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Use-dependent inhibition of the skeletal muscle ryanodine receptor by the suramin analogue NF676

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1 The skeletal muscle Ca^{2+} release channel, the ryanodine receptor, is activated by the trypanocidal drug suramin *via* the calmodulin-binding site. As calmodulin activates and inhibits the ryanodine receptor depending on whether Ca^{2+} is absent or present, suramin analogues were screened for inhibition of the ryanodine receptor.

2 Up to 300 μM, the novel suramin analogue, 4,4′-(carbonyl-bis(imino-4,1-phenylene-(2,5-benzimidazolylene)carbonylimino))-bis-benzenesulfonic acid disodium salt (NF676) was not able to significantly inhibit the basal [^{3}H]ryanodine binding. However, kinetic analysis of the high affinity [^{3}H]ryanodine binding elucidates a time-dependent increment of inhibition by NF676, which is indicative for an open channel blocker.

3 Moreover, the ryanodine receptor was much more sensitive towards inhibition by NF676 when preactivated with caffeine or the nonhydrolysable ATP analogue, adenylyl-imidodiphosphate. Nonetheless, the suramin activated ryanodine receptor was not susceptible towards high-affinity NF676 inhibition, indicating an allosteric hindrance between the binding sites of suramin and NF676.

4 In the line of this finding, NF676 *per se* was not capable to elute the purified ryanodine receptor from a calmodulin-Sepharose, but it prevented the elution by suramin.

5 Other than suramin, NF676 did not inhibit the Ca^{2+} ATPase of the sarcoplasmic reticulum. However, suramin-induced Ca^{2+} release from sarcoplasmic reticulum was completely abrogated by preincubation with NF676.

6 Taken together, we conclude from these data that NF676 represents a novel lead compound as a potent use-dependent blocker of the skeletal muscle ryanodine receptor *via* an allosteric interaction with the suramin-binding site.
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Abbreviations: AMP-PCP, β,γ-methyleneadenosine 5′-triphosphate; AMP-PNP, adenylyl-imidodiphosphate; HSR, heavy sarcoplasmic reticulum; NF307, 8,8′-(piperazine-1,4-diyl-bis(carbonylimino-4,1-phenylenecarbonyl-imino-4,1-phenylene) carbonylimino))-bis-naphthalene-1,3,6-trisulfonic acid hexasodium salt; NF676, 4,4′-(carbonyl-bis (imino-4,1-phenylene-(2,5-benzimidazolylene) carbonylimino))-bis-benzenesulfonic acid disodium salt; RyR1, ryanodine receptor type 1; RyR2, ryanodine receptor type 2

Introduction

Skeletal muscle contraction is under the strict control of the cytoplasmatic Ca^{2+} concentration. Excitation of skeletal muscle results in the rapid Ca^{2+} release from sarcoplasmic reticulum through the ryanodine receptor/Ca^{2+} release channel type 1 (RyR1). The ryanodine receptor is one of the largest ion channels known, organized as a homotetramer consisting of four identical subunits each of a mass of 560 kDa (for a review, see Meissner, 1994; Sutko & Airey, 1996; Franzini-Armstrong & Protasi, 1997). There are several endogenous regulators of the ryanodine receptor. Out of these, calmodulin exerts a dualistic influence on the RyR1 depending on the free Ca^{2+} concentration (Tripathy *et al*., 1995; Rodney *et al*., 2000). In its Ca^{2+}-free form, calmodulin (apocalmodulin) activates the RyR1, but Ca^{2+}-liganded calmodulin is an inhibitor of the channel (Plank *et al*., 1988; Smith *et al*., 1989; Buratti *et al*., 1995; Tripathy *et al*., 1995).

The typanozidal drug suramin is established in the therapy of infections caused by *Onchcerca volvulus*, *Trypanosoma gambiense* and *Trypanosoma rhodensiense*. However, in mammalians, several targets of action have been characterized (Voogd *et al*., 1993; Freissmuth *et al*., 1996). Among these, the RyR1 was identified to be activated by suramin (Emmick *et al*., 1994; Hohenegger *et al*., 1996; Sitsapesan & Williams, 1996). We could previously show that suramin is capable to discriminate between various calmodulin-binding sites in proteins as divergent as the RyR1, the G protein $\beta\gamma$-subunit, the neuronal NO synthase and the glutathion *S*-transferase fusion protein with the COOH-terminal calmodulin-binding domain of the metabotropic glutamate receptor 7A (Klinger *et al*., 2001). These observations made it conceivable that suramin may serve as a lead compound for calmodulin

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antagonism. On the RyR1 suramin and the more potent suramin analogue, 8,8′-(piperazine-1,4-diyl-bis(carbonylimino-4,1-phenylenecarbonyl-imino-4,1-phenylene) carbonyl-imino))-bis-naphthalene-1,3,6-trisulfonic acid hexasodium salt (NF 307) exert their action *via* the calmodulin-binding site, which was mapped to the peptide corresponding to amino acid 3609–3643 (Klinger *et al*., 1999; Papineni *et al*., 2002). Suramin is also a potent activator of the cardiac ryanodine receptor type II (RyR2) in [^{3}H]ryanodine binding and single channel recordings (Hohenegger *et al*., 1996; Sitsapesan & Williams, 1996). Moreover, very recent work of the laboratory of Sitsapesan provides experimental evidence that in the RyR2 suramin may also bind to the calmodulin-binding site of the ion channel (Hill *et al*., 2004).

As calmodulin is capable to activate and inhibit the RyR1 (Buratti *et al*., 1995; Tripathy *et al*., 1995; Fruen *et al*., 2000), we have searched for novel compounds related to suramin but with inhibitory action at the RyR1. The novel suramin analogue NF676 (4,4′-(carbonyl-bis (imino-4,1-phenylene-(2,5-benzimidazolylene) carbonylimino))-bis-benzenesulfonic acid disodium salt) is characterised and shown to act as an use-dependent antagonist of the RyR1 which may highlight a novel drug target binding site.

