was observed. However there seems to be the beginning of a shift toward a higher percentage of longer open events, and therefore 500 nM suramin may be an approximate threshold dose for an increase in open lifetime duration (Fig. 5B). Large increases in open lifetimes can be observed at 5 μM suramin (Fig. 5C), and this includes the introduction of a third long open state with a time constant of 250 ms. At the inactivating concentration of 2 mM suramin, the third long open state is abolished and most of the events occur to a time constant of approximately 18 ms (Fig. 5D). Closed lifetimes are affected differently; 500 nM suramin produces a distinct increase in the duration of closed lifetimes (Fig. 5B), an effect that is reversed at 5 μM suramin (Fig. 5C). Finally, at high, inactivating concentrations of suramin (2 mM), the brief closing events are not apparent, and closings occur only to two long closed states (Fig. 5D).

We have demonstrated previously that suramin affects ion conduction within the RyR in addition to its action on channel gating (Sitsapesan and Williams, 1996). This raises two questions: First, are the effects on conductance and gating caused by the interactions of suramin with a common binding site on RyR? and second, because the triphasic nature of the suramin dose-response relationship indicates that suramin binds to at least three sites on RyR, is the conductance change associated with both the inhibitory effects of suramin (where closed lifetimes are increased) and the activation (where open lifetimes are increased)? In Fig. 6, the current amplitude at the holding potential of 0 mV is shown for control (100 μM Ca²⁺) and for concentrations of suramin that reduce ₃₉₉ (500 nM), increase ₃₉₉ (50 μM), and inactivate ₃₉₉ (2 mM). In the presence of 100 μM Ca²⁺ only, current amplitude was 4.1 ± 0.02 pA (S.E.M.; n = 6) and in the presence of 50 μM and 2 mM suramin, was 5.09 ± 0.03 pA (S.E.M.; n = 7) and 5.01 ± 0.08 pA (S.E.M.; n = 4) respectively. However, at 500 nM suramin, in three of four experiments, no change in conductance was observed. In one of the four experiments, occasional (<10 per minute) channel openings of increased current amplitude (to 5.14 pA) were detected. It was apparent that whenever one of the few increased amplitude events occurred, it was also of longer duration than the other events (Fig. 6, top right). In the other experiments, single-channel current amplitude was no different than that observed with channels activated solely by 100 μM cytosolic Ca²⁺ (Fig. 1, trace 2). It seems likely therefore that the rare, long duration, increased amplitude events are not caused by suramin binding to the high-affinity inhibition sites but by binding to the intermediate-affinity activation sites because 500 nM suramin may be close to the threshold level for binding to these sites.

It is extremely difficult to be certain of the effects of 500 nM suramin on conduction because this concentration of suramin reduces ₃₉₉, and very few events are not truncated by filtering (Fig. 6, top right trace, and Fig. 1, trace 2). The 500 nM suramin trace in Fig. 6 has been chosen to illustrate one of the very few long open events observed. Indeed, even in the control situation, in which the channels have been activated by 100 μM cytosolic Ca²⁺, most of the events are truncated by filtering and are too brief for current amplitude to be measured. To overcome the problem of brief events, which are a characteristic of the sheep cardiac RyR when activated solely by Ca²⁺, we introduced a new experimental protocol to ensure that long open events occur even for controls. We have demonstrated previously that caffeine-induced, Ca²⁺-independent events are much longer than Ca²⁺-activated events (Sitsapesan and Williams, 1990), and this can be observed in Fig. 7. In the top trace, the channel is activated solely by 40 μM cytosolic Ca²⁺, and most events do not seem to reach the fully open channel level. This is because the events are very

