Suramin Interacts with the Calmodulin Binding Site on the Ryanodine Receptor, RYR1*

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Apocalmodulin and Ca2+ calmodulin bind to overlapping sites on the ryanodine receptor skeletal form, RYR1, but have opposite functional effects on channel activity. Suramin, a polysulfonated napthylurea, displaces both forms of calmodulin, leading to an inhibition of activity at low Ca2+ and an enhancement of activity at high Ca2+. Calmodulin binding motifs on RYR1 are also able to directly interact with the carboxy-terminal tail of the transverse tubule dihydropyridine receptor (DHPR) (Sencer, S., Papineni, R. V., Halling, D. B., Pate, P., Krol, J., Zhang, J. Z., and Hamilton, S. L. (2001) J. Biol. Chem. 276, 38237–38241). Suramin binds directly to a peptide that corresponds to the calmodulin binding site of RYR1 (amino acids 3609–3643) and blocks the interaction of this peptide with both calmodulin and the carboxy-terminal tail of the DHPR α-subunit. Suramin, added to the internal solution of voltage-clamped skeletal myotubes, produces a concentration-dependent increase in the maximal magnitude of voltage-gated Ca2+ transients without significantly altering L-channel Ca2+ channel conducting activity. Together, these results suggest that an interaction between the carboxy-terminal tail of the DHPR α-subunit with the calmodulin binding region of RYR1 serves to limit sarcoplasmic reticulum Ca2+ release during excitation-contraction coupling and that suramin-induced potentiation of voltage-gated Ca2+ release involves a relief of this inhibitory interaction.

The skeletal muscle ryanodine receptor (RYR1) functions as a sarcoplasmic reticulum (SR) Ca2+ release channel that plays a central role in excitation-contraction coupling. Two distinct mechanisms are postulated to contribute to the release of Ca2+ from the SR in skeletal muscle. After sarcoplasmic depolarization, SR Ca2+ release channels are initially activated via mechanical coupling with DHPRs (or L-type Ca2+ channels) located in the surface membrane (2). However, morphological data indicate that only every other SR Ca2+ release channel is directly coupled with sarcolemmal DHPRs (3). Adjacent, non-DHPR-coupled release channels (4) are thought to be activated either by Ca2+ released via the mechanically coupled channels (3) or by a coordinated or “coupled-gating” mechanism of activation (5).

RYR1 and DHPR proteins bind CaM in both its Ca2+-bound and Ca2+-free forms (6–9). Overlapping binding sites for apoCaM and Ca2+-CaM are located between amino acids 3614 and 3643 of RYR1 (10, 11). The carboxy-terminal tail of the DHPR α-subunit (12, 13) appears to have binding sites for both forms of CaM. C3635, located within the putative CaM binding region of RYR1, has been postulated to contribute to oxidation-induced intersubunit cross-linking (14) and was recently demonstrated to be the site of CaM-dependent NO modulation of RYR1 (15).

Studies of the interaction of both the DHPR and RYR1 with apoCaM and Ca2+-CaM have been primarily carried out with uncoupled channels, raising the question of whether CaM interacts with either channel when the two proteins are mechanically coupled in intact skeletal muscle. Slavik et al. (12) provided evidence that a sequence within the carboxyl terminus of the DHPR α-subunit interacts strongly with RYR1. This sequence was later shown to also be a CaM binding motif (13). More recently, Sencer et al. (1) demonstrated that the CaM binding site on RYR1 binds directly to the carboxy-terminal tail of the DHPR α-subunit. These findings suggest that the CaM binding motifs on the DHPR and RYR1 proteins may actually function as protein-protein interaction motifs rather than strictly as CaM binding domains. However, the functionally relevant binding partners for these motifs have not yet been identified. If the DHPR and RYR1 proteins utilize CaM binding motifs for binding to one another, then CaM could potentially uncouple the mechanical link formed between the CaM binding site of RYR1 and the carboxy-terminal tail of the DHPR α-subunit. However, the functional consequences of such CaM-mediated uncoupling have yet to be evaluated.

Suramin, a polysulfonated napthylurea is a potent, reversible activator of the RYR, increasing both conductance and P0 of the channel (16). Klinger et al. (17) found that suramin inhibits the RYR1-CaM interaction, possibly by competing for the CaM activating site on RYR1. Suko et al. (18) studied the effects of suramin on the single channel behavior of RYR1 and on [3H]ryanodine binding and found that RYR1 channels reconstituted in planar lipid bilayers are activated by high concentrations (0.3–0.9 mM) of suramin. This effect appeared to be due to an increased affinity of the Ca2+ activating site on RYR1 for Ca2+.

These authors suggested that the complex functional effects of suramin arise from an allosteric regulation of the channel and not from alterations in the binding of endogenous ligands (e.g.)

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§ The abbreviations used are: SR, sarcoplasmic reticulum; CaM, calmodulin; DHPR, dihydropyridine receptor; RYR1, ryanodine receptor skeletal form; MOPS, 3-(N-morpholino)propanesulfonic acid; Alexa CaM, Alexa Fluor® 594-conjugated CaM; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid.

