Activation of the Skeletal Muscle Ryanodine Receptor by Suramin and Suramin Analogs

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SUMMARY

Ca2+ release from skeletal muscle sarcoplasmic reticulum is activated by adenine nucleotides and suramin. Because suramin is known to interact with ATP-binding enzymes and ATP receptors (P2-purinergic receptors), the stimulation by suramin has been postulated to occur via the adenine nucleotide-binding site of the ryanodine receptor/Ca2+-release channel. We tested this hypothesis using suramin and the following suramin analogs: NF037, NF018, NF023, and NF007. The suramin analogs stimulate the binding of [3H]ryanodine binding to sarcoplasmic reticulum membranes with the following rank order of potency: suramin (EC50 = ~60 μM) > NF037 (EC50 = ~150 μM) > NF018 > NF023 > NF007. The suramin-induced stimulation occurs via a myoplasmic binding site on the ryanodine receptor as confirmed by binding experiments and single-channel recordings with the purified protein. This binding site is different than that for ATP, a conclusion that is supported by the following observations: (i) Suramin stimulates the association rate and inhibits the dissociation rate of [3H]ryanodine, whereas ATP analogs increase only the on-rate. (ii) In the presence of suramin but not of ATP analogs, [3H]ryanodine binding is resistant to the inhibitory effect of millimolar Mg2+ and Ca2+. (iii) ATP analogs and suramin have an additive effect on [3H]ryanodine binding. (iv) Affinity labeling of the purified ryanodine receptor with 2',3'-dialdehyde [γ-32P]ATP or after in situ oxidation of [γ-32P]ATP is not affected by suramin. Thus, our results show that suramin acts as a direct and potent stimulator of the ryanodine receptor but that this action is mediated via a binding site different from that for adenine nucleotides.

Cytoplasmic Ca2+ levels are tightly regulated by uptake into and release from intracellular stores as well as by fluxes across the plasma membrane. Signals generated at the plasma membrane are linked to the rapid efflux of Ca2+ from the intracellular stores by two types of release channels: the inositol trisphosphate receptors and the ryanodine receptors. The latter exist in three distinct isoforms, termed the skeletal muscle isoform (type 1), cardiac muscle isoform (type 2), and a ubiquitous isoform (type 3) (1). The regulation of the skeletal muscle ryanodine receptor/Ca2+-release channel is understood in great detail; the receptor resides on the terminal cisternae of the sarcoplasmic reticulum and is intricately linked to the voltage-sensitive L-type Ca2+ channel of the sarcolemmal T-tubular system (2). Although this specialized topology seems to include functional linkage, little is known about the physiological signal activating the ryanodine receptor (3–5). Several physiological and pharmacological ligands regulate the channel gating properties; these include inhibition by millimolar concentrations of Ca2+ or Mg2+ and micromolar concentrations of ruthenium red or ryanodine. Activation is achieved with micromolar concentrations of cytoplasmic Ca2+ and various exogenous ligands, such as the methylxanthine caffeine and the anthracycline doxorubicin (for a review, see Ref. 6). Adenine nucleotides are also involved in the modulation of the ryanodine receptor/Ca2+-release channel activity. Indirectly, ATP acts as the phosphate donor for regulatory phosphorylation of the ryanodine receptor by various protein kinases (7–10). In addition, adenine nucleotides induce Ca2+ release from sarcoplasmic reticulum vesicles. This effect is thought to result from direct interaction with the ryanodine receptor based on the following observations: (i) A stimulation of channel gating by adenine nucleotides is observed in single-channel recordings after insertion of the purified receptor in planar lipid bilayers (11–13). (ii) The purified ryanodine receptor

ABBREVIATIONS: AMP-PCP, β,γ-methylene-ATP; CHAPS, 3-[3-cholamidopropyl]dimethylammonio]propanesulfonate; MOPS, 3-(N-morpholino)propanesulfonic acid; PIPES, pipervazine-N,N'-bis[2-ethanesulfonic acid]; oATP, sodium periodate-oxidized ATP; P, open probability; EGTA, ethylene glycol bis(β-aminoethyl ether)-N,N',N'-tetraacetic acid.

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can be covalently labeled with adenine nucleotide analogs (14–16).

The trypanocidal drug suramin is known to inhibit many ATP-dependent enzymes and to block some classes of ATP receptors (P2X-purinergic receptors). Despite its many other actions, suramin has been used extensively as a pharmacological tool compound (17). Recently, Emmick et al. (18) reported that similar to ATP and other adenine nucleotides, suramin promoted binding of [3H]ryanodine to and triggered Ca2+ release from sarcoplasmic reticulum vesicles. Given the affinity of suramin for ATP-binding sites, the authors proposed that suramin activated the ryanodine receptor directly via interaction with its adenine nucleotide-binding site. We tested this hypothesis by determining the effects of suramin on both the ryanodine receptor in the sarcoplasmic reticulum vesicles and the purified protein. Our observations show that suramin is a potent activator of the ryanodine receptor. However, this stimulation occurs via a site distinct from that mediating the effect of adenine nucleotides.

**Experimental Procedures**

**Materials.** ATP and ADP were purchased from Boehringer-Mannheim Biochemica (Mannheim, Germany). AMP-PCP, ruthe-

nium red, and low-molecular-mass protein standards were from Sigma Chemical (St. Louis, MO). The high-molecular-mass standard kit was from Pharmacia (Vienna, Austria). Lipids were obtained from Avanti Polar Lipids (Birmingham, AL). Ryanodine was from Calbiochem (San Diego, CA). [3H]Ryanodine was from Amersham Buchler (Braunschweig, Germany). α-[32P]ATP and γ-[32P]ATP were from New England Nuclear Research Products (Boston, MA). All other reagents were of analytical grade.

