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Suramin and Suramin Analogs Activate Skeletal Muscle Ryanodine Receptor via a Calmodulin Binding Site

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ABSTRACT

Contraction of skeletal muscle is triggered by the rapid release of Ca^{2+} from the sarcoplasmic reticulum via the ryanodine receptor/calcium-release channel. The trypanocidal drug suramin is an efficient activator of the ryanodine receptor. Here, we used high-affinity [^{3}H]ryanodine binding to sarcoplasmic reticulum from rabbit skeletal muscle to screen for more potent analogs of suramin. This approach resulted in the identification of NF307, which accelerates the association rate of [^{3}H]ryanodine binding with an $EC_{50} = 91 \pm 7$ μM at 0.19 μM calculated free Ca^{2+}. In single-channel recordings with the purified ryanodine receptor, NF307 increased mean open probability at 0.6 μM Ca^{2+} from 0.020 $\pm$ 0.006 to 0.53 $\pm$ 0.07 with no effect on current amplitude and unitary conductance. Like caffeine, NF307 exerts a very pronounced Ca^{2+}-sensitizing effect (EC_{50} of Ca^{2+} shifted ~10-fold by saturating NF307 concentrations). Conversely, increasing concentrations of free Ca^{2+} sensitized the receptor for NF307 ($EC_{50} = 14.6 \pm 3.5$ μM at 0.82 μM estimated free Ca^{2+}). The effects of NF307 and caffeine on [^{3}H]ryanodine binding were additive, irrespective of the Ca^{2+} concentration. In contrast, the effects of calmodulin, which activates and inhibits the ryanodine receptor in the absence and presence of Ca^{2+}, respectively, and of NF307 were mutually antagonistic. If the purified ryanodine receptor was prebound to a calmodulin-Sepharose matrix, 100 μM NF307 and 300 μM suramin eluted the purified ryanodine receptor to an extent that was comparable to the effect of 10 μM calmodulin. We conclude that NF307 and suramin interact directly with a calmodulin binding domain of the ryanodine receptor. Because of its potent calcium-sensitizing effect, NF307 may represent a lead compound in the search of synthetic ryanodine receptor ligands.

The myoplasmic free Ca^{2+} concentration is regulated tightly by uptake into and release from intracellular Ca^{2+} stores. These Ca^{2+} fluxes are the basis for skeletal muscle relaxation and contraction (Fleischer and Inui, 1989). Efflux of Ca^{2+} occurs via the Ca^{2+}-release channel of the sarcoplasmic reticulum, also referred to as ryanodine receptor-1 (RyR_1). Two additional isoforms have been identified (reviewed in Franzini-Armstrong and Protasi, 1997), namely the RyR_2 (cardiac isoform, but also widely expressed in brain) and RyR_3 (thought to be widely expressed at low levels). All isoforms bind ryanodine (as well as related alkaloids from *Ryania speciosa*) with high affinity, and it was this ligand that made the identification of the channel protein possible (Meissner, 1994). The ryanodine receptor/Ca^{2+}-release channel is a homotetramer and resides on the terminal cisternae of the sarcoplasmic reticulum and is closely associated with L-type calcium channel of the sarcolemmal T-tubular system (Melzer et al., 1995). The propagation of the action potential along the surface of the muscle is sensed by the L-type calcium channel, which, upon voltage-dependent activation, provides the triggering signal for gating of the ryanodine receptor.

The ryanodine receptor/Ca^{2+}-release channel is unusually large (molecular mass of one subunit = 565 kDa), and most of its size is accounted for by a large hydrophilic segment at the amino terminus (Meissner, 1994). In skeletal muscle, this domain presumably is involved in the direct interaction with the L-type channel and with additional proteins that maintain the highly organized topology of the triad. The carboxy terminus contains the pore-forming hydrophobic segments (Bhat et al., 1997). In addition, the ryanodine receptor contains multiple binding sites for calmodulin and for Ca^{2+}, the precise number and location of which are still a matter of debate (Franzini-Armstrong and Protasi, 1997). The action of both calmodulin and Ca^{2+} is dualistic; at concentrations in the low-micromolar range, Ca^{2+} promotes channel opening, whereas submillimolar to millimolar concentrations deacti-

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ABBREVIATIONS: HSR, heavy sarcoplasmic reticulum; CHAPS, 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate; MOPS, 3-(*N*-morpholino) ethane sulfonic acid; P_o, open probability.

vate the channel, an effect that is less pronounced in RyR_2 and that presumably is absent in RyR_3 (Franzini-Armstrong and Protasi, 1997). Similarly, in its Ca^{2+}-free form calmodulin increases the open probability of the channel and promotes [^{3}H]ryanodine binding, but Ca^{2+}-liganded calmodulin is a potent inhibitor of the receptor (Meissner, 1986; Plank et al., 1988; Smith et al., 1989); Ca^{2+}/calmodulin directly interacts with the channel protein in a 1:1 stoichiometry (Wagenknecht et al., 1997). The binding sites for ryanodine and ATP have been mapped to the carboxy-terminal fragment (Shoshan-Barmatz and Zarka, 1988; Zarka and Shoshan-Barmatz, 1993). By analogy with other ATP binding proteins, the trypanocidal drug suramin initially was proposed to interact with the ATP binding site of the receptor (Emmick et al., 1994). However, this assignment was not supported by a subsequent analysis. Although both suramin and adenine nucleotides stimulate the ryanodine receptor directly from the cytoplasmic side, they exert different effects on the gating properties of the channel (Hohenegger et al., 1996; Sitsapesan and Williams, 1996); most importantly, suramin does not block the covalent incorporation of an ATP analog into the ryanodine receptor (Hohenegger et al., 1996).

To understand the mechanism by which suramin activates the ryanodine receptor, we have searched for a more potent suramin analog. In the present work, we used stimulation of [^{3}H]ryanodine binding as a screening procedure with a reasonably high throughput. This approach led to the identification of NF307, the affinity of which exceeds that of suramin; in addition, NF307 is substantially more efficacious than suramin in enhancing the Ca^{2+} sensitivity of the ion channel. Finally, our experiments show that NF307 and suramin compete with calmodulin for binding to the ryanodine receptor.

Materials and Methods

Materials. Leupeptin, pepstatin, phenylmethylsulfonyl fluoride, antipain, CsCl (ultrapure), ruthenium red, calmodulin, and low-molecular-mass protein standards were purchased from Sigma (St. Louis, MO); phosphatidylcholine, phosphatidylserine, and phosphatidylethanolamine were purchased from Avanti Polar Lipids (Alabaster, AL); suramin and unlabeled ryanodine were purchased from Calbiochem (San Diego, CA); and Pefabloc was from Boehringer Mannheim (Mannheim, Germany). [^{3}H]Ryanodine was from New England Nuclear (Boston, MA), and reagents for enhanced chemiluminescence and horseradish peroxidase-linked anti-mouse IgG were from Amersham Buchler (Buckinghamshire, UK). The materials for SDS-polyacrylamide gel electrophoresis were obtained from Bio-Rad (Hercules, CA), Calmodulin-Sepharose 4B and molecular mass standards for electrophoresis were obtained from Pharmacia LKB (Uppsala, Sweden). A mouse monoclonal antibody directed against the ryanodine receptor (Airey et al., 1990) was from Biomol (Munich, Germany). Aprotinin was a generous gift from Bayer AG (Wuppertal, Germany). All other reagents were of analytical grade.

Chemical Synthesis. The compounds NF299, NF301, and NF307 are analogs of suramin, in which the central urea bridge of suramin has been displaced by a 1.4-bis(carbamoyl)-piperazine group.

Synthesis of NF299: 10 mmol of 8-(3-(3-aminobenzamido)-4-methylbenzamido)-naphthalene-1.3.5-trisulfonic acid trisodium salt (compound 1), the starting material for the last step of the suramin synthesis, was treated in aqueous solution (100 ml) at pH 4.0 with phenoxycarbonyl chloride (15 mmol). The reaction mixture was extracted exhaustively with diethylether. The aqueous layer was evaporated in vacuum to dryness yielding a white powder (compound 2), the *N*-(phenoxycarbonyl) derivative of compound 1. To a solution of compound 2 (2 mmol) in water (20 ml), a solution of piperazine (1 mmol) in water (20 ml) was added very slowly (4 h). The reaction mixture was extracted exhaustively with diethylether. The aqueous layer was concentrated in vacuum to a small volume (~10 ml). On addition of the same volume of ethanol and after storing at 0°C, NF299 precipitated (yield 87%). The purity of the compound was determined by HPLC using the method described by Kassack and Nickel (1996). In a similar way NF301 and NF307 were synthesized starting from the appropriately aminobenzamido-substituted aminonaphthalenetrisulfonic acids. NF307 precipitated during the reaction and was purified by recrystallization from water. The synthesis of NF449 and of NF503 has been described previously (Hohenegger et al., 1998).