![Fig. 4. The effect of [suramin] on the mean open times (A) and mean closed times (B). Values are mean ± S.D. for n ≥ 3. The broken lines indicates the mean open and closed times of channels activated by 100 μM cytosolic Ca²⁺ alone.](https://example.com/image-url)
Fig. 5. Open and closed lifetime distributions from a typical single cardiac RyR channel activated by 100 μM cytosolic Ca\(^{2+}\) alone (A) and after subsequent additions of 500 nM (B), 5 μM (C), and 2 mM suramin (D). These concentrations of suramin were chosen because they lie on the inhibition, activation, and inactivation regions of the suramin dose-response relationship. This analysis was conducted for three separate experiments at these doses of suramin.
brief and are truncated by filtering. In the middle trace, the cytosolic free [Ca\textsuperscript{2+}] is reduced to subactivating levels (2.5 nM) by the addition of EGTA, and no openings occur. The addition of caffeine (bottom trace) produces long open events, most of which can be fully resolved, and therefore current amplitude can be more accurately measured. The conductance of caffeine-activated channel openings is identical with that of Ca\textsuperscript{2+}-activated channel events (Sitsapesan and Williams, 1990). In the following experiments, we therefore used caffeine to activate RyR channels to a \( P_0 \) of between 0.1 and 0.3 to investigate the relationship between suramin-induced changes in \( P_0 \) and conductance.

The relationship between \( P_0 \) and suramin concentration under these experimental conditions is shown in Fig. 8. Nanomolar levels of suramin did not produce a decrease in \( P_0 \); if anything, slight increases in \( P_0 \) were observed at concentrations of suramin lower than 1 \( \mu \)M. Suramin also caused inactivation at supraoptimal doses as was observed in the presence of activating cytosolic \([\text{Ca}^{2+}]_i\). Figure 9 illustrates that suramin still increases the amplitude of caffeine-activated openings in the absence of activating levels of cytosolic \([\text{Ca}^{2+}]_i\). Current amplitude tended to be highest at the higher concentrations of suramin, at which a greater increase in \( P_0 \) was observed. To investigate whether the suramin-induced increase in current amplitude was related to the increase in \( P_0 \) we plotted current amplitude at 0 mV versus \( P_0 \) (Fig. 10). The figure illustrates that as \( P_0 \) increases, there is also a tendency for current amplitude to increase. A significant positive correlation between \( P_0 \) and current amplitude was obtained, indicating that the changes in the two parameters may not be independent events (Pearson correlation coefficient \( r = 0.7258; p < 0.05 \)).

In skeletal RyR, there is biochemical evidence that CaM and suramin share common binding sites (Klinger et al., 1999; Papineni et al., 2002). Because suramin has been shown previously only to activate RyR channels, it has been assumed that CaM must bind to suramin activation sites. However, we now have shown that suramin produces multiple functional effects that occur via high-, intermediate-, and low-affinity binding sites on cardiac RyR channels. Does CaM produce functional changes to channel gating by binding to any of these sites? Figure 11, A and B, demonstrate that at 100 \( \mu \)M cytosolic \([\text{Ca}^{2+}]_i\), we observed only inhibition of [\text{H}]ryanodine binding or a reduction in the \( P_0 \) of the cardiac RyR by CaM. In fact, 50 nM CaM reduced \( P_0 \) from 0.314 \( \pm \) 0.082 to

### Fig. 6
Comparison of the amplitude of the single-channel events at 0 mV in the absence (top left) and presence of 500 nM, 50 \( \mu \)M, and 2 mM suramin. The dashed lines and O and C illustrate the open and closed channel levels, respectively, of the channel when activated by 100 \( \mu \)M cytosolic \([\text{Ca}^{2+}]_i\) alone. The broken lines indicate the open channel level in the presence of 50 \( \mu \)M suramin. The traces shown for 50 \( \mu \)M and 2 mM suramin show typical gating characteristics. The traces shown for the 100 \( \mu \)M cytosolic \([\text{Ca}^{2+}]_i\) alone and the 500 nM suramin have been chosen to illustrate the very occasional long open events that occur under these experimental conditions and are atypical.

### Fig. 7
Protocol for activating channels with caffeine in the absence of activating cytosolic \([\text{Ca}^{2+}]_i\). In the top trace, the cytosolic \([\text{Ca}^{2+}]_i\) is 40 \( \mu \)M. In the second trace, EGTA was added to give a free [Ca\textsuperscript{2+}] of 2.5 nM, and all channel openings were abolished. In the third trace, caffeine was added to give an approximate \( P_0 \) of between 0.1 and 0.3; in this case, 20 mM caffeine was added. The holding potential was 0 mV. C, closed channel level; O, fully open channel level.