Methods

Synthesis of the suramin analogue NF676

NF676 was synthesized similar to methods recently published (Kassack *et al*., 2004). Briefly, 80 mmol of 4-aminobenzene-sulfonic acid (sulfanilic acid), dissolved in 150 ml water pH 4.5, were acylated with 3,4-dinitrobenzoylchloride (140 mmol, dissolved in 100 ml toluene). The aqueous layer was evaporated in vacuum to dryness yielding a yellow powder (compound 1, yield: 69.2%). In total, 25 mmol of compound 1 was dissolved in 500 ml methanol and hydrogenated using PtO_2 as catalyst (4 bar, 10 h). Subsequently, 26 mmol 4-nitrobenzaldehyde (dissolved in 120 ml methanol) were added immediately and stirred for 24 h. After concentrating the mixture to a volume of $\sim$200 ml, the product was oxidized (O_2: 6 bar, 120°C, 12 h). Recrystallization from ethanol/water yielded 44% of 4-(2-(4-nitrophenyl)benzimidazole-5-carboxamido) benzenesulfonic acid sodium salt (compound 2). In all, 16 mmol of compound 2 was resuspended in a mixture of 350 ml methanol and 150 ml water and hydrogenated using Pd/C as catalyst (4 bar, 10 h) to yield 98% of compound 3 (4-(2-(4-aminophenyl)benzimidazole-5-carboxamido) benzene-sulfonic acid sodium salt). Lastly, 9 mmol of compound 3 were resuspended in a mixture of 120 ml water and 40 ml methanol (pH 7) and treated with 240 mmol phosgene (20% solution in toluene). The complete mixture was evaporated in vacuum to dryness, and the raw product was recrystallized from methanol/water to yield 60% of NF676. Identity of NF676 was confirmed by nuclear magnetic resonance spectroscopy (^{1}H- and ^{13}C-NMR) and electron spray mass spectrometry. Purity of NF676 was demonstrated by elemental analysis (C, H, N) and by a high-performance liquid chromatography method previously published (Kassack & Nickel, 1996). Elemental analysis data were within $\pm$0.4%, and HPLC purity was >95%.

Preparation of heavy sarcoplasmic reticulum (HSR) and purified RyR1

HSR membranes were prepared from rabbit hind leg and back skeletal muscle or heart as described previously (Hohenegger & Suko, 1993; Klinger *et al*., 1999). Skeletal muscle HSR was used as a starting material to purify the RyR1 (Klinger *et al*., 1999). The protein concentration was determined with the BioRad Coomassie-Blue kit (BioRad, Munich, Germany) or the bicinchoninic acid assay (Micro-BCA; Pierce, Rockford, IL, U.S.A.).

[^{3}H]ryanodine binding

Briefly, the HSR membranes (75 μg) were incubated in 50 μl containing 40 mM HEPES (pH 7.4), 200 mM KCl, 10 mM NaCl, 20 nM [^{3}H]ryanodine, 1 μM aprotinin, 1 μM leupeptin, 100 μM pefablock and the drug concentrations indicated in the figure legends. The free calcium concentration was adjusted by the ratio of EGTA and $CaCl_2$. The incubation was carried out for 45 min at 37°C. Under such conditions, the difference between stimulated and inhibited binding is more pronounced. For kinetic experiments, the incubation time was varied from 2 to 180 min and saturation isotherms were incubated between 90 and 120 min. Nonspecific binding was determined in the presence of 100 μM ryanodine. The reaction was terminated by filtration over glass fiber filters (presoaked in 1% polyethyl-enimine) using a Skatron vacuum filtration device. The filters were rinsed with 10 ml ice-cold 10 mM Tris-HCl (pH 7.4) and 500 mM NaCl and the remaining radioactivity on the filters was determined by liquid scintillation counting.

Affinity chromatography and gel electrophoresis

The purified ryanodine receptor (0.1–0.2 mg ml^{-1}) was diluted in 100 μl binding buffer containing 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 and preequilibrated with calmodulin-Sepharose (60 μl of a 50% slurry). After an incubation period of 60 min at 4°C, the suspension was centrifuged for 5 min at 500 $\times$ *g*. The supernatant was removed. The sedimented calmodulin-Sepharose was washed three times and subsequently, the ryanodine receptor was eluted batchwise in three steps with 100 μl binding buffer supplemented with suramin, calmodulin or NF676. The supernatants of the washes and elutions were mixed with Laemmli sample buffer. Similarly, after the last elution step, the calmodulin-Sepharose was boiled in Laemmli sample buffer and centrifuged in order to control for the remaining RyR1 on the gel matrix. The samples were heated to 95°C for 5 min and aliquots were applied onto a discontinuous SDS–polyacrylamide gels (5% stacking and 7% separating gel). The resolved proteins were visualized by silver staining and the intensity of the protein bands quantified by the Quanti-Scan software (Biosoft®, Cambridge, U.K.).

Calcium uptake and calcium release measurements

The photometric Ca^{2+} flux measurements were carried out with HSR (1 mg ml^{-1}) in a medium containing 40 mM MOPS.Tris (pH 7.4), 140 mM KCl, 250 μM $MgCl_2$, 50 μg ml^{-1} creatine kinase, 4 mM creatine phosphate and 30 μM arsenazo

III. The measurements were carried out with a Sigma ZWS II dual-wavelength photometer. The reactions were continuously monitored under permanent stirring at room temperature. Changes in the Ca^{2+} concentration were monitored by subtracting the changes in transmission of arsenazo III at 690 nm from that at 650 nm. The difference in transmission was set zero and thereafter $CaCl_2$ was added stepwise (usually to a final concentration of 60 μM) in order to obtain an estimate for the change in free Ca^{2+} concentration. The Ca^{2+} uptake into HSR was initiated by the addition of 150 μM ATP. The Ca^{2+} release was triggered by injection of the RyR1 agonist suramin directly into the cuvette through a lid using a Hamilton syringe. At the end of each experiment, the virtually Ca^{2+} free condition is defined by the addition of 2 mM EGTA.