Membrane Preparation and Purification of the Ca^{2+}-Release Channel/Ryanodine Receptor. Heavy sarcoplasmic reticulum (HSR) from rabbit white muscle was prepared according to Wyskovsky et al. (1990). The ryanodine receptor was purified as described previously (Suko et al., 1993) with the following modifications (Suko and Hellmann, 1998): HSR (15 mg/ml) was solubilized with 3-[(3-cholamidopropyl) dimethylammonio]-1-propane sulfonate (CHAPS) in a buffer containing 40 mM 3-(*N*-morpholino) ethane sulfonic acid (MOPS)-Tris (pH 7.0), 1 M NaCl, 2 mM dithiothreitol (DTT), 1% (w/v) CHAPS, 0.25% (w/v) phosphatidylcholine, and a cocktail of protease inhibitors (0.5 μg/ml leupeptin, 1.4 μg/ml aprotinin, 1 μg/ml antipain, 1 μM pepstatin, 0.1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine) for 1 h. The soluble proteins were separated from the insoluble material by centrifugation (103,000*g* for 35 min) and subsequently applied onto a linear sucrose gradient from 7.5 to 20% in buffer [40 mM MOPS-Tris, pH 7.0, 300 mM NaCl, 2 mM DTT, 0.5% (w/v) CHAPS, 0.25% (w/v) phosphatidylcholine] and the cocktail of protease inhibitors listed above. After centrifugation (Beckman SW28 rotor; 24,000 rpm, 14 h) the gradient was separated in fractions that were screened by SDS-polyacrylamide gel electrophoresis for the ryanodine receptor. The fractions containing the purified ryanodine receptor were pooled and dialyzed (22–24 h) against a buffer containing 40 mM MOPS-Tris (pH 7.0), 100 mM NaCl, 2 mM DTT, 0.15 mM $CaCl_2$, 0.1 mM EGTA, and the protease inhibitor cocktail. Proteoliposomes were stored in the presence of 200 mM sucrose at −80°C. All steps of the preparation were carried out at 4°C.

[^{3}H]Ryanodine Binding. Sarcoplasmic reticulum membranes (50 μg) were incubated in 50 μl containing 20 mM HEPES (pH 7.4), 20 nM [^{3}H]ryanodine, 1 μM aprotinin, 1 μM leupeptin, 100 μM Pefabloc, and the concentrations of suramin analogs, caffeine, calmodulin, and Ca^{2+} as indicated in the figure legends. Additionally, two salt concentrations were used, either 750 mM KCl or the combination of 200 mM KCl and 10 mM NaCl (referred throughout the text as 0.75 M KCl and 0.2 M KCl buffer). The free Ca^{2+} concentration was adjusted by altering the ratio of EGTA and $CaCl_2$. The incubation was carried out at 30°C for 40 min. For kinetic experiments the incubation time was varied from 3 to 180 min. In saturation experiments, the incubation time was 180 min. The reactions were terminated by filtration over glass-fiber filters (presoaked in 1% polyethylenimine) using a Skatron vacuum filtration device. The filters were rinsed with 10 ml of ice-cold 10 mM Tris-HCl (pH 7.4), 700 mM NaCl, 0.17 mM $CaCl_2$, and 0.2 mM EGTA. Nonspecific binding was determined in the presence of 1000-fold excess of unlabeled ryanodine, which had been added to the incubation mixture before the labeled ligand. None of the compounds investigated affected nonspecific binding. If not otherwise indicated, experiments were carried out in duplicate and each experiment was reproduced at least twice with different protein preparations.

Single-Channel Recordings. Single-channel recordings were carried out after the incorporation of the purified Ca^{2+}-release channel/ryanodine receptor into planar lipid bilayers essentially according to Coronado et al. (1992) and as described previously (Suko and

Hellmann, 1998). Briefly, the bilayer was formed from a lipid mixture (1:1) of phosphatidylserine and phosphatidylethanolamine dissolved at a concentration of 10 mg/ml each in decane. The *cis*- and *trans*-chambers were filled with 0.7 ml and 1.3 ml, respectively. The lipid solution was spread over a 200-μm-diameter aperture in a delrin cup (Warner Instruments Corp., Hamden, CT) separating two aqueous compartments. The *cis*-bath (0.7 ml) and the *trans*-bath (1.3 ml) were connected to the head-stage input of a model EPC-9 amplifier (Heka Elektronik, Lambrecht, Germany); the *trans*-bath was held on virtual ground. Cs^+ was used as a charge carrier to increase the conductance of the channel (Coronado et al., 1992). The *cis*- and *trans*-baths contained 480 mM and 50 mM CsCl, respectively. The buffer composition was 10 mM HEPES-Tris, pH 7.4, 100 μM $CaCl_2$, 80 μM EGTA (i.e., free $Ca^{2+} \approx 20$ μM); at low free Ca^{2+} the *cis* solution contained 0.5 mM EGTA and 0.42 mM $CaCl_2$ (resulting in 0.6 μM calculated free Ca^{2+}). Purified Ca^{2+}-release channel/ryanodine receptor (1.5–2 μg) and other reagents were added to the *cis*-chamber. Recordings were filtered at 4 kHz through a low-pass Bessel filter, digitized at 40 kHz, and subsequently stored on a Macintosh PC. Single-channel events were analyzed with TAC V2.5 software (Skalar Instruments, Inc., Seattle, WA). Mean open probability (P_o) of channels and the corresponding lifetimes (τ) of the open and closed events were identified by a 50% threshold analysis and calculated from data segments of 30- to 90-s duration. NF307 was dissolved at 50 mM in DMSO, carryover of which did not exceed a final concentration of 0.2% in the *cis*-buffer. The holding potentials given in the figure legends were applied with reference to the *trans*-chamber. All experiments were carried out at 22 to 24°C.

Affinity Chromatography and Gel Electrophoresis. The purified ryanodine receptor (2–3 μg) was diluted in 100 μl binding buffer of the following composition: 20 mM HEPES-NaOH (pH 7.4), 200 mM KCl, 10 mM NaCl, 1 mM EGTA, 1.2 mM $CaCl_2$, 0.68% CHAPS, and 0.5% phosphatidylcholine. Alternatively, the free Ca^{2+} concentration in the incubation was reduced by adding only 0.85 mM $CaCl_2$ to the buffer of otherwise identical composition. Preequilibrated calmodulin-Sepharose (40 μl of a 50% slurry) was added. After an incubation period of 60 min at 4°C, the suspension was centrifuged for 5 min at 500*g*; the supernatant was removed, and the sedimented calmodulin-Sepharose was resuspended in 90 μl binding buffer and recentrifuged. This wash step was repeated twice. Subsequently, the ryanodine receptor was eluted batchwise in four steps with 90 μl binding buffer supplemented with 20 μM calmodulin, 300 μM suramin, or 100 μM NF307. As a control, mock elutions were done in parallel with 90 μl binding buffer. The supernatants of the washes and elutions were mixed with Laemmli sample buffer (supplemented with mercaptoethanol and SDS to yield final concentrations of 0.5% and 2.5%, respectively), and the samples were heated to 95°C for 5 min. Similarly, after the last elution, the calmodulin-Sepharose matrix was boiled in Laemmli sample buffer and centrifuged. Aliquots (corresponding to 30% of the of the individual samples) were applied onto discontinuous SDS-polyacrylamide gels (3% stacking and 7% separating gel). Molecular mass standards were myosin (212 kDa), α_2-macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa), and glutamic dehydrogenase (53 kDa). The resolved proteins were visualized by silver staining. For immunoblotting, nitrocellulose blots were incubated with a mouse monoclonal antibody raised against the skeletal muscle isoform of the ryanodine receptor, and the immunoreactive bands were visualized by enhanced chemiluminescence detection.