### Fig. 8
The effect of suramin on the \( P_0 \) of sheep cardiac RyR activated by caffeine (5–30 mM) in the absence of activating cytosolic \([\text{Ca}^{2+}]_i\) (2.5 nM). Data points are mean \( \pm \) S.E.M. of 4 to 11 observations. The broken line indicates the control \( P_0 \) in the presence of 1 to 30 mM caffeine and 2.5 nM \([\text{Ca}^{2+}]_i\). The EC\textsubscript{50} value and Hill coefficient for activation by suramin are 345 \( \mu \)M and 1.4, respectively.
0.031 ± 0.02 (S.E.M.; n = 5). At nanomolar [Ca^{2+}], in agreement with previously published work (Tripathy et al., 1995; Fruen et al., 2000), we found that CaM (up to 2 μM) had no effect on channel gating (Fig. 11C) and could not stimulate \[^{[3H]}\text{ryanodine}\] binding to cardiac SR at nanomolar levels of \[\text{Ca}^{2+}\], whereas CaM did stimulate binding to rabbit skeletal SR (to 120 and 496% of control in the absence and presence of 1 mM ATP, respectively; results not shown). Fruen et al. (2000) suggest that adenine nucleotides are required for most effective activation of the skeletal RyR by CaM. However, even very high concentrations of CaM (5 μM) in the presence of ATP did not increase \(P_o\) at nanomolar cytosolic \[\text{Ca}^{2+}\] (Fig. 11C, bottom trace).

In the presence of 100 μM cytosolic \[\text{Ca}^{2+}\], both CaM and nanomolar suramin reduce \(P_o\). We compared the open and closed lifetime distributions of channels in which \(P_o\) had been reduced by nanomolar suramin or by CaM, and the results are shown in Table 1. After activation of the channel by 100 μM cytosolic \[\text{Ca}^{2+}\], two open and three closed states are observed. Both suramin and CaM have minor effects on the open lifetime distributions, and in both cases, the reduction in \(P_o\) is caused by a similar effect on closed lifetime durations. The two ligands cause an increase in the duration of all three closed states, with a particularly marked increase in the duration of the third longest closed state. The similar mechanism by which suramin and CaM reduce \(P_o\) indicates that they may act via common binding sites on RyR to produce these effects.

It has been shown that suramin can displace CaM from binding sites on skeletal RyR (Klinger et al., 1999; Papineni et al., 2002). We therefore examined whether suramin could displace CaM bound to cardiac SR. Figure 12 illustrates that CaM was associated with the control SR preparation and could be detected by anti-CaM antibody (lane A). Vesicles of cardiac heavy SR were incubated for 30 min on ice with 1 mM suramin and then sedimented to remove displaced CaM from the vesicles. The sedimented pellet (lane B) and supernatant (lane C), corresponding to membrane-bound and displaced CaM, respectively, were then immunoblotted using the anti-CaM antibody. This demonstrated that suramin displaces most of the CaM from its binding sites in SR because most of it was found in the supernatant (lane C). Hence, the results of Western blotting of cardiac SR membranes are consistent with previous reports in skeletal muscle indicating that suramin is very effective at displacing CaM from binding sites on RyR (Klinger et al., 1999; Papineni et al., 2002).

Clearly, suramin does displace CaM from common binding sites in cardiac SR vesicles (Fig. 12), and there is mechanistic evidence from lifetime analysis (Table 1) to suggest that the high-affinity suramin inhibition sites may be CaM binding sites (at least in the presence of micromolar levels of cytosolic \[\text{Ca}^{2+}\]). However, suramin also increases \(P_o\) and single-chan-

Fig. 9. A, amplitude histogram overlay demonstrating the effect of suramin on the current amplitude of a representative cardiac RyR channel at a holding potential of 0 mV. The broken line indicates the control with 30 mM caffeine and 2.5 nM Ca^{2+}. The boldface line indicates the amplitude histogram generated after the addition of 0.6 mM suramin. Note the right shift in mean open channel current amplitude. B, effect of [suramin] on current amplitude at 0 mV of single cardiac RyR channels activated by caffeine (5–30 mM) in the absence of activating levels of cytosolic \[\text{Ca}^{2+}\] (2.5 nM). Data are mean ± S.E.M. of 4 to 11 observations. The absence of error bars indicates a mean of three observations.