Suramin was a generous gift from Bayer AG (Wuppertal, Ger-

many) and alternatively obtained from Calbiochem (Bad Soden, Ger-

many); suramin analogs were kindly provided by Dr. P. Nickel (Bonn, Germany). Their synthesis has been described previously (19): NF037 (8.8'-carbonylbis(imino-3,1-phenylene)carbonylaminol

(3,1-phenylene)bis-(1,3,5-naphthalenetrisulfonic acid)), NF018 (8-[3-(3-nitrobenzamido)benzamido]-1,3,5-naphthalenetrisulfonic acid), NF023 (8-[carbonyl(bis(imino-3,1-phenylene)bis-(1,3,5-

naphthalenetrisulfonic acid)), and NF007 (8-[3-nitrobenzamido]-

1,3,5-naphthalenetrisulfonic acid). oATP was synthesized according to the method of Easterbrook-Smith et al. (20) and purified as previously described (21); ATP or α-[32P]ATP was used as the starting material.

**Membrane preparation and protein purification.** Heavy sar-

coplasmic reticulum from rabbit white hind-leg and back muscle was prepared according to Wyskovsky et al. (22). In some experiments, cardiac sarcoplasmic reticulum was used that was prepared from calf hearts (10). The ryanodine receptor was purified by solubilization of heavy sarcoplasmic reticulum vesicles in CHAPS, followed by su-

crose density gradient centrifugation in the presence of CHAPS and exogenous phospholipids as previously described (23).

**Affinity labeling with oATP.** Sarcoplasmic reticulum mem-

branes (3 mg/ml) were sequentially pretreated with 1 μM oATP for 10 min, 80 mM NaCNBH3 for 1 min, and 40 mM NaBH4 for 30 min in labeling buffer consisting of 50 mM MOPS-NaOH, pH 7.2, 100 mM NaCl, 5 mM NADp, and 100 μM Pefabloc (4-2-aminoethoxy)benzoyl-

sulfonfylfluoride). The control reaction was performed in the absence of oATP. The incubation was carried out at room temperature, whereas the subsequent reduction with NaBH4 was done on ice. To remove free oATP, NaCNBH3, NaBH4, and reduction products, the membranes were centrifuged at 100,000 × g for 30 min and washed three times in labeling buffer. The pellets were resuspended in labeling buffer to give a final protein concentration of 0.8–1.5 mg/ml.

Labeling of the adenine nucleotide-binding site of the purified ryanodine receptor was done as previously described (16). Briefly, the ryanodine receptor (0.5–2.5 μg) was incubated in 20 μl of solution containing 20 mM Tris-HCl, pH 7.4, 30 μM CaC12, 5 mM MgCl2, 10 μM (α-[32P]ATP (specific activity, 12–60 cpm/mmol), and suramin at the concentrations indicated in the figure legends. After 15 min, reduction was initiated by the addition of 100 mM NaCNBH3 for (1 min at 25°) followed by the addition of 30 mM NaBH4 (1 hr on ice). Alternatively, [γ-32P]ATP was incorporated into the ryanodine re-

ceptor after in situ oxidation according to the procedure described for labeling of G protein α subunits with guanine nucleotides (24); briefly, 5 μg of purified ryanodine receptor was incubated in 20 μl of 50 mM MOPS-NaOH, pH 7.2, 100 mM KCl, 5 mM MgCl2, 0.5 mM [γ-32P]ATP (specific activity, 2000 cpm/mmol), and 0.5 mM EGTA with 0.6 mM CaCl2 or 2 mM EGTA for Ca2+-containing and Ca2+-free medium, respectively. After a 10-min incubation at room temperature, oxidation and reduction were achieved by the sequential addition of 4 mM NaIO4 for 1 min, followed by 80 mM NaCNBH3 for 1 min. To remove residual oxidation products, 10 mM NaBH4 was added and allowed to react for 1 hr on ice. The samples were directly diluted in 2X Laemmli sample buffer, to which 1% mercaptoethanol had been added. After the samples were heated at 95° for 2 min, the ryanodine receptor was resolved on discontinuous sodium dodecyl sulfate-poly-

acylamide gels (5% stacking gel and 7% separating gel). Exposure time for autoradiography varied from 6 to 48 hr (Kodak-Xomat film). Molecular mass standards were 212,000 Da for myosin, 170,000 Da for α2-macroglobulin, 116,000 Da for β-galactosidase, 76,000 Da for transferrin, and 53,000 Da for glutamic dehydrogenase.

**[3H]Ryanodine binding.** Sarcoplasmic reticulum membranes (25–50 μg) were incubated in 50 μl containing 25 mM MOPS, pH 7.2, 1 mM NaCl, 100 mM aprotinin, 1 μM leupeptin, 200 μM Pefabloc, and the concentrations of suramin analogs, adenine nucleotides, Mg2+ and [3H]ryanodine indicated in the figure legends. The free Ca2+ concentration was adjusted with CaCl2 and EGTA to give a concentration of 2 μM or it was varied, where applicable, by altering the ratio of EGTA to CaCl2. For [3H]ryanodine concentrations of >50 nM, the original specific activity (60–110 cpm/mmol) was diluted by the addition of unlabeled ligand. If not indicated otherwise, the incubation was carried out at 37° for 2 hr. The reaction was terminated by filtration over glass-fiber filters (presoaked in 1% polyethyleneimine) using a Skatron vacuum filtration device (Lier, Norway). The filters were rinsed with 10 ml of 25 mM MOPS-NaOH, pH 7.2, 1 mM NaCl, 0.48 mM CaCl2, and 0.5 mM EGTA. Nonspecific binding was determined in the presence of 10–100 μM ryanodine, which had been added to the incubation mixture before the radioligand. Suramin did not affect nonspecific binding.