Miscellaneous Procedures. Protein concentration was measured by staining with amido black or with the bicinchonic acid assay (Micro-BCA, Pierce, Rockford, IL) using BSA as the standard. Free Ca^{2+} concentrations were calculated by a computer program using binding constants published by Schoenmakers et al. (1992); we note that these constants and the algorithm yield estimates differ to some extent, in particular at low Ca^{2+} concentrations, from those obtained with the program of Fabiato (1988) that we have used previously. Data were fitted by nonlinear least-squares regression to the appropriate equations describing mono-, bi-, or triexponential decay and association as well as saturation isotherms using the Gauss-Newton or Marquardt-Levenberg algorithm.

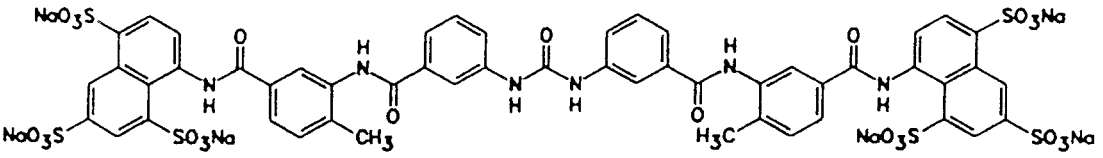
Results

[^{3}H]Ryanodine Binding in the Presence of Suramin Analogs. It is generally appreciated that all compounds and manipulations that promote channel opening accelerate the rate of high-affinity [^{3}H]ryanodine binding; this close correlation indicates that ryanodine interacts with the channel in the open conformation. We therefore have used the initial rate of [^{3}H]ryanodine binding to search for ryanodine receptor activators that are structurally related to suramin. In these screening experiments, the free Ca^{2+} concentration was set below the threshold level required for Ca^{2+}-dependent high-affinity binding, because this biases the search for compounds that are also potentially capable of sensitizing the ryanodine receptor for Ca^{2+}. Suramin is a rigid, symmetric molecule in which two polysulfonated naphtylbenzamide ring systems are connected by a central urea bridge. This is replaced by a piperazine ring in one class of suramin analogs (of several examined), in which we have found a potent activator, NF307. The structures of suramin, NF307, and the two related molecules are shown in Fig. 1. NF307 was found to efficiently promote [^{3}H]ryanodine binding even at the subthreshold Ca^{2+} concentration of 0.45 μM with a calculated EC_{50} of 69 ± 14 μM (Fig. 2A). Under these assay conditions, NF301, suramin, and NF299 (Fig. 2A) were equipotent and promoted [^{3}H]ryanodine binding with comparable efficacy. However, they were clearly much less active than NF307. It is evident from Fig. 1 that the variations in the structure of the compounds are modest. This is true, in particular, for NF301 and NF307, which differ by the position of a single pair of sulfonic acid residues (*meta* versus *para*). We have also tested NF449 and NF503, two analogs that were found to be substantially more selective than suramin in inhibiting individual G protein α-subunits (Freissmuth et al., 1996; Hohenegger et al., 1998). Both, NF449 and NF503 (Fig. 2A) as well as related compounds (not shown) were inactive up to 1 mM.

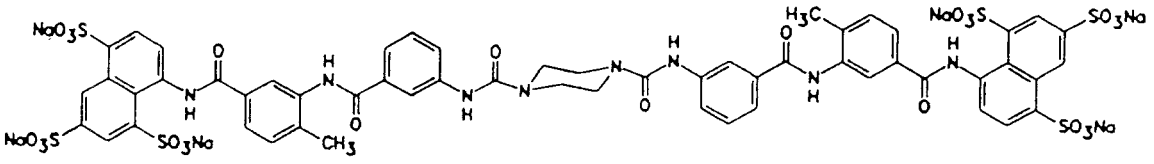
The salt concentration in these binding experiments was 0.75 M KCl. This high ionic strength augments Ca^{2+}-induced single-channel opening and [^{3}H]ryanodine binding (Meissner et al., 1997). We have also verified that the ability of NF307 to promote [^{3}H]ryanodine binding does not rely on preactivation of the ryanodine receptor because of the very high concentration of K^+ by carrying out analogous experiments at lower ionic strength (i.e., at 0.2 M KCl). If preactivation was required, one would expect that the EC_{50} of NF307 is shifted to the right as K^+ is reduced. As can be seen from Fig. 2B, this was not the case; the EC_{50} values were 62.6 ± 1.5 and 45.6 ± 7.0 μM at 0.75 M and 0.2 M KCl, respectively. Lowering the ionic strength raises the calculated free Ca^{2+} concentration to 0.61 μM, and this accounts for the increase in the apparent affinity of NF307 at 0.2 M KCl (see below). As expected, the maximum binding capacity was lower at 0.2 M KCl.

Saturation Isotherms and Kinetic Analysis in the Presence of NF307. The barely detectable levels of basal binding that are observed at submicromolar concentrations of free Ca^{2+} are due to low-affinity binding (K_D = 185 ± 29

Suramin



NF299



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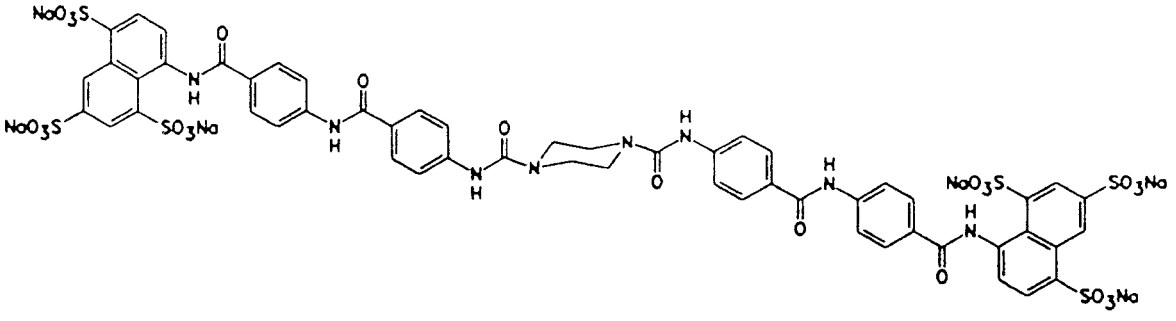

NF307

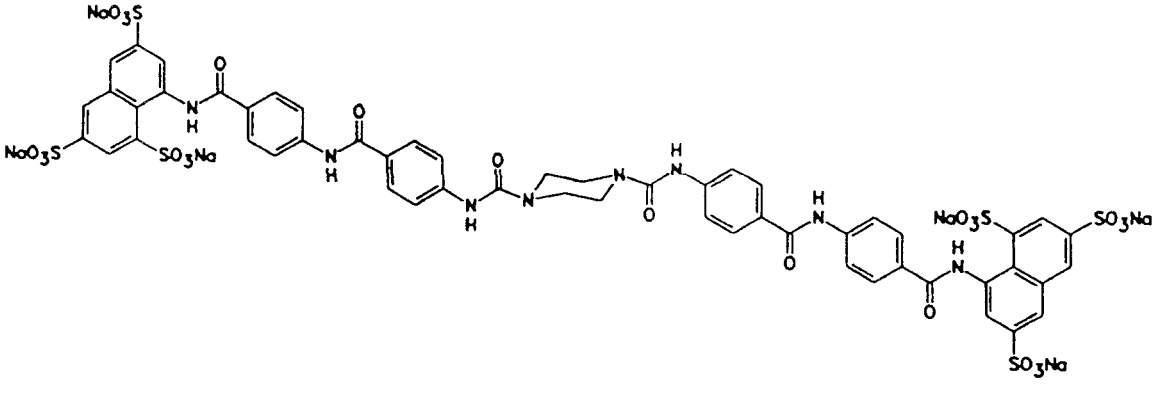

Fig. 1. Chemical structures of suramin, NF299, NF301, and NF307.