Fig. 10. Relationship between current amplitude and \(P_o\) for channels activated by suramin in the presence of caffeine and 2.5 nM Ca^{2+}. Data points are mean values of 3 observations. Error bars are S.E.M. of 4 to 11 observations. The Pearson correlation coefficient of 0.7285 (p < 0.05) indicates a significant, positive correlation between \(P_o\) and current amplitude.
channel conductance by binding to intermediate-affinity sites. Is there any evidence that the intermediate suramin binding sites are also CaM binding sites? We find no evidence that CaM can modify conductance in the presence of 100 μM cytosolic Ca²⁺ (Fig. 11A), although the brief events observed after application of CaM make conductance measurements difficult. We also investigated the effects of CaM on caffeine-activated channel openings by applying the same protocol used in Fig. 7 to produce long open events. We found that CaM did not cause any significant changes in current amplitude over a wide range of CaM concentrations (Fig. 13).

We considered the possibility that endogenous CaM is bound to our channels after incorporation into bilayers and maintains a reduced $P_o$ (and reduced single-channel conductance) and that the ability of suramin to increase $P_o$ (and conductance) simply reflects displacement of the endogenous CaM. Figure 14 indicates that the last suggestion is unlikely. Suramin (100 μM) was added to the cytosolic chamber and left for 10 min. The cytosolic chamber was then perfused out with 10 volume changes of the cytosolic solution. The suramin-induced increase in $P_o$ and single-channel conductance were completely reversible after washout and therefore unlikely to be caused by competing CaM off its binding sites on RyR. It is also unlikely that CaM is tethered to RyR and is not washed away, because this does not fit with the data in Fig. 12 in which CaM was displaced by suramin nor with the fact that we have no evidence that CaM can reduce single-channel conductance (Figs. 11A and 13). The suramin-induced increase in $P_o$ and conductance must result directly from the efficacy of suramin itself.

To further investigate the possibility that the functional effects of CaM and suramin are mediated via common binding sites, we examined the effects of CaM on the binding of [³H]ryanodine to isolated SR at key suramin concentrations identified from the triphasic dose-response curve shown in Fig. 3. The results are shown in Fig. 15. Here, 50 nM suramin produces a decrease in [³H]ryanodine binding by binding to high-affinity suramin inhibition sites, and CaM (5 μM) has no added inhibitory effect. This is expected for ligands competing for the same site (the total inhibitory effect would not be expected to be greater than the maximum inhibitory effect observed with either ligand). At 50 μM suramin, a concentration that produces stimulation of [³H]ryanodine binding but not the maximum effect, CaM causes inhibition. This effect would be expected if the high-affinity inhibition sites were already saturated by the high [suramin] and CaM was competing with suramin at intermediate-affinity activation sites. Finally, at 1 mM suramin (close to the IC₅₀ for the low-affinity inhibitory action of suramin), 5 μM CaM has no effect. Presumably, any effects of CaM are overcome by the 200-fold higher concentration of suramin.

**Discussion**

The triphasic dose-response relationship to suramin exposed in the presence of 100 μM Ca²⁺ indicates that suramin binds to at least three binding sites on the cardiac RyR: high-affinity inhibition sites, intermediate-affinity activation sites, and low-affinity inhibition sites.