Miscellaneous procedures

The experiments were carried out in duplicates and each experiment was repeated at least two times with different protein preparations ($n=6$). All data are presented as mean $\pm$ s.d., if not otherwise stated. The data were fitted by nonlinear least-squares regression to the appropriate equations describing exponential decay, exponential rise and the Hill equation. Statistical significance ($P<0.05$) was determined with Student's *t*-test and for multiple comparison with ANOVA and *post hoc* Scheffe's test.

Results

[^{3}H]ryanodine binding in presence of the suramin analogue NF676

Under physiological conditions (pH 7.4), suramin is an anionic symmetric molecule in which a urea bridge connects two polysulfonated naphthylbenzamide ring systems. The novel suramin analogue NF676 shows modifications in the structure and physicochemical properties compared to suramin (Figure 1). The symmetry is retained in the structure of NF676. However, modifications involve a replacement of the naphthalenetrisulfonic acid by a benzenesulfonic acid residue, a substitution of the 1,3 (meta) connected aromatic residues by 1,4 (para) connection, an exchange of 4-methylbenzamide by benzimidazole, and thus a switch from an anionic compound (suramin) to the zwitterionic NF676.

There is a general consensus that acceleration of the rate of high affinity [^{3}H]ryanodine binding is a measure for RyR activation proportional to channel opening (Chu *et al*., 1990). The novel suramin analogue NF676 is not capable to inhibit the high-affinity [^{3}H]ryanodine binding at concentrations up to 100 μM irrespective of the usage of HSR from cardiac or skeletal muscle (Figure 2a). Nevertheless, close to 1 mM NF676 reduces the binding to approximately 50–60%. In a rough estimate, the high-affinity [^{3}H]ryanodine binding with skeletal muscle HSR was inhibited with an IC_{50} of 850 $\pm$ 41 μM. From the relative comparison of the inhibitory potency of NF676, the skeletal muscle HSR seems to be more sensitive than the cardiac HSR. For this reason the following experiments characterizing the action of NF676 were carried out with HSR from skeletal muscle.

It is possible that NF676 achieves its inhibitory nature by direct competition with the radioligand. Nevertheless, in saturation experiments, NF676 exerts no influence on the high-affinity [^{3}H]ryanodine-binding site (data not shown). The calculated K_d for the high-affinity ryanodine binding was not significantly different, independent whether NF676 was absent or present (control: 4.9 $\pm$ 1.1 nM; 100 μM NF676: 4.2 $\pm$ 2.4 nM; $n=6$). However, at concentrations of above 20 nM ryanodine, the binding was slightly reduced in the presence of NF676.

The kinetic analysis showed that the apparent association rate of [^{3}H]ryanodine in the early time points is not affected by NF676 when five experiments were pooled (Figure 2b). However, after an incubation time above 30 min, NF676 reduced the binding of [^{3}H]ryanodine, which became

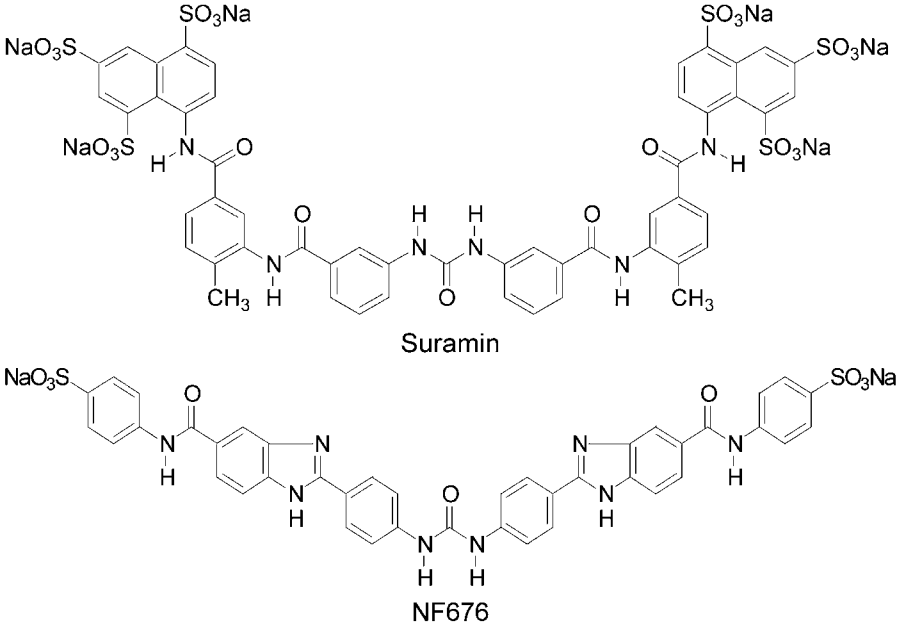

Figure 1 Chemical structure of suramin and NF 676.