In association experiments, the reaction was started by the addition of the sarcoplasmic reticulum to a prewarmed buffer solution containing 5–100 nM [3H]ryanodine and suramin or adenine nucleotides at the concentrations indicated in the figure legends. At the time points indicated, aliquots (50–1 μl aliquots containing 30 μg of protein) were withdrawn, and the reaction was stopped by filtration. In dissociation experiments, [3H]ryanodine (10–100 nM) was preincubated to 60 min with the sarcoplasmic reticulum membranes. Thereafter, dissociation was initiated by diluting the reaction mixture 50-fold (20 μl containing 25 μg of protein/1 ml) with binding buffer containing or lacking suramin or adenine nucleotides. Alternatively, an excess of unlabeled ryanodine (10 or 100 μM) was added in the absence and presence of suramin or adenine nucleotides to the reaction mixture; aliquots containing 30 μg of sarcoplasmic reticulum were withdrawn at the time points indicated in the figure legends, and the reaction was stopped by filtration. All association and dissociation experiments were performed at 37°.
started by the addition of 4 μg of purified ryanodine receptor. After 2 hr at 30°C, the samples were filtered over glass-fiber filters soaked with 5% polyethyleneimine, and the radioactivity trapped on the filter was determined by liquid scintillation counting.

If not otherwise indicated, experiments were carried out in duplicate, and each experiment was repeated at least three times with different protein preparations.

**Planar lipid bilayer measurements.** The purified ryanodine receptor was incorporated into planar lipid bilayers according to Herrmann-Frank et al. (23). Briefly, reconstitution was started in symmetrical buffer solutions containing 250 mM KCl, 150 μM Ca²⁺, 100 μM EGTA, and 20 mM Pipes-KOH, pH 7.2. Small aliquots of the purified ryanodine receptor (<1 μg of protein) were added to the cis side of the bilayer membrane defined as the cytoplasmic side of the channel. Successful incorporation of channels was detected as a steplike increase in current. Electrical signals were filtered at 1 kHz through an eight-pole, low-pass Bessel filter and digitized at 3 kHz. P₀ values were calculated from representative data segments of 20–60-sec duration selected from total recording times of ~8 min for any condition tested. The indicated holding potentials were applied with reference to the trans chamber. Experiments were carried out at 22°C.

**Miscellaneous procedures.** Protein concentration was measured by staining with amido black according to Kaplan and Petersen (25) or with the bichinchonic acid assay (Micro-BCA; Pierce Chemical, Rockford, IL) using bovine serum albumin as the standard. Free Ca²⁺ concentrations were calculated by a computer program using the binding constants of Fabiato (26). Data were fit by nonlinear least-squares regression to the appropriate equations describing monoeponential and biexponential decay and association as well as saturation isotherms using the Gauss-Newton or Marquardt-Levenberg algorithm.

**Results**

**Equilibrium binding of [³H]ryanodine in the presence of suramin.** When added to the incubation mixture, suramin and the nonhydrolyzable adenine nucleotide analog AMP-PCP stimulated the binding of [³H]ryanodine to sarcoplasmic reticulum membranes (Fig. 1). After Scatchard transformation, the data clearly exhibited two ryanodine-binding sites. Although the sum of the ryanodine-binding sites were constrained constant, in the presence of AMP-PCP or suramin, the Bₘₐₓ value for the low affinity site shifted toward the high affinity site (parameters are given in the legend to Fig. 1A). The low affinity ryanodine-binding site in these saturation experiments was determined at ≤500 nm of ryanodine. This concentration is close to the calculated Kᵦ value for this site and therefore is only partially saturated. However, in the presence of suramin, the Kᵦ value of the low affinity ryanodine binding site is decreased by a factor of 2.

Similar effects were seen when suramin was tested on the purified skeletal muscle ryanodine receptor (data not shown). We conducted kinetic experiments to determine whether the ability of suramin to stimulate equilibrium binding of [³H]ryanodine resulted from an effect of suramin on the association or the dissociation rate. Both suramin and AMP-PCP increased the apparent association rate of [³H]ryanodine (Fig. 2A). Similarly, if the ryanodine concentrations were varied over a range of 10–100 nm, the apparent on-rate (kₐpp) for [³H]ryanodine binding was stimulated at each concentration of the radioligand by both suramin and AMP-PCP to approximately equivalent extents (Fig. 2B). The kₐpp for [³H]ryanodine binding increased in a quasilinear manner if the concentration of [³H]ryanodine was raised from 10 to 100 nm

(Fig. 2B, dotted lines). However, the intercept of the regression lines with the y-axis yielded kₐff values of 0.0275 min⁻¹ without the addition and 0.0375 min⁻¹ with the addition of suramin or AMP-PCP. These values did not correspond with the off-rates determined directly in dissociation experiments (Fig. 3A). The apparent dissociation rate constant kₐff values have been added to the plot at the concentration of 0 nm ryanodine (Fig. 2B, open symbols) to illustrate this anomaly, which is consistent with the fact that binding of ryanodine to its receptor deviates from a simple bimolecular reaction (27, 28).
Fig. 2. Association of [3H]ryanodine binding to sarcoplasmic reticulum membranes in the absence and presence of suramin or AMP-PCP. A, Sarcoplasmic reticulum membranes were added to prewarmed buffer containing 20 nm [3H]ryanodine and a free Ca^{2+} concentration of 2 μM in the absence and presence of 2 mM AMP-PCP or 0.3 mM suramin. At the time points indicated, aliquots (50 μL containing 30 μg of sarcoplasmic reticulum membrane protein) were withdrawn and immediately filtered as described in Experimental Procedures. Data are the mean of duplicate determinations in a representative experiment. B, Apparent on-rates (k_{app}) for binding of increasing [3H]ryanodine concentrations were determined in the absence or presence of 2 mM AMP-PCP or 0.3 mM suramin as in A and plotted as a function of the radioligand concentration. Data are the mean ± standard deviation of three independent experiments carried out in duplicate. Dotted lines, linear regression through the three sets of k_{app} values based on the relation k_{app} = k_{on} * L + k_{off}. Data points (open symbols) at 0 nm ryanodine represent the k_{off} rates determined from the experiments in Fig. 3A.