nM; Fig. 3A). Addition of 200 μM NF307 promoted the appearance of high-affinity binding sites for [^{3}H]ryanodine (K_D = 25 ± 9, Fig. 3A); this was seen at low (Fig. 3A) as well as at high ionic strength (not shown). We attribute the pronounced increase in binding to an acceleration of the association rate based on the following observations. In the presence of 0.2 M KCl and of 0.6 μM Ca^{2+}, the basal apparent association rate k_{app} was very low (Fig. 3B); because of the essentially linear relation between time and [^{3}H]ryanodine binding, it was not possible to reliably calculate the rate, but we estimated k_{app} to be below 0.001 min^{-1}. In contrast, upon addition of 200 μM NF307, the binding reaction was described adequately assuming a pseudo-first-order process yielding apparent rate constants of 0.012 ± 0.005 min^{-1} (Fig. 3B). A similar acceleration of [^{3}H]ryanodine binding was seen in the presence of 10 mM caffeine, which as used as a control (k_{app} = 0.012 ± 0.002 min^{-1}, Fig. 3B). Because basal binding at 0.6 μM free Ca^{2+} was too low to assess dissociation kinetics, we have also determined the dissociation rate of [^{3}H]ryanodine binding that was supported by 10 μM free Ca^{2+}; under these conditions, addition of either caffeine or NF307 to the medium use for 100-fold dilution of the assay volume had only modest effects on the dissociation rate (k_{off} = 4.3 ± 0.9, 4.3 ± 0.8, and 6.6 ± 1.2 × 10^{-3} min^{-1}, in the absence and presence of 10 mM caffeine and of 100 μM NF307, respectively).

Single-Channel Recordings of the Purified Ryanodine Receptor in the Presence of NF307. To prove that NF307 directly affected the channel properties of the ryanodine receptor, the purified protein was incorporated into artificial planar lipid bilayers and investigated by single-channel recordings. An example of the effect of NF307 on the purified Ca^{2+}-release channel at a low free calcium concentration is illustrated in Fig. 4. Channel P_o of the control at 20

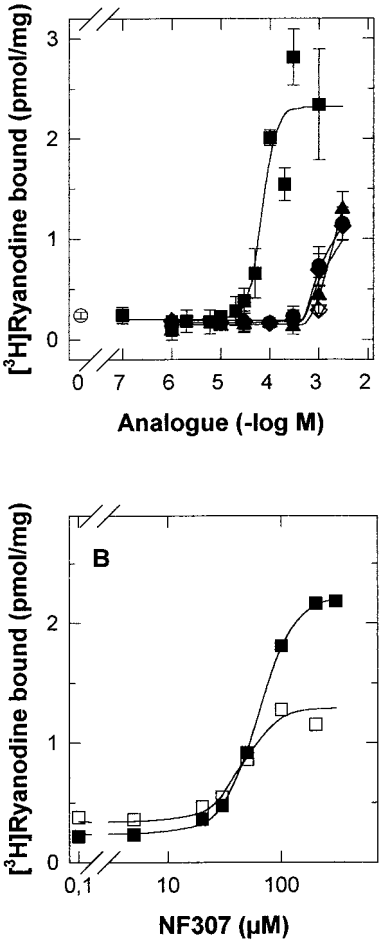

Fig. 2. Stimulation of [^{3}H]ryanodine binding to sarcoplasmic reticulum membranes by suramin and suramin analogs. A, sarcoplasmic reticulum membranes (50 μg) were incubated with 20 nM [^{3}H]ryanodine for 45 min at 30°C in absence (○) or presence of indicated concentrations of NF307 (■), suramin (▲), NF299 (♦), NF301 (●), NF449 (▽), and NF503 (☆) at a ratio of 0.85 mM Ca^{2+}/1 mM EGTA, yielding a calculated free Ca^{2+} concentration of 0.45 μM in presence of 0.75 M KCl. Data are means ± S.D. of three independent experiments carried out in duplicate. B, concentration-response curve for NF307 was determined in parallel in presence of 0.75 M (■) or of 0.2 M KCl (□) and at a ratio of 0.85 mM Ca^{2+}/1 mM EGTA as described for A; however, lowering ionic strength increases calculated free concentration to 0.61 μM. Data points are means of duplicate determinations in a representative experiment that was repeated twice.

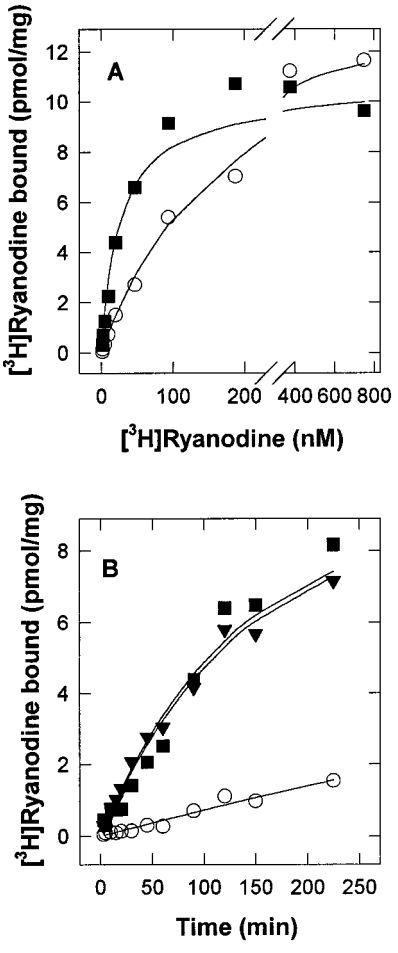

Fig. 3. Saturation isotherms for (A) and association of [^{3}H]ryanodine binding (B) to sarcoplasmic reticulum membranes. A, sarcoplasmic reticulum membranes (50 μg) were incubated in 0.2 M KCl buffer containing 0.69 μM free Ca^{2+} and the indicated concentrations of [^{3}H]ryanodine in absence (○) and presence of 200 μM NF307 (■). Incubation time was 180 min. Lines were drawn by fitting data to an equation describing interaction of radioligand with a single class of binding sites; a two-site model was also tested but did not significantly imporve fit (F-test based on extra sum of squares principle). B, assay conditions were as in A; concentration of [^{3}H]ryanodine was 20 nM. Incubation was done in absence (○) and presence of 200 μM NF307 (■) or 10 mM caffeine (▼). At time points indicated, reaction was terminated by rapid filtration. Data points represent mean of duplicate in representative experiments that were repeated twice.

μM Ca^{2+} was 0.46 (Fig. 4A). When the free calcium concentration on the *cis* side of the bilayer was reduced to 0.6 μM by the addition of EGTA, the Ca^{2+}-release channel was predominantly closed (P_o = 0.01; Fig. 4B). The addition of 100 μM NF307 to the solution on the *cis* side (the cytoplasmic side) increased the open probability (P_o = 0.62, Fig. 4C) as well as the open time constants (Fig. 4, D and E). The NF307 modified Ca^{2+}-release channel was blocked by ruthenium red (not shown). Table 1 summarizes the mean values of P_o, current amplitudes, and the distribution of the open and closed lifetimes of controls and of the same channels modified by 100 μM NF307 in five experiments. The increase in the open probability in the presence of low free calcium of 0.6 μM from 0.02 ± 0.006 (control) to 0.53 ± 0.07 (100 μM NF307; n = 5; means ± S.E.M.) mainly is due to an increase in the frequency of channel openings. The open probability in controls of the same channels in the presence of 20 μM Ca^{2+} was 0.48 ± 0.08 (n = 5; means ± S.E.M.), i.e., the NF307-induced increase in P_o was close to values obtained with 20 μM activating Ca^{2+} in four of five experiments. This corresponds to the range of P_o that has been observed in more than 30 purified Ca^{2+}-release channel preparations with maximally activating Ca^{2+} concentrations (20–100 μM free Ca^{2+}). Moreover, NF307 also increased the open lifetimes at 0.6 μM Ca^{2+}, i.e., in three of five experiments a better fit of the open times was obtained by a three-exponential fit (Fig. 4E). In the other two experiments, the parameters for the third exponential component could not be estimated reliably; hence, Table 1 lists only the estimates derived from the equation describing the sum of two exponential processes.