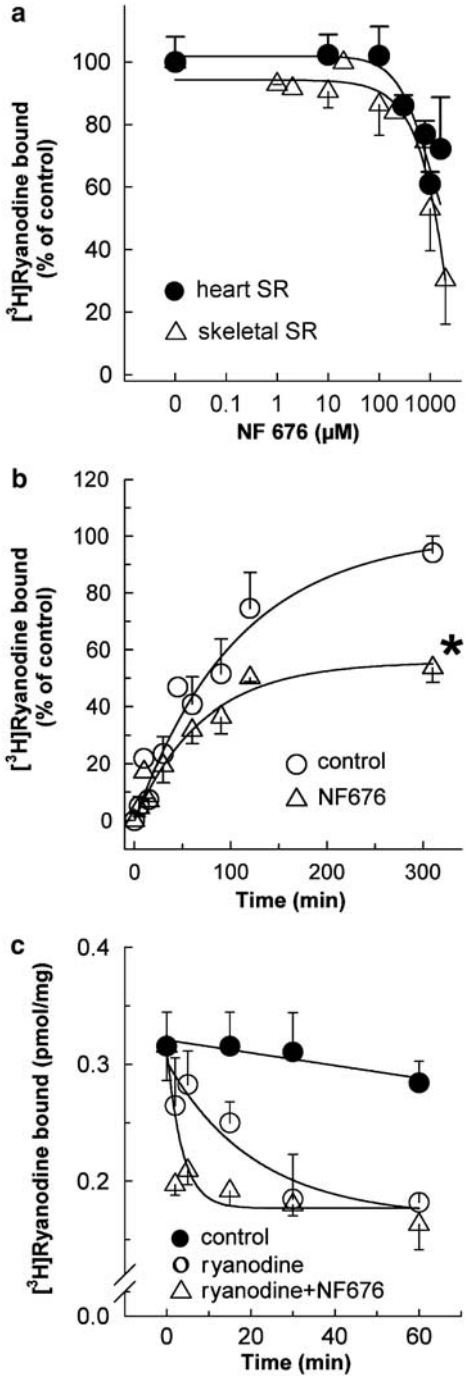

Figure 2 Inhibition of the RyR1 by NF676. (a) Sarcoplasmic reticulum membranes (75 μg) were incubated with 20 nM [^{3}H]ryanodine for 45 min at 37°C in presence of NF676 at a calculated free Ca^{2+} concentration of 1.2 μM. (b) Kinetic analysis of the [^{3}H]ryanodine binding in the absence and presence of 100 μM NF676 of five experiments. Asterisk represents statistical significance *versus* control (*$P=0.015$; Students *t*-test). (c) [^{3}H]ryanodine (20 nM) was allowed to associate to HSR membranes. After 120 min of incubation (time = 0), the dissociation of [^{3}H]ryanodine was triggered by the addition of 20 μM ryanodine in the absence or presence of 100 μM NF676. For comparison, the association was also allowed to proceed (control). The data represent mean ± s.d. of a triplicate experiment, which was repeated twice with similar results.

significant for the late time point (Figure 2b; asterisk: $P=0.015$). The calculated apparent association rate for [^{3}H]ryanodine was $0.0053\pm0.0006\,nM^{-1}\,min^{-1}$ ($n=3$) under control condition, which is lower, but comparable to previously obtained results under similar conditions (Klinger *et al*., 1999). In the presence of NF676, the apparent association rate was calculated to be $0.013\pm0.002\,nM^{-1}\,min^{-1}$ ($n=3$) (Figure 2b). In order to exclude that drug-facilitated heat denaturation is responsible for the reduction of [^{3}H]ryanodine binding by NF676, one would expect that a simple dilution of NF676 in the assay mixture is sufficient to recover [^{3}H]ryanodine binding. We, therefore, incubated HSR vesicles in the absence and presence of 100 μM NF676, under conditions identical to Figure 2b. Compared to the control, the [^{3}H]ryanodine binding was reduced by NF676 to $54.35\pm15.86\%$ ($n=6$) after an incubation for 5 h. Thereafter, aliquots of the incubation were diluted 10-fold, keeping all compounds at a constant concentration, except NF676. Within an additional hour of incubation, a significant recovery of the [^{3}H]ryanodine binding was obtained to $76.94\pm4.38\%$ ($n=9$; $P=0.0012$) of the control. This observation allows for the conjecture that NF676 leads to a reversible inhibition of the ryanodine receptor.

In order to determine dissociation rates, [^{3}H]ryanodine was allowed to bind to HSR vesicles for 2 h and thereafter the dissociation was initiated by a 1000-fold molar excess of cold ryanodine. The dissociation rate was accelerated in the presence of NF676 to $0.2747\pm0.09\,min^{-1}$ ($n=3$) compared to $0.0479\pm0.003\,min^{-1}$ ($n=3$) under control conditions (Figure 2c). Together, the experimentally determined kinetic parameters allow for the assumption that NF676 inhibits the B_{max} of the high-affinity [^{3}H]ryanodine binding by the acceleration of the dissociation of the radioligand (Figure 2b and c).

Pharmacological analysis of the [^{3}H]ryanodine binding in the presence of NF676

The RyR1 is tightly regulated by the cytoplasmic Ca^{2+} concentration (Meissner, 1994). This is documented by a Ca^{2+} dependence of the [^{3}H]ryanodine binding which follows a bell-shaped curve (Figure 3). In the presence of 100 μM NF676, the [^{3}H]ryanodine binding is superimposable with the control at micromolar and millimolar concentrations of free Ca^{2+}. The EC_{50} values for Ca^{2+} activated [^{3}H]ryanodine binding was $4.9\pm0.36\,\mu M$ ($n=4$) in the absence and $4.85\pm0.07\,\mu M$ ($n=4$) in the presence of 100 μM NF676. The Ca^{2+}-dependent inhibition of [^{3}H]ryanodine binding gave an IC_{50} value of 0.96 ± 0.08 mM ($n=4$) under control conditions and 0.94 ± 0.29 mM ($n=4$) in the presence of NF676. This observation is divergent to previous observations with suramin and the more potent suramin analogue NF307, which act in a Ca^{2+}-dependent manner (Klinger *et al*., 1999; Papineni *et al*., 2002). Suramin and NF307 increase the affinity of the RyR1 for Ca^{2+} (Klinger *et al*., 1999). In contrast to NF676, ruthenium red completely inhibited the [^{3}H]ryanodine binding independent of the free Ca^{2+} concentration (Figure 3).