Dissociation of the bound ligand can be initiated in two ways: by the addition of excess unlabeled ligand or by "infinite" dilution. For binding of [3H]ryanodine to the Ca^{2+}-release channel protein, these two methods do not yield equivalent results. When micromolar concentrations of unlabeled ryanodine (10–100 μM) are added at equilibrium, the dissociation of [3H]ryanodine is extremely slow, and the fraction of binding that dissociates with appreciable rates is progressively decreased (see below and Ref. 29). When dissociation was initiated by dilution of the reaction mixture (Fig. 3A), bound [3H]ryanodine was released with a rate constant of 0.011 ± 0.0004 min⁻¹; suramin slowed the rate of disso-

Fig. 3. Dissociation of [3H]ryanodine from sarcoplasmic reticulum membranes in the absence and presence of suramin or AMP-PCP. A, Sarcoplasmic reticulum membranes were allowed to bind 10 nm [3H]ryanodine at a free Ca^{2+} concentration of 2 μM. After 1 hr (time = 0), the incubation was split and diluted 50-fold into ryanodine-free incubation buffer, buffer supplemented with 2 mM AMP-PCP, or buffer supplemented with 0.3 mM suramin. Aliquots of the diluted samples (1 ml containing 25 μg of sarcoplasmic reticulum membrane protein) were filtered at the indicated time points. Data represent the mean ± standard deviation of four independent experiments. Equilibrium binding determined before dilution amounted to 1.9 ± 0.2 pmol/mg and was set 100% to normalize for interassay variation. B, Sarcoplasmic reticulum membranes were allowed to bind 100 nm [3H]ryanodine at a free Ca^{2+}
ciation (0.0065 ± 0.0003 min⁻¹), whereas AMP-PCP had no effect (0.010 ± 0.0007 min⁻¹). Although the difference in the rates was modest, the effect was statistically significant (p = 0.02; paired t test; four experiments). Under all three conditions, the dissociation kinetics followed a monoeXponential decay, resulting in linear plots on logarithmic transformation (Fig. 3A, inset). We cannot rule out that a deviation from this monoeXponential dissociation may have been detected if the dissociation reaction had been observed over a longer incubation period. However, under our experimental conditions, [³H]ryanodine binding progressively declined with incubation times of >3 hr. This made it impossible to differentiate between loss of binding due to dissociation of the ligand and denaturation/degradation of the channel protein.

In contrast, when dissociation experiments were carried out with 100 nM [³H]ryanodine (Fig. 3B), 24 ± 2% and 16 ± 2% of the bound ligand were rapidly released (k_{off} = ~1 min⁻¹) under control conditions (Fig. 3B, ○) and in the presence of AMP-PCP (Fig. 3B, ■), respectively. This is consistent with the occupancy of a sizeable fraction of low affinity sites at 100 nM [³H]ryanodine (see Fig. 1). The remaining radioligand dissociated with rate constants comparable to those determined in the presence of 10 nM [³H]ryanodine (k_{off} = 0.009 ± 0.001 and 0.011 ± 0.001 min⁻¹ under control conditions and in the presence of AMP-PCP, respectively). In contrast, after the addition of suramin (Fig. 3B, ▲), the rapid dissociation was suppressed and virtually undetectable (this fraction was calculated to represent 4% of the ligand bound with an error greater than the parameter estimate). The off-rate for the slow component in the presence of suramin (k_{off} = 0.0065 ± 0.001) was again essentially identical to that determined with 10 nM [³H]ryanodine. The inhibition of fast ryanodine dissociation phase cannot be accounted for by the rebinding of the radioligand after dilution but rather is due to a direct effect of suramin. AMP-PCP, however, was not able to suppress the fast initial dissociation (Fig. 3B), whereas in association experiments, AMP-PCP, like suramin, stimulated the apparent on-rate of the radioligand (Fig. 2B). The increase in affinity for the low affinity ryanodine-binding site in the presence of suramin is possibly not only accounted for by the acceleration of the association of ryanodine but also due to the effect on the fast dissociation kinetics observed in Fig. 3B.

To calculate the K_D value from the kinetics parameters, we used the apparent on-rates of 0.0312 ± 0.0032 min⁻¹ for the control, 0.0432 ± 0.0026 min⁻¹ in the presence of AMP-PCP, and 0.0390 ± 0.0034 min⁻¹ in the presence of suramin, as derived from measurements with 10 nM [³H]ryanodine (Fig. 2B). Based on calculations with the experimentally determined k_{off} parameters, we obtained K_D values of 5, 3, and 2 nM for control conditions, AMP-PCP, and suramin, respectively. These parameters are in agreement with the directly determined K_D values given in the legend to Fig. 1A. In contrast, use of the extrapolated k_{off} values determined graphically (see Fig. 2B) resulted in K_D values that were 15–125-fold higher.

High ryanodine concentrations (100 µM) occlude the ryanodine receptor in a refractory state so that the prebound ligand does not exchange with the added unlabeled ligand (30). We determined whether suramin modifies this reaction (Fig. 3C). When [³H]ryanodine binding was allowed to proceed to equilibrium and 100 µM unlabeled ryanodine was then added to induce dissociation, essentially all of the ligand remained bound (Fig. 3C, ○). Neither suramin (Fig. 3C, ▲) nor AMP-PCP (Fig. 3C, ■) had an effect on this ryanodine-induced inhibition of ligand dissociation. Taken together, these observations indicate that AMP-PCP stimulates high affinity equilibrium binding of [³H]ryanodine primarily by accelerating the association rate, whereas suramin has an additional effect on the off-rate of [³H]ryanodine. Neither AMP-PCP nor suramin prevents the ryanodine-induced occluded state.