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CaM involved in channel gating. In the current study, we demonstrate that suramin binds directly to a peptide corresponding to the CaM binding motif on RYR1 and that suramin blocks the interaction of this peptide with the carboxyl-terminal region of the DHPR α1-subunit. Moreover, suramin potentiates voltage-gated SR Ca\(^{2+}\) release in whole cell voltage-clamped skeletal myotubes, consistent with the idea that suramin disrupts an intrinsic inhibitory interaction between the DHPR carboxyl terminus and the CaM binding domain on RYR1.

EXPERIMENTAL PROCEDURES

Materials—Bovine brain CaM, suramin, and dithiothreitol were obtained from Sigma. Tran\(^{35}\)S-label (>1000 Ci/mmol) was purchased from ICN Biomedicals, Inc. (Irvine, CA). [\(^{3}H\)]ryanodine was purchased from Amersham Biosciences, and unlabeled ryanodine was purchased from Calbiochem. A fluorescent derivative of CaM, Alexa Fluor\(^{488}\) 594-conjugated CaM (Alexa CaM), was purchased from Molecular Probes (Eugene, OR). The peptides were synthesized in the core facility at Baylor College of Medicine (Houston, TX).

BHPR Membrane Preparation—SR and transverse tubule-enriched membranes were prepared from rabbit hind leg and back-strap skeletal muscle and purified using sucrose gradient centrifugation (19). Protein concentrations were estimated by the method of Lowry using bovine serum albumin as the standard (20).

[\(^{35}\)S]Methionine Labeling of CaM—The mammalian CaM was generously provided by Dr. Ruth Altschuld. Metabolic labeling of calmodulin with Tran\(^{35}\)S-label was performed according to a procedure described previously (7).

Equilibrium [\(^{35}\)S]CaM Binding Assay—[\(^{35}\)S]Labeled CaM binding to SR membranes (10 \(\mu\)g) was determined as described previously (7). The binding buffer contained 50 mM MOPS (pH 7.4), 300 mM NaCl, 0.1% CHAPS, and 1 mM EGTA with 1.2 mM CaCl\(_2\), high Ca\(^{2+}\) binding buffer, 200 \(\mu\)M free Ca\(^{2+}\) or 1 mM EGTA alone (low Ca\(^{2+}\) binding buffer, 10 mM free Ca\(^{2+}\)). Samples were incubated for 2 h at room temperature. Bound radioactivity was separated from free by filtration through Whatman GF/F filters and washing 5 times with 3 ml of ice-cold binding buffer.

Equilibrium \([\^{3}\mathrm{H}]\)ryanodine Binding Assay—20-\(\mu\)g SR membranes per assay were incubated with 5 \(\mathrm{nM}\) \([\^{3}\mathrm{H}]\)ryanodine at room temperature for 17 h in binding buffer (300 mM NaCl, 50 mM MOPS (pH 7.4), 0.1% CHAPS, and either 1 mM EGTA or 1.2 mM CaCl\(_2\)), high Ca\(^{2+}\) binding buffer. Nonspecific binding was defined in the presence of unlabeled ryanodine (final concentration of 10 \(\mu\)M). Bound \([\^{3}\mathrm{H}]\)ryanodine was separated from free by filtering through Whatman GF/F glass fiber filters and washing 5 times with 3 ml of ice-cold binding buffer. The radioactivity was quantified by scintillation counting.

Fluorescence Spectroscopy—The interaction of suramin and \(3609-43\) was determined by monitoring the changes in intrinsic tryptophan fluorescence of \(3609-43\). The fluorescence was recorded on an ISS PC1 photon counting spectrophotometer (Champaign, IL). \(3609-43\) in the presence of different concentrations of suramin was excited at 295 nm (UV-solar pass filter), and emission spectra (320–450 nm) were recorded with a "cut-on" filter of 309 nm.

Suramin inhibition of Alexa-CaM-\(3609-43\) binding was determined by monitoring the changes in emission of Alexa CaM recorded on an ISS PC1 photon-counting spectrophotometer. Samples of Alexa CaM (1 \(\mu\)M) alone or in the presence of \(3609-43\) (1 \(\mu\)M) were incubated with 10 \(\mu\)M suramin for 30 min at room temperature. The samples in 100-\(\mu\)l cuvettes (Starlight Cells, Atascadero, CA) were excited at 594 nm (Cut-on filter of 550 nm), and the emission at 622 nm was collected as relative fluorescence units with a cut-on filter of 595 nm.

Nondenaturing Gel Electrophoresis—The electrophoretic mobility of CaM was evaluated by nondenaturating polyacrylamide gel electrophoresis under discontinuous conditions as a modified technique described by Laemmli (21). Nondenaturing gels (20% polyacrylamide) were separated at 30 mA under high Ca\(^{2+}\) conditions (200 \(\mu\)M CaCl\(_2\) in the gel buffers).