Activation-dependent inhibition of the RyR1 by NF676

So far NF676 shows rather low inhibitory potency under basal conditions of [^{3}H]ryanodine binding. However, the inhibition

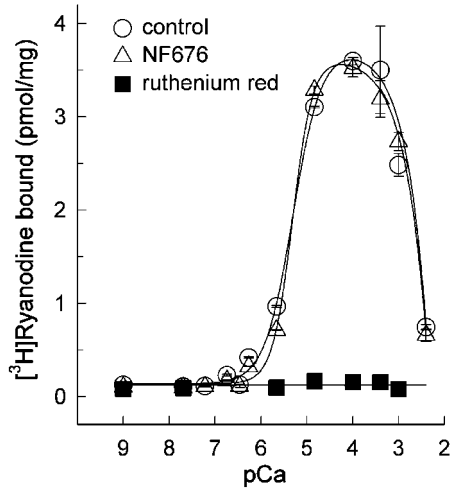

Figure 3 Ca^{2+}-dependent [^{3}H]ryanodine binding in presence of NF676. Sarcoplasmic reticulum membranes (75 μg) were incubated with 20 nM [^{3}H]ryanodine under conditions described in legend to Figure 2a. The free Ca^{2+} concentrations were adjusted by variation of the concentration of EGTA and $CaCl_2$. The incubation buffer containing only 1 mM EGTA but no supplemented $CaCl_2$ was set to be 1 nM free Ca^{2+}. Incubations were carried out in the absence and presence of 100 μM NF676 or 20 μM ruthenium red. The data represent mean $\pm$ s.e.m. of a triplicate experiment, which was repeated three times.

became significantly pronounced at longer incubation times (Figure 2b). Activating micromolar Ca^{2+} concentrations were not sufficient to enhance inhibition of the basal [^{3}H]ryanodine binding. Therefore, the RyR1 was stimulated with 10 mM caffeine which increased the [^{3}H]ryanodine binding, and also the potency of NF676 to inhibit the RyR1 (Figure 4a and b). The concentration–response curve is dramatically shifted to the left as this is clearly seen for the normalized data (Figure 4b). The calculated IC_{50} for NF676 in the absence of caffeine was approximately 850 μM (cf. Figure 2a) and is reduced to 3.6 ± 0.96 μM ($n=3$) in the presence of 10 mM caffeine (Figure 4b). These data are in accordance with the assumption that NF676 got full access to its binding site only in the activated conformation of the RyR1. Therefore, facilitation of the open conformation by the RyR1 agonist caffeine had a pronounced effect on the potency of NF676. Similar results were obtained with the nonhydrolysable ATP analogues, AMP-PCP (β, γ-methyleneadenosine 5′-triphosphate) or AMP-PNP. The nucleotide AMP-PNP was used at a concentration of 0.5 mM, which stimulated the ryanodine binding approximately half maximally. Nevertheless, this activation of the RyR1 was sufficient to facilitate the inhibition by NF676 (Figure 4c and d). The normalized concentration–response curve shifted to the left (Figure 4d) which was reflected by IC_{50} values of 809 ± 44 μM in the absence of AMP-PNP and 107.4 ± 53.4 μM ($n=3$) in the presence of AMP-PNP. Similar results were obtained with AMP-PCP (data not shown). Compared to controls, the shift in the IC_{50} of NF676 was highly significant in the presence of caffeine ($P<0.003$) or AMP-PNP ($P<0.005$; ANOVA and *post hoc* Scheffe's test). Thus, NF676 can be considered as an use-dependent inhibitor of the RyR1.

Suramin, which is known to activate the RyR1, may possibly interact with the inhibitory NF676-binding site in a competitive manner. If this is true, one would expect that in the presence of NF676, the affinity for suramin is shifted to the right. Surprisingly, such a shift was not detectable in the concentration–response curve of suramin (Figure 5a and b). Moreover, the amplitude of the suramin induced stimulation of [^{3}H]ryanodine binding is diminished progressively by increasing concentrations of NF676, which may indicate that NF676 precludes further action of suramin (Figure 5a). Conversely, the potency of NF676 was not significantly altered by the presence of suramin (Figure 5b). These observations are compatible with a noncompetitive behavior between suramin and NF676. Most likely, NF676 interacts allosterically with the suramin binding.

Interaction of NF676 with the suramin-binding site

In order to confirm an allosteric hindrance between suramin and NF676, the purified RyR1 was immobilized on a calmodulin-Sepharose matrix and eluted by calmodulin interacting agents. The eluted RyR1 was visualised by silver staining, which represents a semiquantitative experimental approach. In accordance to our earlier observations, calmodulin and suramin were capable to elute the immobilized RyR1 from the calmodulin-Sepharose in the presence of 200 μM Ca^{2+} (Figure 6a and b). NF676 at a concentration of 1 mM was not capable to disrupt the binding of the RyR1 to calmodulin, thus nearly all of the RyR1 was trapped on the Sepharose matrix (Figure 6c). Moreover, the successive elution steps significantly reduced the intensity of the corresponding RyR1 band compared with the wash step. Furthermore, the suramin-induced dissociation of the RyR1–calmodulin complex was abrogated by NF676. Consequently, no RyR1 was found in the supernatant of the elution steps (Figure 6d). Thus, the accessibility of the calmodulin-binding site by suramin is antagonized by NF676, although NF676 *per se* had no influence on the interaction of the RyR1 with calmodulin.

However, it is possible that NF676 may develop calmodulin interacting properties only at the activated RyR1 (cf. Figure 2). To test this possibility, the purified RyR1 was also exposed to 10 mM caffeine or 0.5 mM AMP-PNP in the presence of 1 mM NF676. In all cases, the RyR1 was not eluted from the calmodulin-Sepharose independent whether caffeine or AMP-PNP were used alone or in combination with NF676 (data not shown). Therefore, a conformation-dependent interaction of NF676 with the calmodulin-binding site is unlikely.