Structure-function relationship of suramin analogs. A panel of suramin analogs was previously characterized as inhibitors of P_2-purergic receptors and of the plasma membrane ecto-ATPase (19). We selected four analogs of varying size and charge and tested their ability to promote [³H]ryanodine binding (Fig. 4). The rank order of potency was suramin (EC_{50} = 57 ± 18 µM) > NF037 (didemethylated suramin; EC_{50} = 148 ± 15 µM) > NF018 (the half-molecule of NF037) > NF023 (NF037 lacking one benzamide ring on both sides of the central urea bridge) > NF007 (the half molecule of NF023, which was essentially ineffective). These experi-

Fig. 4. Structure-activity relationship of suramin analogs for stimulation of [³H]ryanodine binding to sarcoplasmic reticulum membranes. Increasing concentrations of suramin, NF037, NF023, NF018, and NF007 were added to sarcoplasmic reticulum membranes (30 µg) at 10 nM [³H]ryanodine and 2 µM free Ca^{2+}. After 45 min at 37°C, the samples were filtered. Data are mean ± standard deviation from three independent experiments carried out in duplicate. Binding determined in the absence of suramin analogs (●) amounted to 1.2 ± 0.3 pmol/mg and was set 100% to normalize for interassay variation.

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**Suramin-Induced Activation of the Ryanodine Receptor**

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**Fig. 3C.**
ments were carried out under nonequilibrium conditions [i.e., the incubation lasted 45 min (see Fig. 2A)], which gave a more favorable signal-to-noise ratio to detect effects of weakly active analogs. However, the EC₅₀ value for suramin was identical if measured under equilibrium conditions (EC₅₀ = 63 ± 21 μM; three experiments).

We also determined the EC₅₀ value for suramin on the purified ryanodine receptor and obtained a value of 43 ± 23 μM (three experiments; data not shown). In addition, the effect of suramin was investigated on ryanodine binding to cardiac sarcoplasmic reticulum membranes prepared from calf hearts. When the vesicles were incubated in the presence of 10 nM [³H]ryanodine and a free Ca²⁺ concentration of 2 μM, suramin stimulated binding by a factor of ~2 (0.52 ± 0.25 and 1.1 ± 0.3 pmol/mg in the absence and presence of 0.3 mM suramin, respectively). Therefore, the effect of suramin is not restricted to the skeletal isoform of the ryanodine receptor.

Effect of regulatory ligands in the presence of suramin. The dissociation experiment (see Fig. 3A) indicated that suramin and AMP-PCP induced different effects on the binding characteristics of the ryanodine receptor. We therefore tested whether suramin affected the interaction of other regulatory ligands with the ryanodine receptor. At Mg²⁺ concentrations of >1 mM, channel gating and ryanodine binding are progressively suppressed (12, 29) (Fig. 5A). Half-maximal inhibition of [³H]ryanodine binding was observed at concentrations of 13.2 ± 2.4 and 16.3 ± 2.0 mM Mg²⁺ in the absence and presence of AMP-PCP, respectively. However, in the presence of suramin, this Mg²⁺-dependent inhibition was relieved so that at a concentration of 100 mM, Mg²⁺ caused only a modest reduction in [³H]ryanodine binding of ~30% from the stimulated value (Fig. 5A). In contrast to Mg²⁺, the inhibition of the ryanodine binding by ruthenium red was not appreciably affected by suramin (Fig. 5B).

Channel gating of the Ca²⁺-release channel and ryanodine binding are highly dependent on the free Ca²⁺ concentration. Under the assay conditions that we used, essentially no high affinity ryanodine binding was observed unless the Ca²⁺ concentrations were >0.5 μM. The concentration-response curve for Ca²⁺ was steep and bell shaped; at concentrations of ≥1 mM, Ca²⁺ inhibited [³H]ryanodine binding in the absence and presence of 1 mM AMP-PCP (Fig. 6A, A and B). The Ca²⁺ dependency of [³H]ryanodine binding was not altered by the ATP analog (Fig. 6A) (31). In contrast, suramin had two effects on the concentration-response curve for Ca²⁺ (Fig. 6A, A). First, the inhibition at high Ca²⁺ concentrations was absent; second, ryanodine binding was promoted at low Ca²⁺ concentrations, whereas the Ca²⁺-stimulated component of ryanodine binding was only modestly affected. This phenomenon is exemplified in Fig. 6B. High affinity binding of [³H]ryanodine was observed in a nominally Ca²⁺-free solution (0 mM CaCl₂ and 0.5 mM EGTA) in the presence of suramin (B), whereas it was virtually undetectable in its absence (●). Furthermore, suramin had no effect on the nonspecific [³H]ryanodine binding under these conditions (Fig. 6B, open symbols).