In the presence of 20 μM free Ca^{2+}, i.e., a high, activating concentration, NF307 increased the open probability of the Ca^{2+}-release channel from 0.47 ± 0.07 to 0.82 ± 0.04 (n = 6, means ± S.E.M.) (Fig. 5 and Table 2). Upon activation by NF307, the conductance, which was determined from the same channel as shown in Fig. 5, was not affected (control: 529; NF307: 518 pS). The increase in the open time constants is illustrated in Fig. 5, C and D. Means of P_o, current amplitudes and the distribution of the open and closed lifetimes of controls and of the same channels modified by 100 μM NF307 in the presence of 20 μM Ca^{2+} are given in Table 2. In all experiments, the best fit in the open lifetimes of the NF307-stimulated channel was obtained by three exponentials [control: τ_{o1} = 0.28 ± 0.05 ms (68%); τ_{o2} = 0.57 ± 0.04 ms (32%); NF307: τ_{o1} = 0.29 ± 0.06 ms (41%); τ_{o2} = 1.10 ± 0.15 ms (47%); τ_{o3} = 3.60 ± 1.09 ms (12%)] (Table 2, n = 6, means ± S.E.M.).

NF307 appears to be more active than the parent compound suramin. In five experiments (carried out under conditions given in Fig. 5, in the presence of 20 μM activating

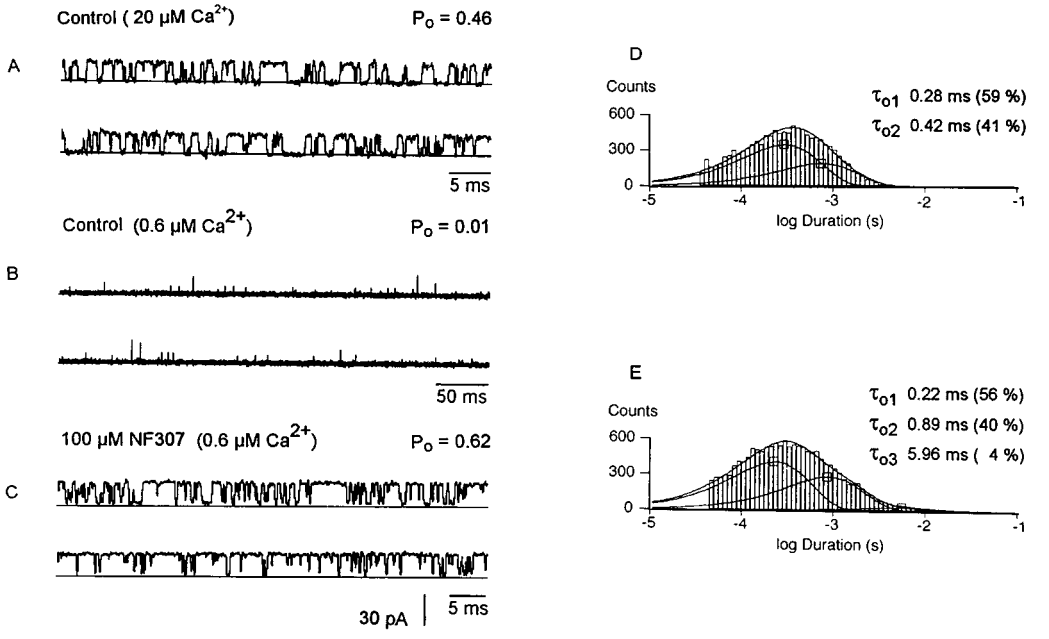

Fig. 4. Effect of NF307 on a single purified skeletal muscle calcium-release channel at low free calcium (0.6 μM). Single-channel currents, shown as upward deflections, were recorded at 0-mV holding potential with 480 mM/50 mM CsCl (*cis/trans*). Baselines are indicated by solid lines. Control and test records (A–C) are from same channel. A, control (20 μM Ca^{2+}), P_o = 0.46. B, control (0.6 μM Ca^{2+}), P_o = 0.01. C, activation of channel by 100 μM NF307 added to the *cis* side (0.6 μM Ca^{2+}), P_o = 0.62. Calibration bars represent 20 pA and 5 ms or 50 ms. Lifetime histograms of open times and channel-opening time constants (τ_o) of control and NF307-modified channel are displayed in D and E, respectively. Channel open probabilities (P_o) and τ_o were calculated from 22,825 events (control, 20 μM Ca), 2,530 events (control, 0.6 μM Ca^{2+}), and 29,160 events (100 μM NF307).

TABLE 1

Means of P_o, current amplitudes, and mean open and closed channel lifetimes of controls and stimulation by 100 μM NF307 at 0.6 μM Ca^{2+}

Channel open probabilities (P_o), cumulative mean open and closed channel time constants (τ_o, τ_c), and values of channel represented by a time constant (as percentage) for the purified Ca^{2+}-release channel/ryanodine receptor activated by 0.6 μM free *cis* Ca^{2+} in absence or presence of 100 μM NF307. Values given are means ± S.E.M. from five channels included in analysis and were calculated from data segments of 30- to 90-s duration. Calcium release channels were recorded at 0-mV holding potential with 480 mM/50 mM CsCl (*cis/trans*).

Treatment	P_o	τ_o (ms), Weight	τ_c (ms), Weight	Amplitude
		%	%	*pA*
Control	0.48 ± 0.08	τ_{o1} 0.33 ± 0.06 (74%)	τ_{c1} 0.41 ± 0.08 (96%)	19.8 ± 1.01
20 μM Ca^{2+}		τ_{o2} 0.73 ± 0.12 (26%)	τ_{c2} 1.57 ± 0.49 (4%)	
Control	0.02 ± 0.006	τ_{o1} 0.10 ± 0.01 (89%)	τ_{c1} 1.15 ± 0.51 (23%)	19.8 ± 0.76
0.6 μM Ca^{2+}		τ_{o2} 0.36 ± 0.07 (11%)	τ_{c2} 7.65 ± 1.16 (63%)	
NF307+	0.53 ± 0.07	τ_{o1} 0.34 ± 0.07 (85%)	τ_{c1} 0.27 ± 0.03 (83%)	19.8 ± 0.71
0.6 μM Ca^{2+}		τ_{o2} 1.42 ± 0.42 (15%)	τ_{c2} 1.04 ± 0.23 (23%)	

Ca^{2+}), 300 μM suramin caused an increase in the open probability from 0.40 ± 0.05 to 0.67 ± 0.06 (means ± S.E.M.; n = 5). The requirement of high suramin concentrations to affect P_o also has been noted previously (Sitsapesan and Williams, 1996; Hohenegger et al., 1996). In single-channel recordings of skeletal muscle HSR or cardiac HSR, a modest suramin-induced increase in current amplitude has been described (Sitsapesan and Williams, 1996). However, under the conditions used in the present study, NF307 and suramin did not affect the current amplitudes. This discrepancy may be due to different experimental conditions (10 μM/50 mM calcium *cis/trans* in the experiments of Sitsapesan and Williams, 1996). There is no evidence for an interaction of NF307 with FK506 binding proteins. Inhibition of a FK506-binding protein and the ensuing dissociation from the ryanodine receptor result in the appearance of subconductance states (Brillantes et al., 1994). However, these were observed only very rarely in the presence of NF307.

Ca^{2+} Dependence of the NF307 Effect. At concentrations exceeding the threshold concentration, i.e., in the low micromolar range, Ca^{2+} promotes high-affinity [^{3}H]ryanodine binding and the concentration-response relation is very steep; in contrast, above 100 μM free Ca^{2+}, [^{3}H]ryanodine binding to RyR_1 is inhibited, resulting in a bell-shaped curve (Franzini-Armstrong and Protasi 1997; see also Fig. 6). This inhibition was also seen in the presence of 10 mM caffeine and 100 μM NF307 (Fig. 6). In accordance with our previous observations (Hohenegger et al., 1996), the suramin-induced stimulation of the ryanodine receptor was only modestly inhibited by high Ca^{2+} concentrations (Fig. 6A). More importantly, NF307 and caffeine shifted the stimulatory limb of the Ca^{2+} curve to the left irrespective of whether the experiment was carried out in a 0.75 M (Fig. 6A) or in 0.2 M KCl buffer (Fig. 6B). However, basal binding, i.e., binding supported by the sole addition of micromolar concentrations of Ca^{2+}, was enhanced at high ionic strength (EC_{50} for Ca^{2+} = 1.20 ± 0.20 and 0.72 ± 0.08 μM at 0.75 and at 0.2 M KCl, respectively); hence, the shift induced by NF307 appeared more pronounced at 0.2 M KCl (cf. Fig. 6, A and B). Nevertheless, in the presence of NF307 the EC_{50} values for Ca^{2+} were comparable at low (0.12 ± 0.01 μM) and high ionic strength (0.11 ± 0.02 μM).