Ca^{2+} uptake and Ca^{2+} release measurements

Taken together, the above-described results provide substantial evidence that NF676 inhibits the RyR1 in the activated conformation with high affinity *via* a binding site related to that of suramin. In order to confirm this interpretation and expand it by functional analysis, we have employed photometric Ca^{2+} measurements with arsenazo III in order to circumvent the fluorescent behavior of suramin and NF676. This experimental approach allows for the simultaneous observation of Ca^{2+} uptake and Ca^{2+} release in HSR vesicles (Wyskovsky *et al*., 1990). Suramin has been shown to release Ca^{2+} from passively loaded SR vesicles (Emmick *et al*., 1994). This holds true also for actively loaded HSR in the presence of an ATP regenerating system (Figure 7a). However, it is evident that after suramin addition Ca^{2+} reuptake is dramatically

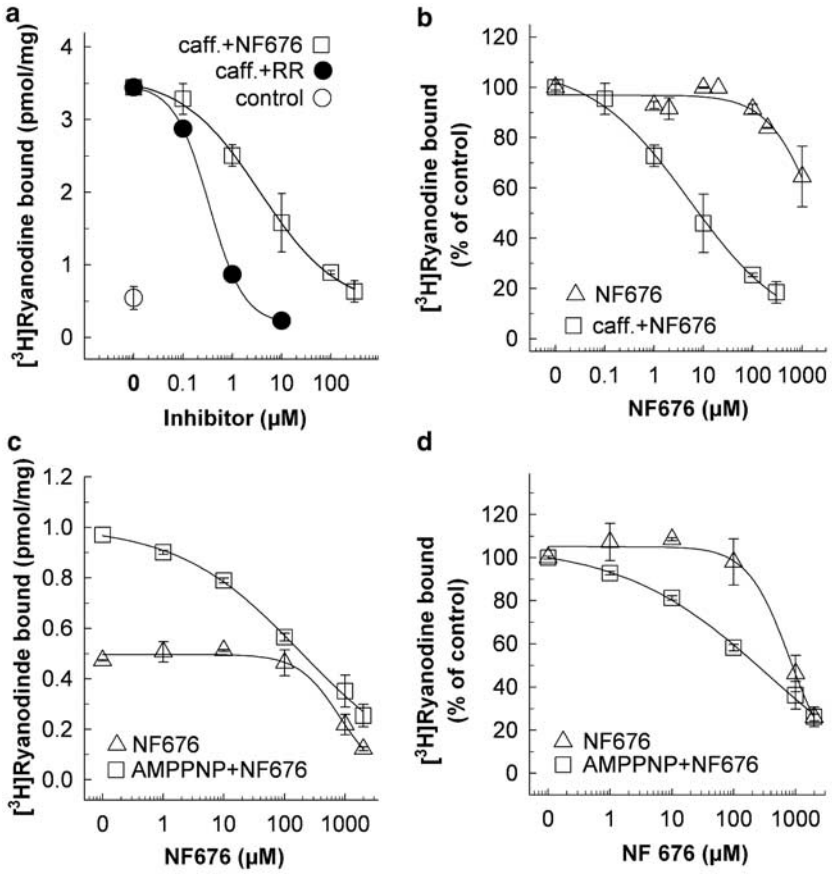

Figure 4 NF676 blocks the activated RyR1. (a) Under conditions described for Figure 2a, the basal [^{3}H]ryanodine binding (control) was stimulated with 10 mM caffeine (caff.) and inhibited with the indicated concentrations of NF676 or ruthenium red (RR). (b–d) The [^{3}H]ryanodine binding was inhibited with NF676 in absence or presence of 10 mM caffeine (b) or 0.5 mM AMP-PNP (c and d). Panel (b) and (d) depict the normalized [^{3}H]ryanodine binding. The data represent mean $\pm$ s.d. of a duplicate experiment, which was repeated two times.

reduced. This is compatible with the finding that suramin is also a potent inhibitor of the Ca^{2+} ATPase of the sarcoplasmic reticulum (Emmick *et al*., 1994). Therefore, 100 μM NF676 was added prior to 150 μM ATP in order to detect a possible inhibition of the Ca^{2+} ATPase. As depicted from Figure 7b, the addition of 100 μM NF676 caused an increase in transmission which is not due to dilution of the suspension (1.5%). Nevertheless, upon administration of 150 μM ATP, the Ca^{2+} uptake rate was not altered, the traces were superimposable to controls and the steady-state plateau was reached (cf. also Figure 7c). As a rough estimate, the initial pseudo-linear Ca^{2+} uptake phase was calculated to be 47.0 ± 17.3 a.u. min^{-1} ($n = 3$) in the absence of NF676 and 33.2 ± 13.7 a.u. min^{-1} ($n = 4$; $P = 0.48$) in the presence of 100 μM NF676. Spontaneous Ca^{2+} releases or the occurrence of spontaneous Ca^{2+} oscillations was not observed in the presence of NF676. A simple addition of 100 μM NF676 at the plateau phase of the Ca^{2+} uptake curve had no effect on the suramin-induced Ca^{2+} release (Figure 7d). At the plateau phase of the Ca^{2+} uptake curve, the RyR1 is virtually closed and NF676 may have no excess to its binding site. Therefore, the peak amplitude of the suramin-induced Ca^{2+} release (150 μM) was 2.97 ± 0.06 a.u. in the absence of NF676 and 2.77 ± 0.35 a.u. in the presence of 100 μM NF676 ($n = 3$; $P = 0.38$). In contrast, addition of 100 μM NF676 prior to the ATP application resulted in a complete abrogation of the suramin-induced Ca^{2+} release (Figure 7c). Analogously, caffeine-induced Ca^{2+} release was also inhibited by NF676 (data not shown). We therefore conclude that NF676 is a novel and potent RyR1 antagonist.

Discussion

We have previously shown that suramin and its analogue NF307 are potent agonists of the RyR1 *via* the calmodulin-binding site (Hohenegger *et al*., 1996; Klinger *et al*., 1999; 2001; Suko *et al*., 2001). Here, we show that modifications in the structure of suramin convert the molecule into a potent RyR1 antagonist. This suramin analogue, NF676, has only weak effects on the basal ryanodine binding to HSR from cardiac or skeletal muscle (Figure 2a). Conversely, under conditions of RyR1 activation, NF676 turns into a potent antagonist. The conclusion that NF676 is a use-dependent inhibitor of the RyR1 is based on two observations. First, NF676 inhibited [^{3}H]ryanodine binding which becomes significantly pronounced with increasing incubation times (Figure 2b). Second, the potency of NF676 to inhibit [^{3}H]ryanodine binding was significantly increased when the RyR1 was activated by AMP-PNP or caffeine (Figure 4). In both cases, it is assumed that cumulative channel openings