Effects of the combined addition of adenine nucleotides and suramin. If suramin exerted its effect on [³H]ryanodine binding via the adenine nucleotide-binding site of the ryanodine receptor, any effect of an adenine nucleotide should be precluded at saturating concentrations of suramin; as can be seen from Fig. 7A, this was clearly not the case. In

![Fig. 5. Inhibition of [³H]ryanodine binding by (A) Mg²⁺ and (B) ruthenium red in the absence and presence of suramin. A, Sarcoplasmic reticulum membranes (50 μg) were incubated with 10 nM [³H]ryanodine at 2 μM free Ca²⁺ and the indicated concentrations of MgCl₂ in the (○) absence and presence of (●) 1 mM AMP-PCP or (▲) 0.3 mM suramin. The incubation lasted 90 min. Data represent the mean of duplicate determinations in a single experiment. B, Sarcoplasmic reticulum membranes (30 μg) were incubated with 20 nM [³H]ryanodine at 2 μM free Ca²⁺ for 90 min and the following ligands: no addition (control), 20 μM ruthenium red (ru 20), 100 μM suramin (sur), 100 μM suramin plus 2 μM ruthenium red (sur+ru 2), and 100 μM suramin plus 20 μM ruthenium red (sur+ru 20). Data represent the mean of two experiments carried out in duplicate. Error bars, standard deviation.](image-url)
were of concentrations Ca2+

**Fig. 6.** Effect of Ca2+ on [3H]ryanodine binding in the absence and presence of suramin. A, Sarcoplasmic reticulum membranes (50 μg) were incubated with 20 nm [3H]ryanodine for 90 min. The indicated free Ca2+ concentrations were adjusted with EGTA and CaCl2. Ca2+ concentrations exceeding 100 μM were adjusted only by the addition of CaCl2. The incubation was performed in the absence and presence of 1 μM AMP-PCP or (A) 0.3 mM suramin. Data represent the mean of two experiments carried out in duplicate. Error bars, standard deviation. B, [3H]Ryanodine binding was performed in buffer containing 0.5 mM EGTA and no added CaCl2. Sarcoplasmic reticulum membranes (30 μg) were incubated for 90 min in the absence and presence of 0.3 mM suramin. Total binding. C and D, Non-specific binding. Data represent the mean of duplicate determinations in a single experiment that was reproduced twice.

concentration of suramin did not preclude the caffeine-induced stimulation of [3H]ryanodine binding. Similar to the combination of AMP-PCP and suramin, the combination of suramin and caffeine resulted in additive stimulation of [3H]ryanodine binding (data not shown).

We have previously shown that the ATP analog oATP binds to the ryanodine receptor in a quasi-irreversible manner via Schiff's base formation with lysine residues in the adenine nucleotide-binding pocket. Subsequent reduction with NaCNBH3/NaBH4 results in covalent incorporation of oATP (16). We exploited this property of oATP through an alternative approach to show that suramin and adenine nucleotides acted independently (Fig. 7B). The sarcoplasmic reticulum was preincubated in the absence and presence of oATP followed by the addition of NaCNBH3/NaBH4. The reaction products were subsequently removed through centrifugation. When the preincubation was done in the absence of oATP, both AMP-PCP and suramin stimulated [3H]ryanodine binding (Fig. 7B, control); this stimulation was not affected by the sole addition of NaCNBH3/NaBH4 (Fig. 7B, NaBH4). In contrast, when the preincubation was carried out with oATP and the reducing agents NaCNBH3/NaBH4 were subsequently added, [3H]ryanodine binding was enhanced and no additional stimulation was observed in the presence of AMP-PCP (Fig. 7B, oATP/NaBH4). This was to be expected because the adenine nucleotide-binding site was occu-
plied by covalently incorporated oATP. More importantly, this incorporation did not block the stimulation by suramin.

**Affinity labeling of the ryanodine receptor.** Based on their functional effects on [3H]ryanodine binding, adenine nucleotides and suramin do not seem to share a common binding site on the Ca2+-release channel protein. To obtain more direct evidence for this interpretation, we labeled the purified ryanodine receptor with radioactive oATP in the absence and presence of suramin. Two methods were used. The purified protein was incubated in the presence of 10 μM \( \alpha-32P \) oATP followed by reduction with NaBH\(_4\) (Fig. 8A); we have previously shown that this labeling is specific because it is blocked by unlabeled ATP in a competitive manner (16). Alternatively, the incubation was carried out in the presence of 0.5 mM \( \gamma-32P \) ATP, and in situ oxidation and reduction were achieved by the sequential addition of NaI\(_4\), NaC- NBH\(_4\), and NaBH\(_4\) (Fig. 8B). We rule out that labeling under the latter condition was due to phosphorylation by a contaminating protein kinase because radioactive labeling was not observed when NaI\(_4\) and the reducing agents were omitted (not shown). The gel slices corresponding to the ryanodine receptor were also excised, and the radioactivity was determined by liquid scintillation counting. Radioactive labeling determined in this manner remained unaffected by the addition of suramin. Therefore, regardless of the method used, suramin did not affect the incorporation of oATP into the ryanodine receptor. This confirms that suramin does not block the interaction of adenine nucleotide with the binding site on the Ca\(^{2+}\)-release channel protein. A minor band is visible in the autoradiogram of Fig. 8A; this corresponds to the top of the separating gel, where some of the radioactivity is retained. It is unclear whether this is due to retention of labeled protein or simply due to interaction of the radioactive substrate with the gel matrix.

**Activation of the isolated Ca\(^{2+}\)-release channel/ryanodine receptor complex.** To investigate a possible direct interaction of suramin with the ryanodine receptor, the purified protein was incorporated into planar lipid bilayers. The effect of suramin on the gating properties of the reconstituted Ca\(^{2+}\)-release channel was studied in the presence of 0.9 μM cytoplasmic (cis) Ca\(^{2+}\) (i.e., under conditions in which the channel is essentially inactive). As shown in Fig. 9, the channel was activated in a concentration-dependent manner when suramin was added to the cis bilayer chamber. Suramin did not exert an activating effect when applied to the luminal (trans) side of the channel. Suramin activation was characterized by alternating periods of high and low activity that lasted 60–90 sec. Periods of low activity revealed \( P_0 \) values similar to those in the absence of suramin. The \( P_0 \) values calculated from periods of high activation were used to obtain the dose-response curve shown in Fig. 9B, whereas the periods of low activity were excluded from the analysis. This approach is based on the following consideration: If we calculated the \( P_0 \) value for segments, including low and high activity, the values would represent neither the low nor the high activity state of the channel. In addition, calculated open-time constants would be misleading. Because in the absence of suramin only one open state was found but two open states were found during the high activity periods induced by suramin, open-time constants calculated from segments including both types of activity would not reflect the true gating property of the channel. Using this approach, we estimated that suramin activated the channel half-maximally at 207 μM. This apparent affinity of suramin is lower than that obtained if [3H]ryanodine binding was used as the readout system to measure the effects of suramin (EC\(_{50}\) = 40–80 μM). This discrepancy can be explained by the different free Ca\(^{2+}\) concentrations used in the two approaches. Increasing the concentration of free Ca\(^{2+}\) 5 μM in the single-channel recordings shifted the concentration-response curve to the left, with EC\(_{50}\) values comparable to those determined in binding experiments; this suggests that the apparent affinity of suramin is dependent on the free Ca\(^{2+}\) concentration (data not shown).