DHPR-RYR Interaction—Pull-down assays were performed using a biotinylated \(3609-43\) peptide and streptavidin beads. Briefly, transverse tubule-enriched membranes (1 mg/ml in 150 mM KCl, 25 mM NaCl, 100 \(\mu\)M CaCl\(_2\), 50 mM MOPS (pH 7.4)) were solubilized with 1% CHAPS. Aliquots (50 \(\mu\)l) of the solubilized membranes were added to streptavidin beads (50 \(\mu\)l) that had been preincubated for 1 h with 40 \(\mu\)M \(3609-43\). After gently mixing for 1 h at room temperature the beads were pelleted for 1 min in a low speed centrifuge, and the supernatant was removed. An additional 300 \(\mu\)l of buffer was then added, the sample was vortexed, and the beads were once again pelleted. After the addition of 100 \(\mu\)l of H\(_2\)O, beads were extracted with SDS and electrophoresed on a 7.5% SDS-polyacrylamide gels and either stained or Western-blotted with the indicated antibodies.

\[\text{FIG. 1. Suramin inhibits apoCaM and Ca}^{2+}\text{-CaM binding to RYR1. SR membranes were incubated with 5 nM Tran}^{35}\text{S-labeled CaM and increasing concentrations of suramin at either 200 \(\mu\)M free Ca}^{2+} \text{ (closed circles) or 10 mM free Ca}^{2+} \text{ (closed squares) as described under "Experimental Procedures." Data (mean \pm S.E.) on the ordinate are plotted as the amount of radioligand bound normalized to the amount bound in the absence of the competing ligand (B/B_o).}\]

\[\text{FIG. 2. Inhibition of apoCaM and Ca}^{2+}\text{-CaM binding to R3609-43 by suramin. The interactions of the fluorescent derivative of CaM, Alexa CaM, with R3609 at 10 nM or 200 \(\mu\)M free Ca}^{2+} \text{ were assessed in the presence and absence of suramin (10 \(\mu\)M). The figure shows the change in steady-state Alexa CaM fluorescence at 10 nM free Ca}^{2+} \text{ (panel A) and 200 \(\mu\)M free Ca}^{2+} \text{ (panel B). A, Alexa CaM (1 \(\mu\)M); b, Alexa CaM and R3609-43 (1 \(\mu\)M each); c, Alexa CaM (1 \(\mu\)M) and suramin (10 \(\mu\)M); d, Alexa CaM (1 \(\mu\)M) in the presence of R3609-43 (1 \(\mu\)M) and suramin (10 \(\mu\)M). Data represent the mean \pm S.E. for three independent experiments. The asterisk (*) indicates p < 0.001 compared with bar a, and the double asterisk (**) indicates p < 0.001 compared with bar b. AU, absorbance units.}\]
The ability of suramin to inhibit CaM binding to RYR1 or DHPR peptides. The effects of suramin on the interaction between CaM and R3609–43 or D1665–1685 were assessed by nondenaturing gel electrophoresis. Peptides (1.5 μM) and CaM (1.5 μM) in 200 μM free Ca²⁺ were incubated in the presence of increasing concentrations of suramin followed by electrophoresis and Coomassie Brilliant Blue staining. Panel A, Coomassie-stained gel. The arrow with a solid line indicates the CaM band bound to the peptide, and the dotted arrow indicates the uncomplexed CaM band. lane a, no suramin; lane b, 100 nM suramin; lane c, 1.25 μM suramin; lane d, 2.5 μM suramin; lane e, 5 μM suramin; lane f, 10 μM suramin; lane g, 20 μM suramin; lane h, 40 μM suramin; lane i, 80 μM suramin. B, densitometric analysis of uncomplexed CaM band in the presence of peptide R3609–43 (closed circles) and D1665–1685 (open circles) and increasing concentrations of suramin (0, 100 nM, and, 1.25, 2.5, 5, 10, 20, 40, and 80 μM).

R3609–43-D1393–1527 Interaction by Nickel Chelate Plate Assay—Analysis of the interaction between R3609–43 and the recombinantly expressed carboxyl-terminal fragment of DHPR α₁-subunit was determined as follows. The cDNA encoding the skeletal DHPR amino acids α₁-1393–1527 (D1393–1527) was used to express His-tagged recombinant protein as described by Sencer et al. (1). The His-tagged protein was coupled to nickel chelate assay plates as recommended by the manufacturer (BD Biosciences). 1 μM biotinylated R3609–43 (150 μM KCl, 25 mM NaCl, 100 μM CaCl₂, 50 mM MOPS (pH 7.4)) in the presence of various concentrations of suramin was added to the wells, and the plates were mixed at 100 rpm for 1 h at room temperature. The bound biotinylated R3609–43 was calorimetric-assayed using avidin-conjugated alkaline phosphate enzyme (1:1000), and p-nitrophenyl phosphate as a substrate/chromogen. The reaction was measured at 405 nm on a SpectraMax microplate spectrophotometer ( Molecular Devices Corp., Sunnyvale, CA).

Whole Cell Patch Clamp Measurements of Voltage-gated L-currents and Intracellular Calcium Transients in Mouse Myotubes—Myotubes were prepared from the skeletal muscle of newborn mice as previously described (22–24). Voltage-gated Ca²⁺ currents and Ca²⁺ transients were recorded using the whole cell patch clamp technique. The pipette solution for all experiments was 145 mM cesium aspartate, 0.1 mM Cs₂-EGTA, 1.2 mM MgCl₂, 5 mM MgATP, 0.2 mM K₅-Fluo-3, 10 mM HEPES, pH 7.4. For suramin experiments, suramin (5 or 50 μM) was also included in the internal solution. The external solution for all experiments was 145 mM triethylammonium chloride, 10 mM CaCl₂, 10 mM HEPES, 0.003 mM tetrodotoxin (pH 7.4).