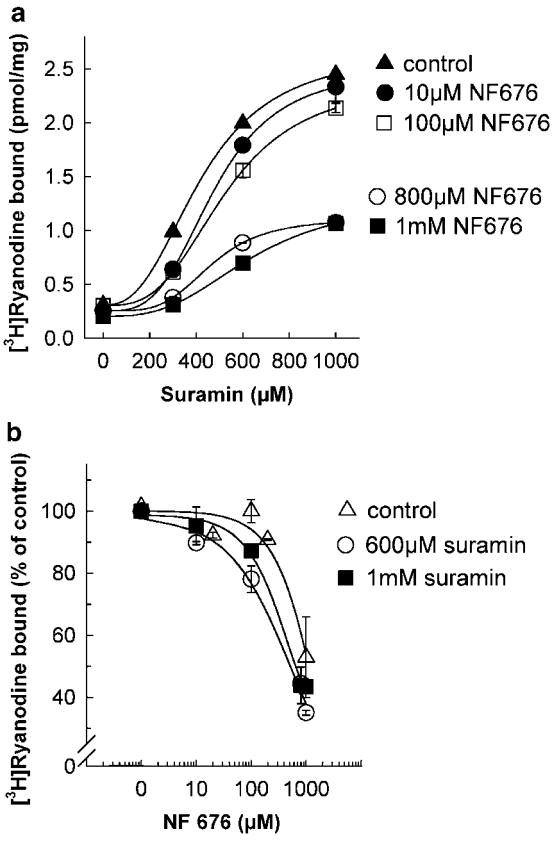

Figure 5 Inhibition of suramin stimulated [^{3}H]ryanodine binding by NF676. (a) Sarcoplasmic reticulum membranes (75 μg) were incubated under conditions given in legend to Figure 2a. The binding was carried out in the absence and presence of the indicated concentrations of NF676. (b) Inhibition of high-affinity [^{3}H]ryanodine binding by NF676 in the absence or presence of 600 μM or 1 mM suramin. The data represent mean $\pm$ s.d. of a duplicate experiment, which was repeated two times.

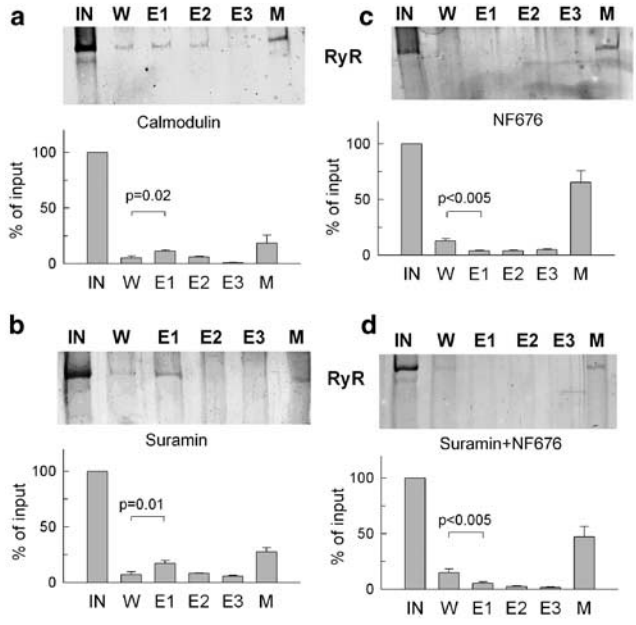

Figure 6 Modulation of the binding of calmodulin to the purified RyR1. (a–d) Elution of the purified RyR1 from a calmodulin-Sepharose matrix. The purified RyR1 was incubated with equilibrated calmodulin-Sepharose in presence of 200 μM free Ca^{2+} for 60 min at 4°C. The affinity matrix was washed three times and subsequently, RyR1 was eluted with buffer containing 10 μM calmodulin (a), 100 μM suramin (b), 1 mM NF676 (c) or 100 μM suramin plus 1mM NF676 (d). Residual protein trapped in the matrix is labelled 'M'. Aliquots of input (lanes labelled 'IN'), of last wash step (lanes labelled 'W') and of the three implemented elution steps (lanes labelled 'E1–E3'), were applied onto SDS–polyacrylamide gels. The proteins were visualized by silver staining and the high molecular mass range is depicted of the RyR1 indicated by 'RyR'. The intensity of the RyR1 band was quantified by the Quanti Scan® software.

increase with time. Therefore, a possibly hidden or protected binding site for NF676 may become accessible. We have previously demonstrated that suramin does not interact with the adenine nucleotide-binding site of the RyR1 (Hohenegger *et al*., 1996). The same is true for NF676. Over the complete range, the concentration–response curve for NF676 is significantly shifted to the left in the presence of AMP-PNP (Figure 4d). This is not compatible with a competitive interaction *via* the adenine nucleotide-binding site of the RyR1.

Our data suggest that NF676 may interact allosterically with the suramin-binding site of the RyR1. The suramin activated RyR1 was progressively inhibited by increasing concentrations of NF676, but the IC_{50} values were not significantly changed compared to control (Figure 5a and b). *Vice versa*, in the presence of NF676, the concentration–response curves for suramin were not shifted to the right. Such a noncompetitive behavior is corroborated by the following findings: (i) suramin is capable to elute the RyR1 from a calmodulin-Sepharose while NF676 is not (Figure 6b and c). The calmodulin-binding site is therefore not accessible for NF676 such that the RyR1 is not displaced from the calmodulin-Sepharose. (ii) In the presence of NF676, the elution of the RyR1 by suramin is abrogated (Figure 6d). Thus, based on these results, the conclusion is drawn that NF676 interferes with the suramin-binding site *via* an allosteric interaction, but has no access to the calmodulin-binding site. This mechanism is very efficient, since suramin-induced Ca^{2+} release is completely blocked by preincubation with NF676 (Figure 7c). Moreover, the mechanism of NF676-induced inhibition of the [^{3}H]ryanodine binding is reversible. Dilution of NF676 in the binding buffer from 100 to 10 μM recovers [^{3}H]ryanodine binding significantly from 54 to 77% of control.