**High-Affinity Suramin Inhibition.** When suramin binds to its high-affinity sites, it causes a reduction in $P_o$ by producing an increase in the duration of closed lifetimes. At subactivating cytosolic Ca²⁺, when caffeine levels were titrated to bring $P_o$ to 0.1 to 0.3, nanomolar suramin, unexpectedly, did not reduce $P_o$ even at concentrations as low as 1 nM. In fact, slight activation was observed. The inhibitory effects of suramin mediated via the high-affinity inhibition sites are, therefore, Ca²⁺-dependent. This is confirmed by the fact that the suramin-induced reduction in $P_o$ is obvious at 100 μM Ca²⁺ but is difficult to detect at 10 μM Ca²⁺. The
predominant effect of cytosolic Ca$^{2+}$ on the gating of sheep cardiac RyR is to increase the frequency of channel openings with only slight increases in duration of open lifetimes (Zahradniková and Palade, 1993; Sitsapesan and Williams, 1994; Saftenku et al., 2001). Because the main effect of suramin at the high-affinity inhibition sites is to reduce the frequency of channel openings, it is possible that the binding of suramin to these sites affects the Ca$^{2+}$ activation of the channel by lowering the Ca$^{2+}$ sensitivity of the channel.

**Intermediate-Affinity Suramin Activation.** At 10 and 100 µM cytosolic Ca$^{2+}$, suramin increases $P_o$ higher than control levels in the micromolar dose range. The increase in $P_o$ is brought about by an unusually marked increase in open lifetime duration and is accompanied by an increase in conductance. Thus, both activation and conductance changes seem to be mediated by the interactions of suramin with intermediate-affinity activation sites.

The experiments carried out in the presence of caffeine at subactivating [Ca$^{2+}$] confirmed that the suramin-induced increases in $P_o$ and conductance are Ca$^{2+}$-independent. The effects of suramin on current amplitude and $P_o$ seem to be related because the increase in current amplitude is significantly correlated with an increase in $P_o$ (Fig. 10). This is not the first report in which an increase in current amplitude seems to depend on ligand concentration. Smoothly graded, dose-dependent increases in conductance have been reported with only slight increases in duration of open lifetimes (Zahradniková and Palade, 1993; Sitsapesan and Williams, 1994; Saftenku et al., 2001). Because the main effect of suramin at the high-affinity inhibition sites is to reduce the frequency of channel openings, it is possible that the binding of suramin to these sites affects the Ca$^{2+}$ activation of the channel by lowering the Ca$^{2+}$ sensitivity of the channel.

**Low-Affinity Suramin Inhibition.** Inactivation at high suramin levels occurs in the presence and absence of activating levels of cytosolic Ca$^{2+}$ and therefore is not strictly Ca$^{2+}$-dependent. Typically, inactivation occurs at millimolar concentrations; hence, low-affinity suramin binding sites are involved. Binding to the low-affinity inhibition sites does not lead to a reversal of the increase in conductance, at least up to 4 mM suramin.

**Does CaM Mediate Inhibition of the Cardiac RyR Channel via Suramin Binding Sites?** In broad agreement with work published previously (Tripathy et al., 1995; Moore et al., 1999; Fruen et al., 2000; Rodney et al., 2000; Balshaw et al., 2001), we found that at 100 µM cytosolic Ca$^{2+}$, CaM decreases $P_o$ and reduces $[^3H]$ryanodine binding but has no significant effect on the channel at subactivating cytosolic Ca$^{2+}$. We also demonstrated by Western blot analysis that CaM bound to cardiac heavy SR can be displaced by suramin. Thus, as for RyR1 (Papineni et al., 2002), it seems that CaM can be competitively displaced from RyR2 by suramin, indicating that the two ligands share common binding sites on RyR2. Displacement experiments, of course, provide no information about whether the functional effects of CaM and suramin on RyR conductance and gating are mediated via common binding sites, and this information can only be obtained from single-channel experiments or deduced indirectly from other functional assays such as the $[^3H]$ryanodine binding experiments. At 100 µM cytosolic Ca$^{2+}$, the effect of CaM on the gating of the channels was very similar to that caused by nanomolar concentrations of suramin. Both nanomolar suramin and CaM decreased $P_o$ by decreasing the frequency of channel openings and did not reduce the duration of open states significantly. The similar mechanism for the inhibition may indicate a common binding site and therefore that the high-affinity suramin inhibition site could be a

### Table 1

Comparison of the effects of nanomolar [suramin] and CaM on the open and closed lifetime distributions of single cardiac RyR channels in the presence of 100 µM cytosolic Ca$^{2+}$ Values are mean ± S.D. for n = 3.