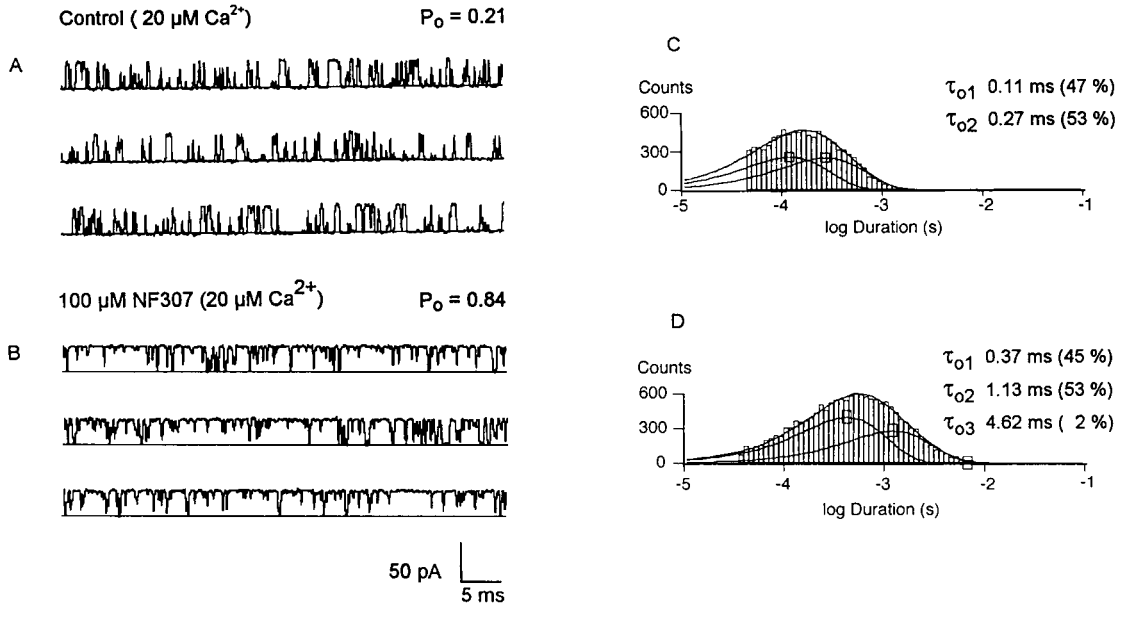

Fig. 5. Effect of 100 μM NF307 on a single purified skeletal muscle calcium-release channel at 20 μM activating Ca^{2+}. Single-channel currents, shown as upward deflections, were recorded at +20 mV holding potential with 480 mM/50 mM CsCl (*cis/trans*). Baselines are indicated by solid lines. Control and test records are from same channel. A, control: 20 μM Ca^{2+}, P_o = 0.21. B, activation of channel by 100 μM NF307 added to *cis* side (20 μM Ca^{2+}), P_o = 0.84. Slope conductances were 529 pS (control) and 518 pS (NF307). Lifetime histograms of open times and channel-opening time constants (τ_o) of control and NF307-modified channel are displayed in C and D, respectively. Solid lines represent fit according to two or three exponentials. Channel open probabilities (P_o) and τ_o were calculated from 18,100 events (control) and 27,950 events (100 μM NF307), respectively. Calibration bars represent 50 pA and 5 ms.

TABLE 2

Mean open and closed channel lifetimes in presence of 20 μM free Ca^{2+} and after stimulation by 100 μM NF304 at 20 μM

Channel open probabilities (P_o), cumulative mean open and closed channel time constants (τ_o, τ_c), and values of percentage of the channel represented by a time constant for purified Ca^{2+}-release channel/ryanodine receptor activated by 20 μM *cis* Ca^{2+} in absence or presence of 100 μM NF307. Values given are means ± S.E.M. from six channels included in analysis and were calculated from data segments of 30- to 90-s duration. Ca^{2+}-release channels were recorded at 0-mV holding potential with 480 mM/50 mM CsCl (*cis/trans*).

Treatment	P_o	τ_o (ms), Weight	τ_c (ms), Weight	Amplitude
		%	%	*pA*
Control	0.47 ± 0.07	τ_{o1} 0.28 ± 0.05 (68%)	τ_{c1} 0.36 ± 0.05 (88%)	20.3 ± 0.73
20 μMCa^{2+}		τ_{o2} 0.57 ± 0.04 (32%)	τ_{c2} 1.32 ± 0.51 (12%)	
NF307	0.82 ± 0.04	τ_{o1} 0.29 ± 0.06 (41%)	τ_{c1} 0.28 ± 0.02 (99%)	19.8 ± 2.1
20 μMCa^{2+}		τ_{o1} 1.10 ± 0.15 (47%)		
		τ_{o3} 3.60 ± 1.09 (12%)		

NF307 sensitizes the ryanodine receptor to the stimulatory effect of Ca^{2+}; conversely, Ca^{2+} ought to modulate the affinity of NF307 for the ryanodine receptor. We have verified this prediction by comparing the EC_{50} of NF307 in the presence of two different Ca^{2+} concentrations taken from the activating limb of the Ca^{2+} concentration-response curve. At a free Ca^{2+} concentration of 0.19 μM, NF307 stimulated [^{3}H]ryanodine binding with an EC_{50} of 91 $\pm$ 7 μM (Fig. 6C). In contrast, suramin was barely effective under these experimental conditions (Fig. 6C). If the free Ca^{2+} concentration was raised to 0.82 μM, the concentration-response curve of NF307 was shifted to the left (EC_{50} = 14.6 $\pm$ 3.5 μM; Fig. 6C). This provides unequivocal evidence for Ca^{2+}-dependent sensitization of the ryanodine receptor to the stimulatory effect of NF307. Moreover, Ca^{2+} also enhanced the stimulatory action of suramin (Fig. 6C).

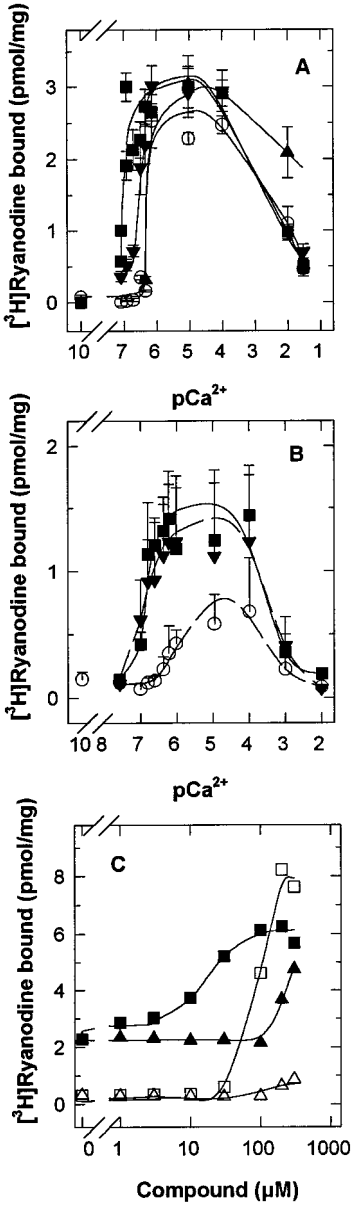

Fig. 6. Ca^{2+}-dependent [^{3}H]ryanodine binding in presence of NF307 and caffeine. A and B, [^{3}H]ryanodine binding was determined in 0.75 M KCl buffer (A) or 0.2 M KCl buffer (B) in absence (O) and presence of 100 μM NF307 (■), 10 mM caffeine (▼), or 300 μM suramin (▲ in A). Incubation conditions were as outlined in legend to Fig. 2. Data represent mean of three experiments carried out in duplicate; error bars indicate S.D. C, increasing concentrations of NF307 (■,□) or suramin (▲,△) were added to sarcoplasmic reticulum membranes incubated in presence of 0.75 M KCl buffer; free Ca^{2+} concentration was adjusted to 0.19 μM (■,△) or to 0.82 μM (■,▲). Data points are means of duplicate determinations in a representative experiment that was repeated twice.