Peak L-currents were normalized to cell capacitance (pA/picofarads), plotted as a function of test potential and fitted according to the equation,

\[ I = G_{\text{max}} \times (V_m - V_{\text{rev}}) / [1 + \exp(V_m - V_{\text{rev}})/k_F] \]  

where \( G_{\text{max}} \) is the maximal L-channel conductance, \( V_m \) is test potential, \( V_{\text{rev}} \) is the extrapolated reversal potential, \( V_{\text{rev}} \) is the potential for half-maximal activation of \( G_{\text{max}} \), and, \( k_F \) is a slope factor.

Relative changes in cytosolic Ca²⁺ were measured using the Ca²⁺ indicator K₅-Fluo-3 as described previously (24). Fluorescence traces were analog-filtered (τ = 0.5 ms) before digitization and expressed as \( \Delta F / F_0 \), where \( F_0 \) is the base-line fluorescence immediately before depolarization, and \( \Delta F \) represents the fluorescence change from base line. Fluorescence amplitudes at the end of each test pulse are plotted as a function of test potential and fitted according to the equation,

\[ \Delta F / F_0 = \Delta F / F_{\text{max}} / [1 + \exp(V_m - V_{\text{rev}})/k_F] \]  

where \( \Delta F / F_{\text{max}} \) is the calculated maximal change in fluorescence for each test potential (\( V_m \)), \( V_{\text{rev}} \) is the potential for half-maximal activation of \( \Delta F / F_{\text{max}} \), and, \( k_F \) is a slope factor.

Data Analysis—Inhibition of ³⁵S-labeled CaM binding to SR membranes was analyzed by non-linear regression (Sigma Plot 2000; Jandel Scientific, San Rafael, CA) using the equation,

\[ y = B_{\text{max}} \times (1 + K_a/[L]) / (1 + x/K_i) \]  

where \( B_{\text{max}} \) is the number of binding sites, \( K_a \) is the apparent dissociation constant for ³⁵S-labeled CaM binding, \( [L] \) is the concentration of ³⁵S-CaM, \( y \) is the concentration of suramin, \( x \) is the amount bound, and, \( K_i \) is the apparent inhibitory constant for suramin.

In gel shift assays, densitometry was performed on the peptide-bound CaM band. Optical density data obtained in the presence of suramin were normalized to the optical density of the peptide-CaM band alone and plotted as a function of suramin concentration. The data described are the mean ± S.E. for at least three independent determinations.
RESULTS

Suramin Inhibits Ca\(^{2+}\)-bound and Ca\(^{2+}\)-free CaM Binding to RYR1—Both RYR1 and CaM are Ca\(^{2+}\)-binding proteins. Our studies comparing the interactions of a Ca\(^{2+}\) binding site mutant of CaM and wild type CaM with RYR1 demonstrated that the affinities of both forms of CaM for RYR1 are greater at nM than at \(>10\) nM Ca\(^{2+}\) concentrations, indicating that Ca\(^{2+}\) binding to RYR1 increases the affinity for CaM (7). Suramin inhibits CaM binding to RYR1 (17). To determine the affinity of the interaction of suramin with RYR1 we analyzed the concentration dependence for suramin inhibition of \(^{35}\)S-labeled CaM binding to SR membranes at both nM and \(>10\) nM Ca\(^{2+}\) concentrations (Fig. 1). 

\[ K_i = 1.7 \pm 0.1 \ (n = 3) \text{ and } 1.3 \pm 0.14 \ (n = 3), \] respectively. These results indicate that suramin inhibits the interaction of both apoCaM and Ca\(^{2+}\)-CaM with RYR1 in a manner that is not altered by Ca\(^{2+}\) binding to RYR1. The complete inhibition of \(^{35}\)S-labeled CaM binding suggests that suramin binding to RYR1 is competitive with CaM. This is further supported by our finding that the rate of dissociation of \(^{35}\)S-labeled CaM from RYR1 is not altered by suramin (data not shown). The measured dissociation rate constants for \(^{35}\)S-labeled CaM bound to SR membranes are 0.33 \pm 0.001 min\(^{-1}\) (with CaM) and 0.35 \pm 0.002 min\(^{-1}\) (in the absence and presence of suramin, respectively.

Suramin Binds to Peptide R3609–43 and Inhibits Its Interaction with CaM—The studies of Klinger et al. (17) indicated that suramin binds competitively to the CaM binding domain on the RYR. However, Suko et al. (18) suggested that suramin effects on RYR channel function involve an allosteric mechanism that occurs in the absence of direct effects on endogenous ligands involved in channel gating. To address whether the suramin interaction with RYR1 is competitive or noncompetitive, we examined the ability of suramin to inhibit the interaction of a peptide corresponding to the CaM binding domain of RYR1 (R3609–3643) with CaM at both high and low Ca\(^{2+}\) concentrations (Fig. 2). At 200 \(\mu\)M and \(<10\) nM free Ca\(^{2+}\) concentrations, suramin displaces CaM from R3609–3643. The inhibition was assessed by the changes in the fluorescence of Alexa CaM in the presence or absence of the peptide (R3609–43). These results suggest a competitive interaction between suramin and CaM with the R3609–43 region of RYR1.