<table>
<thead>
<tr>
<th></th>
<th>r1</th>
<th>Area</th>
<th>r2</th>
<th>Area</th>
<th>r3</th>
<th>Area</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Open lifetime parameters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 µM Ca$^{2+}$</td>
<td>2.3 ± 0.7</td>
<td>93.0 ± 3.8</td>
<td>17.1 ± 2.0</td>
<td>6.7 ± 3.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>500 nM suramin</td>
<td>2.5 ± 0.5</td>
<td>86.7 ± 2.0</td>
<td>28.6 ± 12.8</td>
<td>13.3 ± 2.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 nM CaM</td>
<td>1.8 ± 0.9</td>
<td>93.0 ± 3.6</td>
<td>22.5 ± 17.9</td>
<td>7.0 ± 3.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Closed lifetime parameters</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>100 µM Ca$^{2+}$</td>
<td>3.9 ± 0.6</td>
<td>65.5 ± 14.4</td>
<td>11.5 ± 2.0</td>
<td>28.7 ± 15.1</td>
<td>30 ± 9.76</td>
<td>5.7 ± 2.5</td>
</tr>
<tr>
<td>500 nM suramin</td>
<td>5.0 ± 1.7</td>
<td>48.6 ± 8.5</td>
<td>32.6 ± 9.9</td>
<td>39.3 ± 3.5</td>
<td>223 ± 156</td>
<td>12.0 ± 5.0</td>
</tr>
<tr>
<td>50 nM CaM</td>
<td>5.8 ± 1.3</td>
<td>33.0 ± 20.8</td>
<td>45.5 ± 12.6</td>
<td>48.3 ± 7.5</td>
<td>232 ± 103</td>
<td>18.7 ± 13.3</td>
</tr>
</tbody>
</table>
CaM binding site. The [$^3$H]ryanodine binding experiments add weight to this hypothesis. Both CaM and nanomolar suramin cause partial inhibition of the 100 μM Ca$^{2+}$-stimulated [$^3$H]ryanodine binding to cardiac SR, and the inhibition is of the same magnitude (approximately 25–35% maximum inhibition) by both compounds. Moreover, the simultaneous presence of both ligands cannot further increase the level of inhibition obtained by an optimum concentration of either ligand (Fig. 15). Thus, CaM and suramin act as though they are competing for the same binding site, the high-affinity suramin inhibition site, to produce a decrease in $P_o$. Given the other supporting evidence, this seems a likely interpretation of the [$^3$H]ryanodine binding results, although it is possible that allosteric effects of either CaM or suramin acting at separate sites could produce the same effect. High-affinity inhibition by suramin and by CaM is abolished by lowering the cytosolic free [Ca$^{2+}$], and therefore it seems likely that inhibition at this site is brought about by reducing the sensitivity of the channel to cytosolic Ca$^{2+}$. This hypothesis is supported by the competition experiments performed in the presence of 100 μM Ca$^{2+}$. The effect of suramin on [$^3$H]ryanodine binding to cardiac heavy SR in the absence and presence of CaM concentrations of suramin were chosen that correspond to the high-, intermediate-, and low-affinity suramin binding. Error bars are S.E.M. ($n = 4$). $^*$, CaM significantly decreased [$^3$H]ryanodine binding relative to the corresponding [suramin] in the absence of CaM ($p < 0.05$).

Fig. 12. Suramin displaces CaM bound to cardiac heavy SR. Western blotting was used to determine the presence of CaM in heavy SR. In the absence of suramin, CaM was associated with SR vesicles and could be detected by anti-CaM antibody (lane A). After incubation for 30 min on ice with 1 mM suramin and subsequent centrifugation at 20,000g, the pellet and supernatant, corresponding to membrane-bound and displaced CaM, respectively, were also immunoblotted. In the presence of suramin, most of the CaM was displaced from its binding sites in SR (lane B, pellet) because the majority of the CaM was found in the supernatant (lane C).