The analysis of the gating kinetics revealed that the activating effect of suramin is accompanied by an increase in the duration of channel open events. Representative open-time histograms in the presence of Ca\(^{2+}\) and suramin are shown in Fig. 10. At a low Ca\(^{2+}\) concentration, the corresponding open-time histograms could be adequately fit by a single exponential function with a time constant of 0.3 msec. When the channel was activated by suramin, a second exponential component had to be introduced (\( \tau_1 = 1.3 \) msec; \( \tau_2 = 4.6 \) msec). Compared with the activation in the presence of 50 μM Ca\(^{2+}\), both time constants were increased 2–3-fold. The addition of ATP also resulted in an increase of the two open-time constants but did not induce long periods of high and low activity as were observed in the presence of suramin (11).

At concentrations in the low micromolar range, ryanodine
transferred the skeletal and cardiac Ca\textsuperscript{2+}-release channel into a characteristic long open state of reduced conductance (30, 32, 33). This property was retained by the suramin-activated channel (Fig. 9A). At ~5 min after the addition of 0.6 μM ryanodine, the suramin-activated channel was transferred into the typical long open half-conductance state (Fig. 9A, bottom trace), which was not further modified by other known modulators of the ryanodine receptor. The applied suramin-induced Activation of the Ryanodine Receptor

**Fig. 9.** Activation of the isolated Ca\textsuperscript{2+}-release channel by suramin. A, Representative single-channel K\textsuperscript{+} currents of the purified protein in the presence of the indicated modulators [suramin (Sur) and ryanodine (Rya)] successively added to the cis side of the channel. The free Ca\textsuperscript{2+} concentration was adjusted by the addition of EGTA. P\textsubscript{o} values calculated from data segments of 20–60-sec duration were 0.202 (50 μM Ca\textsuperscript{2+}), 0.003 (0.9 μM Ca\textsuperscript{2+}), 0.356 (plus 300 μM Sur), and 0.690 (plus 500 μM Sur). The holding potential was −41 mV. Downward deflections, open events; bottom trace, typical subconductance state induced 5 min after the addition of 0.6 μM ryanodine in the presence of 500 μM suramin. B, Dependence of P\textsubscript{o} value on applied suramin concentration. P\textsubscript{o} values were calculated from single-channel recordings in the presence of 0.9 μM Ca\textsuperscript{2+} and indicated concentrations of suramin. Error bars, mean ± standard error from five independent experiments.

Ryanodine concentration of 0.6 μM is lower than the effective concentration range described for ryanodine induced modification of the Ca\textsuperscript{2+}- and ATP-activated channel (30, 32, 33). This observation suggests that suramin renders the Ca\textsuperscript{2+}-release channel more sensitive to ryanodine. Channel activity was also inhibited by micromolar concentrations of ruthenium red (data not shown), indicating that the basic pharmacological properties of the Ca\textsuperscript{2+}-release channel are preserved in the presence of suramin.

**Discussion**

We characterized the interaction of suramin with the ryanodine receptor. Our observations show that suramin is a direct activator of the channel protein. This conclusion is based on two sets of observations. First, suramin promoted binding of [3H]ryanodine to both the channel protein in sarcoplasmic reticulum membranes of skeletal and cardiac muscle and the protein purified from skeletal muscle. Second,
suramin increased the $P_r$ value of the purified channel protein after insertion into a planar lipid bilayer. These findings confirm and extend those of a previous report (18). More importantly, we designed a series of experiments to test the working hypothesis put forth previously (18): that suramin and adenine nucleotides exert similar effects on the ryanodine receptor because they interact with a common binding site.

Our experiments unequivocally demonstrate that suramin does not interact with the adenine nucleotide-binding site of the ryanodine receptor for the following reasons. (i) ATP and suramin have distinct actions on the kinetics of ryanodine binding; both adenine nucleotides and suramin stimulate the apparent on-rate, whereas only suramin slows the dissociation rate. (ii) The effects of suramin and adenine nucleotides are not mutually exclusive but rather are additive; saturating concentrations of suramin do not occlude the stimulation by AMP-PCP, and stimulation by suramin persists after irreversible activation with oATP. (iii) Suramin does not block affinity labeling by the ATP analog oATP. (iv) Mg$^{2+}$ inhibits the AMP-PCP-induced stimulation of ryanodine binding, whereas it is virtually ineffective with suramin. Suramin may either sterically block the access of the Mg$^{2+}$ to the regulatory site or induce a conformation rendering the protein refractory to the inhibitory effect. In contrast, the inhibitory effects of ruthenium red and high ryanodine concentrations were not altered by suramin.