NF307 and Suramin Interact with a Calmodulin-Binding Site. Caffeine has long been known to sensitize the ryanodine receptor for Ca^{2+} (Sitsapesan and Williams, 1990; see also Fig. 6, A and B); because NF307 exerted a similar effect, NF307 and caffeine may share a common binding site on the protein. This conjecture appeared unlikely, because the structures of NF307 and caffeine have little in common. An alternative candidate site of action may be the site(s) by which Ca^{2+}-free calmodulin stimulates the ryanodine receptor (Buratti et al., 1995; Tripathy et al., 1995). Calmodulin is acidic, and the polyanionic nature of NF307 is evident from Fig. 1. To test these two hypotheses, binding experiments were performed in a nominally Ca^{2+}-free medium (2 mM EGTA, no added Ca^{2+}). As expected, the apparent affinity of NF307 is reduced under these conditions ($EC_{50} \sim 400$ μM; Fig. 7A). However, if 10 mM caffeine was combined with increasing amounts of NF307, the stimulation induced by the combination was strictly additive over the entire range of the NF307 concentration-response curve (Fig. 7A); this finding is incompatible with an action of the two compounds via a common site. In contrast, addition of 2 μM calmodulin blunted the effect of NF307 (Fig. 7A). Because of the large effect of NF307, the *y*-axis in Fig. 7A covers a range that is too wide to illustrate the smaller stimulatory effect that calmodulin exerted per se. The latter is more readily appreciated in Fig. 7B. More importantly, Fig. 7B also shows that the combination of caffeine and calmodulin resulted in overadditive stimulation. The experiments summarized in Fig. 7, A and B were done at high ionic strength. Analogous results were obtained if the incubation medium contained 0.2 M KCl (data not shown).

Contrary to Ca^{2+}-free calmodulin, Ca^{2+}-liganded calmodulin is a potent inhibitor of the ryanodine receptor (Meissner, 1986); accordingly, in the presence of 100 μM free Ca^{2+}, calmodulin suppressed high-affinity [^{3}H]ryanodine binding with an IC_{50} of 45 $\pm$ 12 nM (Fig. 7C). The concentration-response curve of Ca^{2+}/calmodulin was shifted progressively to the right by increasing concentrations of NF307 in a manner that is consistent with competitive antagonism (Fig. 7C). Similarly, the inhibition induced by Ca^{2+}/calmodulin was abolished in the presence of 300 μM suramin (Fig. 7C).

Taken, together, these findings provide functional evidence for the assumption that binding of NF307 and of calmodulin to the ryanodine receptor is mutually exclusive. This interpretation was verified by immobilizing the purified ryanodine receptor on calmodulin-Sepharose. Addition of 10 μM calmodulin, 300 μM suramin, or 100 μM NF307 resulted in the elution of comparable amounts of protein (Fig. 8). This was seen irrespective of whether the experiment was done at a free-Ca^{2+} concentration of 200 μM (Fig. 8A) or 0.6 μM (Fig. 8B). However, in the presence of 0.6 μM Ca^{2+}, the ryanodine receptor apparently was eluted less efficiently; in all experiments carried out at 0.6 μM Ca^{2+}, the second and third eluate contained ryanodine receptor. In contrast, if 200 μM Ca^{2+} was used, the first eluate contained essentially the bulk of the protein that could be released (cf. lanes e1–e3 in Fig. 8,

A and B). The ryanodine receptor is highly susceptible to proteolysis during purification (Inui et al., 1987), and a doublet was visualized in the range of 300 to 350 kDa upon silver staining. We have verified that both the upper and the lower band corresponded to the ryanodine receptor by immunoblotting with a monoclonal antibody that recognizes RyR_1 (Fig. 8C). If the ryanodine receptor was preincubated with calmodulin, suramin, or NF307 before addition of calmodulin-Sepharose, no protein was retained by the matrix (data not shown).

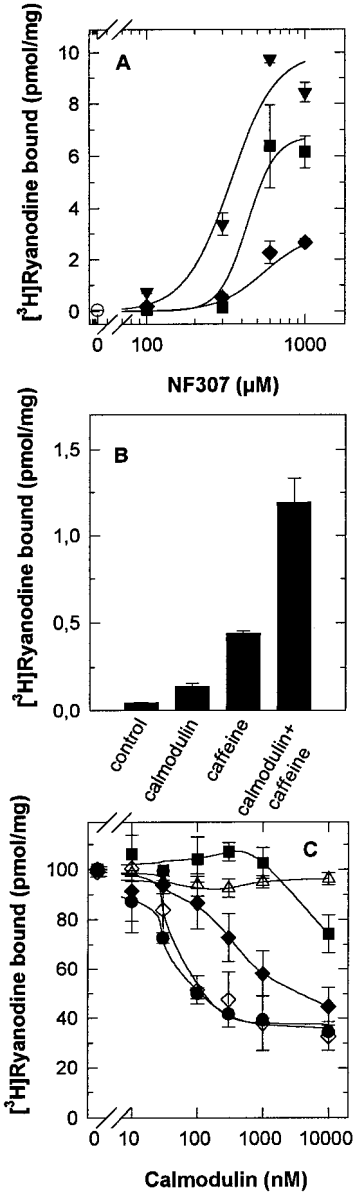

Fig. 7. Stimulation and inhibition of [^{3}H]ryanodine by the combined addition of NF307, calmodulin, and caffeine. A, sarcoplasmic reticulum membranes (50–70 μg) were incubated in nominally Ca^{2+}-free buffer (2 mM EGTA in absence of added $CaCl_2$) containing 0.75 M KCl, 20 nM [^{3}H]ryanodine, and increasing concentrations of NF307 in the absence (■) and presence of 10 mM caffeine (▼) or of 2 μM calmodulin (♦). B, [^{3}H]ryanodine binding was determined under same ionic conditions as in A in absence (control) and presence of 2 μM calmodulin, 10 mM caffeine, or combination of calmodulin and caffeine. C, assay conditions were similar to those in A, but free Ca^{2+} concentration was raised to 100 μM. Sarcoplasmic reticulum membranes were incubated with increasing concentrations of calmodulin in absence (◇) and presence of 10 μM (●), 30 μM (♦), and 100 μM (■) NF307 or 300 μM suramin (△). To avoid a reduction in free Ca^{2+} concentration, calmodulin stock solution was prepared in presence of a 4-fold molar excess of $CaCl_2$ and serial dilutions made therefrom. Binding observed in absence of calmodulin was set to 100% to normalize for interassay variation and stimulatory effect of NF307 and suramin (~1.3-fold at highest concentration of NF307; see also Fig. 6A). Data are means of three to five independent experiments carried out in duplicate; error bars represent S.D.

Discussion

The Ca^{2+}-binding protein calmodulin regulates the activity of the ryanodine receptor by at least three mechanisms, i.e., direct activation, direct inhibition, and indirect modulation. The latter is achieved via phosphorylation of the channel protein by Ca^{2+}/calmodulin-dependent kinase II (Witcher et al., 1991; Hohenegger and Suko, 1993; Suko et al., 1993). Calmodulin binds directly to the ryanodine receptor; in the