Competitive inhibition by suramin of the binding of CaM was confirmed by non-denaturing polyacrylamide gel electrophoresis. As shown in Fig. 3, the ability of R3609–3643 to bind CaM on non-denaturing gels is completely inhibited at suramin concentrations above 10 \(\mu\)M. CaM binding to a peptide (D1665–1685) corresponding to the CaM binding domain of the carboxyl-terminal region of the DHPR \(\alpha_1\)-subunit (13) is not affected by suramin (Fig. 3). These results indicate that suramin inhibition of the binding of CaM is relatively selective to the CaM recognition sequence found in RYR1.

To demonstrate direct binding between suramin and R3609–3643, we analyzed changes in intrinsic tryptophan fluorescence (excitation at 295 nm) of R3609–3643 in the presence and absence of 10 \(\mu\)M suramin (Fig. 4). The addition of suramin to R3609–43 peptide (Fig. 4A) induced a shift in tryptophan emis-
Fig. 6. **Endogenous calmodulin bound to RYR1 is displaced by suramin.** A, the presence of CaM was determined by Western blotting. 30 μg of SR membranes (0.2 mg/ml) was incubated with 0 and 10 μM suramin in 0.2 mM CaCl₂, 300 mM NaCl, 50 mM MOPS (pH 7.4) for 30 min on ice. Soluble proteins were separated from membranes by centrifugation in a Beckman Airfuge for 5 min at 30 p.s.i. and resolved by 15% SDS-PAGE. CaM was detected using monoclonal anti-CaM antibodies Research Diagnostics Inc. (Flanders, NJ) and visualized by ECL. Lane a, pellet (no suramin); lane b, supernatant (no suramin); lane c, pellet (10 μM suramin); lane d, supernatant (10 μM suramin). B, endogenous CaM bound to RYR1 was determined by separating SR membranes (10 μg, 0.2 mg/ml) in 0.1% CHAPS, 0.2 mM CaCl₂, 300 mM NaCl, 50 mM MOPS (pH 7.4) by filtration through Whatman GF/F filters with 5 × 3-ml washes with the above buffer followed by Western blotting. The filters were incubated with Lamin marker sample buffer. The proteins extracted from the filters were subjected to SDS-PAGE electrophoresis followed by transfer onto Immobilon-P membranes (Millipore Bedford, MA). Anti-CaM antibodies (Research Diagnostics) were used to detect CaM. The densities of CaM band are shown in the absence (a) and presence (b) of 10 μM suramin. AU, absorbance units. C, SR membranes (1.2 mg) were prewashed once in low Ca²⁺ buffer (300 mM NaCl, 1 mM EGTA, 50 mM MOPS (pH 7.4)) followed by a prewash in high Ca²⁺ buffer (300 mM NaCl, 1.2 mM CaCl₂, 50 mM MOPS (pH 7.4)). Membranes (20 μg) in the presence (open circles) or absence (closed circles) of CaM (1 μM) were used to determine the effect of suramin on [³H]ryanodine binding.

Effects of Suramin on [³H]Ryanodine Binding in the Presence and Absence of CaM—We assessed the ability of suramin to act as a competitive antagonist to Ca²⁺-CaM and apoCaM modulation of RYR1. [³H]Ryanodine binding is widely used to screen for effects of modulators on RYR activity. ApoCaM, like most RYR activators, increases [³H]ryanodine binding to RYR1, whereas Ca²⁺-CaM like other channel inhibitors decreases [³H]ryanodine binding to RYR1 (7). As expected, a decrease in [³H]ryanodine binding to RYR1 was observed in the presence of Ca²⁺-CaM (Fig. 5A), whereas apoCaM enhanced [³H]ryanodine binding to RYR1 (Fig. 5B). These effects of CaM were overcome by increasing concentrations of suramin (from 0.1 to 10 μM), suggesting that suramin competes with CaM for a binding site on RYR1 over this concentration range. Higher suramin concentrations (>100 μM) resulted in both the displacement of endogenous FKBP12 and enhanced [³H]ryanodine binding to RYR independent of the presence of CaM (data not shown).
**Suramin and RYR1**

This was inferred from the amounts of CaM detected in the proteins extracted from the filters routinely used for binding assays. Moreover, the suramin-induced changes in [³H]ryanodine binding in the absence of added CaM was not observed when SR membranes were pre-washed extensively (Fig. 6C).

**Modulation of DHPR-R3609–43 Interaction by Suramin—**

The CaM binding motif of RYR1 physically interacts with the carboxyl terminus of the DHPR α₁-subunit (1). We analyzed the effect of suramin on this interaction by assessing the ability of R3609–43 to pull down the DHPR from detergent-solubilized T-tubule membranes. In these experiments a biotinylated derivative of R3609–3643 (R3609–3643-biotin) and streptavidin beads was used to pull down DHPRs. Western blotting with anti-α₁-DHPR antibodies of the pull-down fractions demonstrated that 50 μM suramin blocked the pull down of the DHPR α₁-subunit (Fig. 7A). The effect of suramin on the interaction of R3609–43 and a recombinant protein fragment of carboxyl terminus of the DHPR α₁-subunit (His-tagged D1393–1527) expressed in *Escherichia coli* was also analyzed. The interaction was completely inhibited by 10 μM suramin (Fig. 7B).