Fig. 13. Effects of CaM on the current amplitude at 0 mV of cardiac RyR channels activated by caffeine (5–30 mM) in the absence of activating cytosolic Ca$^{2+}$ (2.5 nM). A, an example of typical open events in the absence (left trace) and presence of 1 nM CaM (middle trace) and 4 μM CaM (right trace) are shown. C, closed channel level; O, fully open channel level. B, the current amplitude at 0 mV is shown for a range of CaM concentrations. Data points are mean ± S.E.M. ($n = 4$). The broken line indicates control values.

Fig. 14. Example of the reversibility of the effects of suramin on cardiac RyR channel function. On the left, current fluctuations in the presence of 10 μM cytosolic Ca$^{2+}$ are shown. Suramin (100 μM) added to the cytosolic chamber increases $P_o$ and current amplitude (middle trace). After exposure of the channel to suramin for 10 min, the cytosolic chamber was perfused with the control solution to remove suramin and any displaced CaM. C, closed channel level; O, fully open channel level.

Fig. 15. Competition experiments performed in the presence of 100 μM Ca$^{2+}$. The effect of suramin on [$^3$H]ryanodine binding to cardiac heavy SR is shown in the absence (□) and presence (□) of 5 μM CaM. Concentrations of suramin were chosen that correspond to the high-, intermediate-, and low-affinity suramin binding. Error bars are S.E.M. ($n = 4$). $^*$, CaM significantly decreased [$^3$H]ryanodine binding relative to the corresponding [suramin] in the absence of CaM ($p < 0.05$).
esis also fits with the published work of other investigators who have shown that CaM causes changes to the Ca\textsuperscript{2+}-activation profile of RyR channels (Fruen et al., 2000, 2003; Balshaw et al., 2001).

To investigate whether the suramin activation sites are also CaM binding sites, we investigated the effects of CaM in the presence and absence of activating cytosolic Ca\textsuperscript{2+} and examined whether CaM could modulate the effects of suramin. CaM itself was not able to increase \( P_o \) or conductance either in the presence or absence of activating Ca\textsuperscript{2+}, indicating that if CaM was binding to intermediate-affinity suramin sites, it must have very low efficacy. At 100 \( \mu \text{M} \) cytosolic Ca\textsuperscript{2+}, CaM antagonized the activating effects of 50 \( \mu \text{M} \) suramin on \(^3\text{H}\)ryanodine binding to cardiac SR (Fig. 15). This antagonistic effect of CaM cannot be the result of CaM binding to the inhibition sites because these sites are already saturated by this dose of suramin. The most likely explanation for the inhibition is that Ca\textsuperscript{2+}-bound CaM is binding to suramin activation sites but because CaM exhibits no efficacy itself, it is acting as an antagonist.

In summary, by manipulating cytosolic [Ca\textsuperscript{2+}] we have revealed the complex effects of suramin on the gating and conductance of the cardiac RyR. We have described high-affinity suramin inhibition sites that mediate a reduction in \( P_o \) and may be identical with CaM inhibition sites. The intermediate-affinity suramin binding sites mediate increases in both conductance and \( P_o \), whereas the low-affinity suramin binding sites are responsible for inactivation at high suramin concentrations. It is possible that CaM also binds to the intermediate-affinity suramin sites but is without any measurable efficacy; however, we have no evidence that the low-affinity suramin sites are also CaM binding sites. We also show for the first time that suramin can produce a graded increase in RyR single-channel conductance and that this effect is related to channel \( P_o \). Further studies are required to determine how RyR conductance can be linked to \( P_o \).

References
Fruen BR, Black DJ, Bloomquist RA, Bardy JM, Johnson JD, Louis CF, and Balag EM (2003) Regulation of the RyR1 and RyR2 Ca\textsuperscript{2+} release channel isoforms by Ca\textsuperscript{2+}-insensitive mutants of calmodulin. Biochemistry 42:2740–2747.
Safsten E, Williams AJ, and Sitsapesan R (2001) Markovian models of low and high activity levels of cardiac ryanodine receptors. Biophys J 80:2727–2741.

Address correspondence to: Dr. R. Sitsapesan, University of Bristol, Department of Pharmacology, School of Medical Sciences, University Walk, Bristol BS8 1TD, United Kingdom. E-mail: r.sitsapesan@bris.ac.uk