Thus, these results substantially expand our previous knowledge on suramin and suramin analogues. Importantly, suramin and the related agonist NF307 bind to the calmodulin-binding site of the RyR1 but mimic only the stimulatory effect of calmodulin (Klinger *et al*., 1999; Papineni *et al*., 2002). Apocalmodulin acts as an activator, whereas Ca^{2+} bound to the COOH-terminal end of calmodulin switches the protein to an inhibitor (Plank *et al*., 1988; Smith *et al*., 1989; Buratti *et al*., 1995; Tripathy *et al*., 1995; Rodney *et al*., 2001). The calmodulin-binding site was mapped to a peptide stretched from RyR1 amino acids 3609–3643 (Moore *et al*., 1999; Rodney *et al*., 2000; 2001). Apocalmodulin and Ca^{2+}-bound calmodulin bind to this binding site with their COOH-terminal loop; however, apocalmodulin additionally requires the amino acids between 3634 and 3643 (Xiong *et al*., 2002). These findings are in agreement with the structural alignments

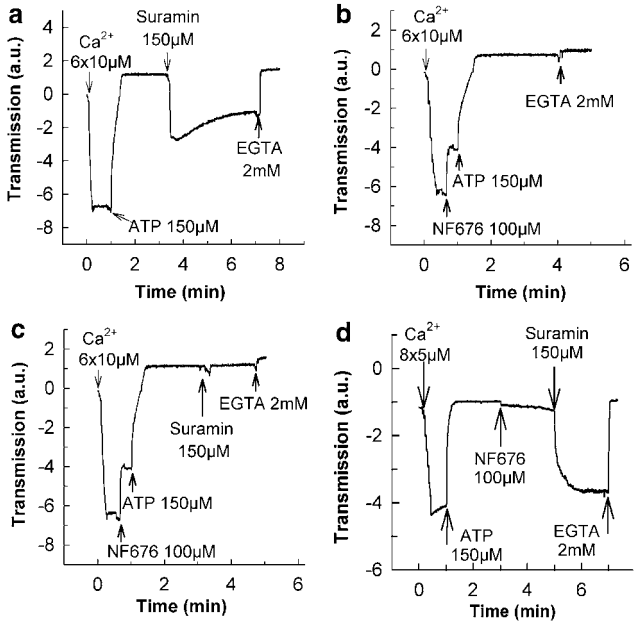

Figure 7 Effect of NF676 on Ca^{2+} uptake and Ca^{2+} release with HSR vesicles. (a–d) Ca^{2+} uptake and Ca^{2+} release were continuously monitored with a dual-wavelength photometer using 30 μM arsenazo III as a Ca^{2+}-sensitive dye. Ca^{2+} uptake and suramin-induced Ca^{2+} release (a, c and d) were investigated in the absence (a) and presence of NF676 (b, c and d). The Ca^{2+} transients depict representative experiments ($n = 3$–7).

investigating calmodulin binding to the RyR1 in the absence and presence of Ca^{2+} (Samso & Wagenknecht, 2002). The three-dimensional reconstructions of the two conformations of the calmodulin–RyR1 complex show distinct but overlapping binding sites for calmodulin. The novel antagonist NF676 interacts with the RyR1 in a calcium-independent mechanism. Interestingly, Ca^{2+}-dependent activation and inhibition of the [^{3}H] ryanodine binding of the RyR1 was not altered by NF676 at any free Ca^{2+} concentration (Figure 3). This is surprising, because suramin and NF307 have been found to increase the sensitivity of the RyR1 for Ca^{2+} and by doing so this mechanism essentially contributes to the stimulatory effect of suramin and NF307. When the RyR1 is activated by caffeine, AMP-PNP or suramin, NF676 is a potent inhibitor of the RyR1 (Figures 4 and 5). We are not able to explain this behavior at the moment, but possibly the conformation of the Ca^{2+}-activated RyR1 may not give full access to the NF676-binding site (cf. also Figure 7d). Therefore, a simple opening or activation of the RyR1 by Ca^{2+} is not sufficient to enable NF676-mediated inhibition.

The calmodulin-binding site of the RyR1 is conserved in the cardiac isoform (RyR2) with a homology of more than 90%. Moreover, mutation or deletion of the putative cardiac calmodulin-binding site abrogated calmodulin binding and inhibition by calmodulin in single channel recordings (Yamaguchi *et al*., 2003). Nevertheless, the RyR2 is not activated by calmodulin at submicromolar Ca^{2+} concentrations. This functional diversity of the RyR isoforms has not been clarified on the molecular level.

Recent observations highlight the essential pathophysiological role of the RyR2 in heart failure and arrhythmia (Scoote & Williams, 2002; Wehrens & Marks, 2003). Owing to sympathetic stimulation of the heart, the RyR2 is hyperphosphorylated which results in augmented channel opening and Ca^{2+} depletion of sarcoplasmic reticulum. Consequently, the myocardial contractility is reduced and clinical features like heart failure are observed. Mutations in the RyR2 have been linked to heart failure and exercise-induced sudden cardiac death. It is worth mentioning that a use-dependent inhibitor of the RyR2 would represent a rational new therapeutic concept for the treatment of these common diseases of the heart. Suramin is capable to activate RyR1 and the RyR2 (Hohenegger *et al*., 1996; Sitsapesan & Williams, 1996). However, suramin has the drawback that it inhibits efficiently the Ca^{2+} ATPase of the sarcoplasmic reticulum *via* the ATP-binding site (Figure 7a) (Emmick *et al*., 1994). This is clearly not the case for NF676 (Figure 7b). Although, not investigated in great detail, the basal [^{3}H]ryanodine binding was also inhibited by NF676 in cardiac HSR and thereby indicates the RyR2 as a drug target (Figure 2a). Moreover, NF676 at physiological pH shows zwitterionic behaviour, which may facilitate bioavailability. The suramin analogue NF676 represents, therefore, an important novel lead compound in the search for a novel class of use-dependent inhibitors of the RyR.

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