The carboxyl terminus of the DHPR α₁-subunit (D1393–1527) has been shown to inhibit ryanodine binding to RYR1 (1). D1393–1527 was isolated using nickel-chelated beads after thrombin cleavage to determine whether suramin could prevent inhibition of ryanodine binding. The D1393–1527 preparation contained His tag as a contaminant. 10 μM suramin completely attenuated the inhibition of [³H]ryanodine binding by D1393–1527 (Fig. 7C). The His tag by itself has no effect on the [³H]ryanodine binding to RYR1 (data not shown). Together these results indicate that suramin effectively uncouples the physical interaction between the carboxyl terminus of the DHPR α₁-subunit and a peptide corresponding to the CaM binding site of RYR1.

**Effect of Suramin on Excitation-Contraction Coupling in Mouse Myotubes—**
The results reported here demonstrate that suramin disrupts binding of both CaM and the carboxyl terminus of the DHPR α₁-subunit to a peptide corresponding to the CaM binding domain of RYR1. To determine whether these effects alter excitation-contraction coupling in intact skeletal muscle cells, we measured L-type Ca²⁺ channel currents and voltage-gated SR Ca²⁺ release in normal myotubes in the presence and absence suramin. Because suramin is not membrane-permeant, 50 μM suramin was included in the patch pipette internal solution (with buffer alone used as control), and recordings were made 5 min after establishing the whole cell configuration. For these experiments, 50 μM suramin was used because this concentration of suramin was found in biochemical experiments to maximally inhibit CaM binding to RYR1 (Figs. 1 and 3) and would, therefore, be likely to overcome potential limitations with regard to suramin dialysis and accessibility to junctional RYR1 proteins in patch-clamped myotubes. Maximal L-type Ca²⁺ channel conductance (G(max)) and the voltage required for half-maximal activation of G(max) (V(G1/2)) were similar in the presence and absence of 50 μM internal suramin (Fig. 8A and Table I). The lack of an effect of suramin on L-type Ca²⁺ channel activity is consistent with the finding reported here that suramin does not interact with the CaM binding region of the skeletal muscle DHPR. However, 50 μM suramin caused a significant increase (67.9 ± 0.2%, n = 13; p < 0.05) in the maximal ΔF/F₀ without altering V(G1/2), the voltage required for half-maximal release (Fig. 8B and Table I). The effects of suramin on maximal voltage-gated SR Ca²⁺ release were concentration-dependent since a smaller increase in maximal ΔF/F₀ was observed at a lower concentration (5 μM suramin: 39.7 ± 0.2%, n = 16; p < 0.05). The increase in maximal voltage-gated Ca²⁺ release could arise from either a direct

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**Fig. 7. Suramin inhibits DHPR-RYR interaction.** A, pull-down assays were performed using biotinylated R3609–43 and streptavidin beads in the presence of control buffer and either 5 μM CaM or 50 μM suramin as described under “Experimental Procedures.” After SDS-PAGE and Western blotting, the blots were probed for the α₁-subunit of the DHPR using an antibody (Affinity Bioreagents, Golden, Co) specific for the α₁-subunit. a, pull down with 3609–43 alone; b, pull down in the presence of CaM; c, pull down in the presence of suramin. Data represent the mean plus the S.E. of the mean for three determinations. B, binding between the RYR peptide (R3609–43) and DHPR fragment (His-tagged D1393–1527) were analyzed using nickel chelate plate assay described under “Experimental Procedures.” Data represent the mean plus the S.E. of the mean for three determinations, a, control; b, suramin, 1 μM; c, suramin 10 μM. C, [³H]ryanodine binding to RYR1 was performed in the presence of expressed carboxyl-terminal fragment of DHPR (D1393–1527). The preparation includes His tag as a contaminant. SR membranes were washed in low Ca²⁺ buffer (300 mM NaCl, 1 mM EGTA, 50 mM MOPS (pH 7.4)) and then high Ca²⁺ buffer (300 mM NaCl, 1.2 mM CaCl₂, 50 mM MOPS (pH 7.4)). [³H]Ryanodine binding to prewashed membranes (20 μg) was performed in the presence of 0 μM (a), 0.75 μM (b and c), and 1 μM (d and e) of D1393–1527. Samples b and d were added with 10 μM of suramin. AU, absorbance units.