The binding of $[^3H]$ryanodine is complex, and the apparent affinity constants derived from equilibrium binding experiments depend on the assay conditions. In general, two affinity binding sites can be derived in saturation experiments with sarcoplasmic reticulum membranes: a high affinity site with a $K_D$ value of $\sim 5$ nm and a low affinity site with a $K_D$ value in the submicromolar range (34). Affinity estimates consistent with these values were also calculated from the current saturation experiments. However, a detailed kinetic analysis has revealed as many as four interdependent affinity states/binding sites (27), and our observations are in line with these findings. First, the increment in the apparent on-rate with increasing concentrations of ligand suggests a quasilinear relationship between $[^3H]$ryanodine (10–100 nm) and $k_{app}$; however, if binding occurred via a simple bimolecular reaction, extrapolation to zero ligand concentration ($L$) should yield $k_{off}$ based on the following relation: $k_{app} = k_{on} \cdot L + k_{off}$. This clearly was not the case. The kinetically determined dissociation constant $K_D$ thus depends on which ligand concentration is used to derive this parameter from the association and dissociation rate constants. In addition, the fact that the addition of an excess of unlabeled ryanodine failed to displace the radioligand bound at equilibrium (Fig. 3C and Refs. 30 and 35) demonstrates that multiple non-equivalent ryanodine-binding sites exist within the tetrameric protein and that these are linked in a cooperative manner. Finally, high affinity binding of $[^3H]$ryanodine is too slow (by 3–4 orders of magnitude) to be a simple diffusion-limited reaction. It is therefore plausible to postulate that the rate-limiting step is the access of ryanodine to the binding site and that this reaction requires the open-channel conformation (36, 37); under these assumptions, suramin and other agonists (e.g., adenine nucleotides and Ca$^{2+}$ at micromolar concentrations) promote binding of ryanodine primarily by accelerating the association rate via channel opening. This interpretation is consistent with the increased $P_r$ value observed in single-channel recordings in the presence of suramin. We have no mechanistic explanation for the modest effect of suramin on the dissociation rate of ryanodine.

Study of the effect of suramin on the purified Ca$^{2+}$-release channel reconstituted into planar lipid bilayers proved that suramin directly interacts with the ryanodine receptor. Suramin activated the Ca$^{2+}$-release channel in a concentration-dependent manner without changing the conductance of the channel (Fig. 9). Comparable to the effect of ATP (11–13), the suramin-induced activation was accompanied by an increase in the duration of open-channel events (Fig. 10). Fitting the channel open-time histograms suggested the presence of one open state in the presence of 0.9 $\mu$M Ca$^{2+}$. In the presence of suramin, a second open-time constant was necessary to fit the data, indicating the presence of at least two open states. In addition, suramin increased the number of open events of a 20-sec file from 60 to 650 events. The same data set revealed that suramin increased the $P_r$ value of the channel from ~1% to 44% (Fig. 9B). In conclusion, suramin exerts its activating effects by increasing the number of open events and by inducing a second open state of the channel. However, the general activation pattern was different from that typically observed in the presence of ATP. In contrast to ATP, suramin induced a transient activation of the Ca$^{2+}$-release channel. Periods of high activation that lasted ~2 min were followed by periods of low activity comparable to those occurring in the absence of suramin. Although we did not investigate the simultaneous activation of the isolated channel by adenine nucleotides and suramin, these data strongly support our conclusion that ATP and suramin act on different binding sites of the ryanodine receptor. Unlabeled ryanodine occludes $[^3H]$ryanodine prebound to the receptor rather than causing dissociation of the radioligand. In single-channel recordings of the ryanodine receptor, high concentrations of ryanodine induce a long-lasting subconductance state of the channel that switches into a closed state on further increases in ryanodine concentration (Fig. 9) (30, 32, 33). Both phenomena, which may reflect the same ryanodine-induced conformational change of the channel protein, are unaffected by suramin.

Suramin has pleiotropic effects and binds to many receptors and enzymes (17). It is popular among pharmacologists because it is used to subclassify $P_2\_x$-purinergic receptors; suramin is a competitive antagonist for ATP at the $P_2\_x$- and $P_2\_y$-purinoceptors (38). It is clear that binding of suramin requires positive charges on the surface of the proteins (39, 40). Nevertheless, differences in the structure-activity relation for the suramin analogs that we used are readily apparent if their affinity for $P_2\_x$-purinergic receptors and ecto-ATPases (19) as well as G proteins (41, 42) is compared with their ability to promote $[^3H]$ryanodine binding. This is best exemplified with NF023; this compound is a high affinity inhibitor of G proteins of the $G_{\alpha}/G_{\alpha_{o}}$ group (41) and a potent antagonist at $P_2\_x$-purinergic receptors (19). However, NF023 (with six negative charges) has only a very modest effect on $[^3H]$ryanodine binding, and NF018 (the half-molecule of NF037 with only three negative charges) is more active. In addition, NF037 (didemethylated of suramin) has a 3-fold lower apparent affinity than suramin. This indicates that the negative charges are not the sole determinants of activation of the ryanodine receptor.
We conclude from our findings that the suramin-binding site is distinct from that for other regulatory ligands, such as ATP, ryanodine, and activating Ca$^{2+}$. Ryanodine receptors play a crucial role as molecular switches in the phasic release of Ca$^{2+}$ and in the amplification of Ca$^{2+}$-induced Ca$^{2+}$ release. We therefore believe that this class of intracellular channels may represent a potential target for drug action, in particular, in pathophysiological situations associated with cellular Ca$^{2+}$ overload (43). A search for inhibitory ligands that target the suramin-binding site on the various ryanodine receptor isoforms may yield therapeutically useful drugs.

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References

11. Lai, F. M. Miura, L. Xu, H. F. Smith, and G. Meissner. The ryanodine receptor Ca$^{2+}$ release channel complex of skeletal muscle sarco(endo)plasmic reticulum: evidence for a chloride:Ca$^{2+}$ antisomotropic, negatively charged homo

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