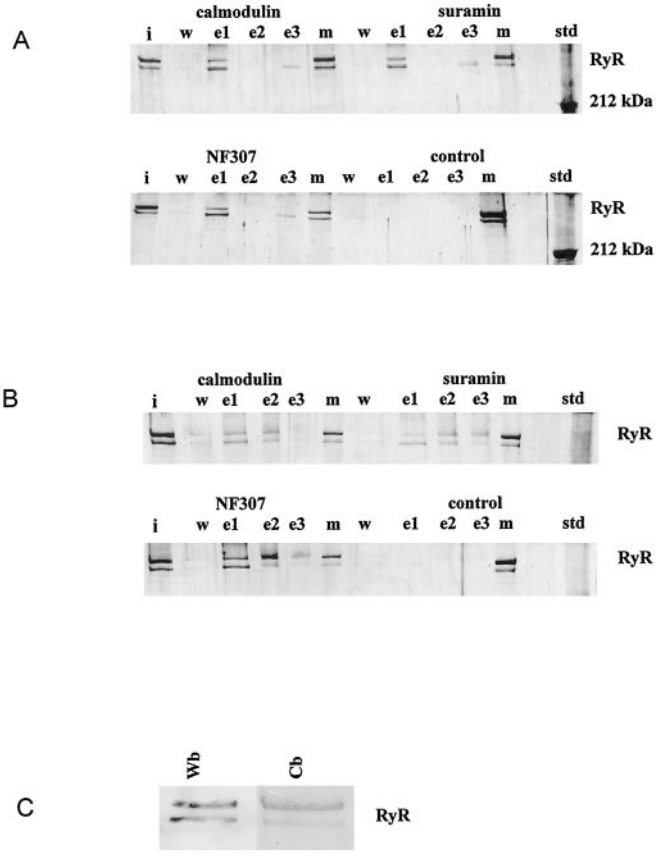

Fig. 8. Elution of the purified ryanodine receptor from a calmodulin-Sepharose matrix by calmodulin NF307 and suramin. Purified ryanodine receptor (1–2 μg/incubation) was incubated with appropriately equilibrated calmodulin-Sepharose in presence of 200 μM (A) or 0.6 μM free Ca^{2+} (B) as described under *Materials and Methods*. Affinity matrix was washed four times with incubation buffer. Subsequently, ryanodine receptor was eluted batchwise with buffer containing 20 μM calmodulin, 300 μM suramin, or 200 μM NF307 as indicated. Mock elution was done with buffer alone (lanes labeled "control"); residual protein trapped in matrix was released by boiling in Laemmli sample buffer. Aliquots corresponding to one-third of input (lanes labeled "i"), of last wash step (lanes labeled "w"), of three successive elution steps (lanes labeled "e1–e3"), and of material released by denaturation of calmodulin-Sepharose matrix (lanes labeled "m") were applied onto SDS-polyacrylamide gels. Proteins were visualized by silver staining. Position of molecular mass standard is indicated (lanes labeled "std"). Two additional experiments have similar results. C, after electrophoresis, purified ryanodine receptor preparation used in A and B was visualized by staining with Coomassie blue (lane Cb) or transferred to nitrocellulose and detected by immunoblotting (lane Wb).

absence of Ca^{2+}, this interaction results in increased channel opening (Buratti et al., 1995; Tripathy et al., 1995); in contrast, Ca^{2+}-liganded calmodulin suppresses the activity of the channel (Meissner, 1986; Plank et al., 1988; Smith et al., 1989). In the present work, we show that the suramin analog NF307 is a potent and direct activator of the ryanodine receptor and that its effect results from an interaction with a calmodulin-binding domain. This conclusion is supported by the mutually antagonistic effects that NF307 and calmodulin exert on [^{3}H]ryanodine binding and by the ability of NF307 to elute the channel protein from a calmodulin-based affinity matrix. Multiple potential calmodulin-binding sites have been predicted based on the deduced primary sequence of the skeletal muscle ryanodine receptor RyR_1; however, the precise number is still a matter of debate (Franzini-Armstrong and Protasi, 1997) and evidence for up to 24 sites/tetrameric receptor has been reported (Yang et al., 1994). At least three binding sites have been identified by overlaying heterologously expressed portions of RyR_1, which had been fused to carrier proteins, with radiolabeled calmodulin (Chen and McLennan, 1994; Menegazzi et al., 1994; Buratti et al., 1995); the relation of these sites to the regions to which calmodulin may bind to the intact protein has been questioned (Tripathy et al., 1995). After reconstitution of the purified ryanodine receptor into phospholipid vesicles, calmodulin was observed to bind with a stoichiometry of three to four molecules and one molecule per monomer in the absence and presence of Ca^{2+}, respectively (Tripathy et al., 1995). Kinetic arguments suggest that the site that is occupied by calmodulin in the absence of Ca^{2+} also binds calmodulin in the presence of Ca^{2+} (Tripathy et al., 1995). It presumably is this high-affinity binding site for Ca^{2+}/calmodulin that has been visualized by electron cryomicroscopy (Wagenknecht et al., 1997); however, at present it is difficult to gain mechanistic insights from these three-dimensional reconstructions, because the four molecules of Ca^{2+}/calmodulin bound per tetramer are located at a 10-nm distance from the putative ion pore (Wagenknecht et al., 1997).

The concentration-response curves for NF307 are steep, the Hill-coefficient being consistently >1 irrespective of the free Ca^{2+} concentration. Given the tetrameric nature of the channel, the steep slope may reflect either cooperativity between several binding sites on one monomer or the cooperative activation of the tetramer via a single binding site on each monomer, and it currently is not possible to differentiate between the two possibilities. Although it is conceivable that binding of NF307 allosterically inhibits the interaction of calmodulin with the ryanodine receptor, the available evidence is consistent with a simpler model of mutual competition for a common site on the ryanodine receptor; this interpretation is supported by the observation that the concentration-response curve of calmodulin is progressively shifted to the right by increasing the concentration of NF307. In contrast to calmodulin, NF307 elicits only stimulatory effects on the ryanodine receptor. The basis for this discrepancy is unknown. Calmodulin is a bipartite, pseudosymmetric molecule; the globular Ca^{2+}-binding domains at the amino and at the carboxy terminus are connected by an α-helix. Genetic evidence suggests that the two lobes subserve nonequivalent functions in ion-channel regulation. Mutations in the calmodulin gene of *Paramecium* that alter the carboxy terminus disrupt regulation of one class of ion channels, whereas those affecting the amino terminus abrogate modulation of the other class (for review, see Saimi and Kung, 1994). Given that one binding site on the ryanodine receptor monomer is occupied by calmodulin both in the absence and presence of Ca^{2+} (Tripathy et al., 1995), it is attractive to speculate that the dual actions of calmodulin on channel gating is mediated by different parts of the molecule and that NF307 only mimics the portion of calmodulin that activates the ryanodine receptor.

Apart from its trypanocidal and anthelminthic effects, suramin has multiple pharmacological actions in mammalian organisms (Voogd et al., 1993). However, our experiments show that structural determinants, which go beyond merely providing a rigid backbone for multiple negative charges, are important for the potency and efficacy of suramin analogs at the ryanodine receptor. This is exemplified by the finding that closely related analogs are less active or entirely inactive. Furthermore, suramin analogs that we have reported recently to be more selective G protein antagonists than suramin (Freissmuth et al., 1996; Hohenegger et al., 1998) are inactive when tested on the ryanodine receptor. Finally, apart from the distinct potencies, NF307 and suramin differ in their mode of action at the ryanodine receptor; suramin, but not NF307, blunts the inhibition of the ryanodine receptor that is elicited by millimolar concentrations of Ca^{2+}. In contrast, NF307 is considerably a more efficient Ca^{2+}-sensitizer than suramin, and the magnitude of its effect is comparable to that of caffeine.

The ryanodine receptor is notorious for its role in side effects occurring in clinical pharmacotherapy (for review, see Zucchi and Ronca-Testoni, 1997). In malignant hyperthermia, halogenated volatile anesthetics (e.g., halothane) alone or in combination with depolarizing neuromuscular-blocking agents (e.g., succinylcholine) trigger uncontrolled Ca^{2+} release from the sarcoplasmic reticulum, muscle contracture, and a hypermetabolic state that precipitates a life-threatening elevation in body temperature. In many cases (presumably $\geq$50%), the underlying disturbance is associated with point mutations in the ryanodine receptor (McLennan and Philipps, 1992). The mechanism by which these point mutations cause susceptibility to malignant hyperthermia is not known; however, an analogous disease exists in domestic pigs (referred to as porcine stress syndrome). The ryanodine receptor of animals homozygous for the mutation is more susceptible to the stimulatory effect of calmodulin (O'Driscoll et al., 1996). Similarly, oxygen radicals activate the cardiac ryanodine receptor by relieving the inhibition imposed by calmodulin (Kawakami and Okabe, 1998); this may account for Ca^{2+} overload in cardiac myocytes as a result of oxidative stress that occurs during pathophysiological conditions such as ischemia and reperfusion. Hence, compounds that act at the calmodulin-binding site(s) of the ryanodine receptor are clearly of interest for understanding the mechanism of ryanodine receptor regulation by calmodulin. Ultimately, these ligands also may be useful therapeutically.

Acknowledgments

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