Suramin at concentrations greater than 1 μM increases [³H]ryanodine binding to RYR1 even in the absence of added CaM (Fig. 5A). The increase in [³H]ryanodine binding is likely due to displacement of endogenous calmodulin in these membranes by suramin. Consistent with this, Western blotting of these membranes with an antibody to calmodulin shows the presence of endogenous calmodulin that is partially displaced by 10 μM suramin (Fig. 6A). Endogenous CaM bound to RYR was, however, completely displaced by 10 μM suramin (Fig. 6B).
FIG. 8. Suramin potentiates voltage-gated SR Ca\(^{2+}\) release in mouse myotubes. A, average peak I-V plot for mouse myotubes dialyzed with internal solutions containing buffer alone (closed circles) or 50 \(\mu M\) suramin (open circles)-treated myotubes. Data from each cell were fit according to Equation 1 and the average parameters given in Table I (I-V). The solid line through the data represents a fit of the average data according to Equation 1 (control: \(G_{\text{max}} = 211\) nanosiemens per nanofarad, \(V_{\text{III}} = 15.8\) mV, \(k = 7.0\) mV; Suramin: \(G_{\text{max}} = 193\) nanosiemens/nanofarads, \(V_{\text{III}} = 18.3\) mV, \(k = 6.3\) mV). B, average peak \(\Delta F/\Delta F_{\text{max}}\) values for control (closed circles) and suramin (open circles)-dialyzed myotubes. Data were fit according to Equation 2, and the solid line is a fit of the average data (Table 1, F-V) (control: \(\Delta F/\Delta F_{\text{max}} = 2.7\), \(V_{\text{FRM}} = -2.8\) mV, \(k = 6.2\) mV; suramin: \(\Delta F/\Delta F_{\text{max}} = 4.2\), \(V_{\text{FRM}} = 3.0\) mV, \(k = 5.6\) mV). In A and B, peak currents and transients were elicited by 200-ms depolarizations to the indicated test potentials. Representative I-currents (C) and Ca\(^{2+}\) transients (D) for control and suramin dialyzed-myotubes are shown. Currents and transients for each condition are from the same cell.

Table 1

|        | Parameters of I-V and F-V curves |
|--------|----------------------------------|
|        | I-V                             | F-V                             |
|        | \(n\) | \(G_{\text{max}}^{a}\) \(\text{nS/}\mu\text{F}\) | \(V_{\text{III}}\) \(\text{mV}\) | \(k\) \(\text{mV}\) | \(n\) | \(\Delta F/\Delta F_{\text{max}}\) | \(V_{\text{FRM}}\) \(\text{mV}\) | \(k\) \(\text{mV}\) |
| Control | 12  | 210 ± 10  | 16.1 ± 1.7 | 6.1 ± 0.2 | 12 | 2.7 ± 0.3 | -2.9 ± 1.9 | 5.2 ± 0.4 |
| Suramin | 13  | 206 ± 20  | 18.9 ± 1.6 | 5.3 ± 0.4 | 13 | 4.5 ± 0.5\(^b\) | 1.8 ± 2.0 | 5.3 ± 0.8 |

\(^a\) nS/\mu F, nanosiemens nanofarads.  
\(^b\) \(p < 0.05\).

Suramin potentiates volt-
age-gated SR Ca\(^{2+}\) release in mouse myotubes. A, average peak I-V plot for mouse myotubes dialyzed with internal solutions containing buffer alone (closed circles) or 50 \(\mu M\) suramin (open circles)-treated myotubes. Data from each cell were fit according to Equation 1 and the average parameters given in Table I (I-V). The solid line through the data represents a fit of the average data according to Equation 1 (control: \(G_{\text{max}} = 211\) nanosiemens per nanofarad, \(V_{\text{III}} = 15.8\) mV, \(k = 7.0\) mV; Suramin: \(G_{\text{max}} = 193\) nanosiemens/nanofarads, \(V_{\text{III}} = 18.3\) mV, \(k = 6.3\) mV). B, average peak \(\Delta F/\Delta F_{\text{max}}\) values for control (closed circles) and suramin (open circles)-dialyzed myotubes. Data were fit according to Equation 2, and the solid line is a fit of the average data (Table 1, F-V) (control: \(\Delta F/\Delta F_{\text{max}} = 2.7\), \(V_{\text{FRM}} = -2.8\) mV, \(k = 6.2\) mV; suramin: \(\Delta F/\Delta F_{\text{max}} = 4.2\), \(V_{\text{FRM}} = 3.0\) mV, \(k = 5.6\) mV). In A and B, peak currents and transients were elicited by 200-ms depolarizations to the indicated test potentials. Representative I-currents (C) and Ca\(^{2+}\) transients (D) for control and suramin dialyzed-myotubes are shown. Currents and transients for each condition are from the same cell.

**Discussion**

Our results indicate that suramin inhibits both Ca\(^{2+}\)-CaM and apoCaM binding to RYR1 with nearly identical \(K_c\) values (\(\sim 1-2\) \(\mu M\)), suggesting that inhibition by suramin is independent of Ca\(^{2+}\) binding to both CaM and RYR1. A recent study (17) reported that a suramin IC\(_{50}\) for inhibition of Ca\(^{2+}\)-CaM binding to RYR1 is \(\sim 10\)-fold higher than we are reporting. The reason for the different apparent affinity of suramin for RYR1 is not clear but may be related to the use of \(^{125}\text{I}\)-labeled CaM in their study versus \(^{35}\text{S}\)-labeled CaM in the current study. We have previously demonstrated that the iodination of CaM using the Bolton-Hunter reagent can produce substantial artifacts in the analysis of CaM binding to RYR1 (10).

Suramin was found to bind to and competitively inhibit CaM binding to a peptide (R3609–43) that corresponds to the CaM binding region of RYR1. Therefore, suramin would be expected to compete with CaM for binding to RYR1 proteins that are not interacting with the DHPR \(\alpha_1\)-subunit and the CaM binding domain of RYR1 (see “Discussion”).

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Suramin and RYR1

that very high levels (0.3 – 1.0 mM) of suramin are required to activate RYR1, concentrations that are 6–20 times greater than that found to alter voltage-gated Ca2+ release in intact skeletal myotubes. Our observation of a potentiation of voltage-gated Ca2+ release by a relatively low concentration of suramin is even more striking considering potential limitations with regard to junctional accessibility of suramin introduced into myotubes via internal dialysis of patch-clamped myotubes. Suramin at concentrations higher than 100 μM displaces FKBP12 bound to RYR1 (data not shown), which may lead to an increase in release channel activity via a mechanism distinct from what has been suggested here.

In summary, our studies indicate that suramin inhibits both Ca2+/CaM and apoCaM binding/regulation of RYR1 by competing for the CaM binding sequence on RYR1 (encoded by RYR1 residues 3609–3643). In addition, suramin increases the magnitude of the voltage-gated Ca2+ release in skeletal myotubes, possibly by disrupting an inhibitory interaction between the carboxyl terminus of the DHPR α1-subunit and the CaM binding region of RYR1. Future analysis of the effects of suramin analogs and their binding/regulation of RYR1 is likely to provide better tools for probing the role of CaM in the regulation of RYR1 and for investigating the functional role of the DHPR carboxyl terminus in the regulation of skeletal muscle excitation-contraction coupling.

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REFERENCES

1. Sence, S., Papini, R. V., Halling, D. B., Patel, P., Kole, J., Zhang, J. Z., and Hamilton, S. L. (2001) J. Biol. Chem. 276, 38237–38241
2. Beeler, G. W., Jr., and Reuter, H. (1970) J. Physiol. (Lond.) 207, 191–209
3. Franzini-Armstrong, C., and Protasi, F. (1997) Physiol. Rev. 77, 699–729
4. Frizzoni-Armstrong, C., and Kish, J. W. (1995) J. Muscle Res. Cell Motil. 16, 319–324
5. Marx, S. O., Ondrias, K., and Marks, A. R. (1998) Science 281, 818–821
6. Tripathy, A., Xu, L., Mann, G., and Meisner, G. (1995) Biophys. J. 69, 106–119
7. Rodney, G. G., Williams, B. Y., Strasburg, G. M., Beckingham, K., and Hamilton, S. L. (2000) Biochemistry 39, 7867–7872
8. Zühlke, R. D., Pitt, G. S., Deisseroth, K., Tsien, R. W., and Reuter, H. (1999) Nature 399, 159–162
9. Petersen, B. Z., DeMaria, C. D., Adelman, J. P., and Yue, D. T. (1999) Neuron 22, 549–558
10. Moore, C. P., Rodney, G., Zhang, J. Z., Santacruzo-Toledo, I., Strasburg, G., and Hamilton, S. L. (1999) Biochemistry 38, 8532–8537
11. Hamaguchi, N., Xin, C., and Meisner, G. (2001) J. Biol. Chem. 276, 22575–22585
12. Slavik, K. J., Wang, J. P., Aghdasi, B., Zhang, J. Z., Mandel, F., Malouf, N., and Hamilton, S. L. (1997) Am. J. Physiol. 272, C1475–C1481
13. Pate, P., Mocha-Morales, J., Wu, Y., Zhang, J. Z., Roden, G. G., Serysheva, I. I., Williams, B. Y., Andersen, M. E., and Hamilton, S. L. (2000) J. Biol. Chem. 275, 39786–39792
14. Zhang, J. Z., Wu, Y., Williams, B. Y., Roden, G. G., Mandel, F., Strasburg, G. M., and Hamilton, S. L. (1999) Am. J. Physiol. 276, C46–C53
15. Sun, J., Xin, C., Yu, J., Stamler, J. S., and Meisner, G. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 11158–11162
16. Sitsapesan, R., and Williams, A. J. (1996) J. Membr. Biol. 153, 93–102
17. Klinger, M., Bollard-Cordona, E., Mayer, B., Nansoff, C., Freimuth, M., and Hohenegger, M. (2001) Biochem. J. 355, 827–833
18. Suko, J., Hellmann, G., and Drobny, H. (2001) Mol. Pharmacol. 59, 543–556
19. Hawkes, M. J., Diaz-Munoz, M., and Hamilton, S. L. (1989) Membr. Biochem. 8, 133–145
20. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) J. Biol. Chem. 193, 265–275
21. Laemmli, U. K. (1970) Nature 227, 680–685
22. Nakai, J., Dirksen, R. T., Nguyen, H. T., Bassam, A. I., Beams, K. G., and Allen, P. D. (1996) Nature 380, 72–75
23. Avila, G., and Dirksen, R. T. (2000) J. Gen. Physiol. 114, 467–480
24. Avila, G., O’Connell, M. S., Groom, L., and Dirksen, R. T. (2001) J. Biol. Chem. 276, 17732–17738
25. Klinger, M., Freimuth, M., Nickel, P., Stabler-Schwambart, M., Kassack, M., Suko, J., and Hohenegger, M. (1999) Mol. Pharmacol. 55, 462–472