

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

MARTIN HOHENEGGER, MARINA MATYASH, KATI POUSSU, ANNEGRET HERRMANN-FRANK, SANDOR SARKÖZI,<sup>1</sup> FRANK LEHMANN-HORN, and MICHAEL FREISMUTH

*Institute of Pharmacology, University of Vienna, A-1090 Vienna, Austria (M.H., M.M., K.P., M.F.), and the Department of Applied Physiology, University of Ulm, D-89069 Ulm, Germany (A.H.-F., S.S., F.L.-H.)*

Received April 1, 1996; Accepted August 23, 1996

## SUMMARY

$\text{Ca}^{2+}$  release from skeletal muscle sarcoplasmic reticulum is activated by adenine nucleotides and suramin. Because suramin is known to interact with ATP-binding enzymes and ATP receptors ( $P_2$ -purinergic receptors), the stimulation by suramin has been postulated to occur via the adenine nucleotide-binding site of the ryanodine receptor/ $\text{Ca}^{2+}$ -release channel. We tested this hypothesis using suramin and the following suramin analogs: NF037, NF018, NF023, and NF007. The suramin analogs stimulate the binding of [ $^3\text{H}$ ]ryanodine binding to sarcoplasmic reticulum membranes with the following rank order of potency: suramin ( $\text{EC}_{50} = \sim 60 \mu\text{M}$ ) > NF037 ( $\text{EC}_{50} = \sim 150 \mu\text{M}$ ) > NF018 > NF023 > NF007. The suramin-induced stimulation occurs via a myoplasmic binding site on the ryanodine receptor as confirmed by binding experiments and single-channel recordings with the purified protein. This

binding site is different than that for ATP, a conclusion that is supported by the following observations: (i) Suramin stimulates the association rate and inhibits the dissociation rate of [ $^3\text{H}$ ]ryanodine, whereas ATP analogs increase only the on-rate. (ii) In the presence of suramin but not of ATP analogs, [ $^3\text{H}$ ]ryanodine binding is resistant to the inhibitory effect of millimolar  $\text{Mg}^{2+}$  and  $\text{Ca}^{2+}$ . (iii) ATP analogs and suramin have an additive effect on [ $^3\text{H}$ ]ryanodine binding. (iv) Affinity labeling of the purified ryanodine receptor with 2',3'-dialdehyde [ $\alpha$ - $^{32}\text{P}$ ]ATP or after *in situ* oxidation of [ $\gamma$ - $^{32}\text{P}$ ]ATP is not affected by suramin. Thus, our results show that suramin acts as a direct and potent stimulator of the ryanodine receptor but that this action is mediated via a binding site different from that for adenine nucleotides.

Cytoplasmic  $\text{Ca}^{2+}$  levels are tightly regulated by uptake into and release from intracellular stores as well as by fluxes across the plasma membrane. Signals generated at the plasma membrane are linked to the rapid efflux of  $\text{Ca}^{2+}$  from the intracellular stores by two types of release channels: the inositol trisphosphate receptors and the ryanodine receptors. The latter exist in three distinct isoforms, termed the skeletal muscle isoform (type 1), cardiac muscle isoform (type 2), and a ubiquitous isoform (type 3) (1). The regulation of the skeletal muscle ryanodine receptor/ $\text{Ca}^{2+}$ -release channel is understood in great detail; the receptor resides on the terminal cisternae of the sarcoplasmic reticulum and is intricately linked to the voltage-sensitive L-type  $\text{Ca}^{2+}$  channel of the sarcolemmal T-tubular system (2). Although this specialized topology seems to include functional linkage, little is known

about the physiological signal activating the ryanodine receptor (3–5). Several physiological and pharmacological ligands regulate the channel gating properties; these include inhibition by millimolar concentrations of  $\text{Ca}^{2+}$  or  $\text{Mg}^{2+}$  and micromolar concentrations of ruthenium red or ryanodine. Activation is achieved with micromolar concentrations of cytoplasmic  $\text{Ca}^{2+}$  and various exogenous ligands, such as the methylxanthine caffeine and the anthracycline doxorubicin (for a review, see Ref. 6). Adenine nucleotides are also involved in the modulation of the ryanodine receptor/ $\text{Ca}^{2+}$ -release channel activity. Indirectly, ATP acts as the phosphate donor for regulatory phosphorylation of the ryanodine receptor by various protein kinases (7–10). In addition, adenine nucleotides induce  $\text{Ca}^{2+}$  release from sarcoplasmic reticulum vesicles. This effect is thought to result from direct interaction with the ryanodine receptor based on the following observations: (i) A stimulation of channel gating by adenine nucleotides analogs is observed in single-channel recordings after insertion of the purified receptor in planar lipid bilayers (11–13). (ii) The purified ryanodine receptor

This work was supported by Grant FWF P-10871 from the Austrian Science Foundation (M.F.) a grant from the University of Vienna "Förderung von Auslandsbeziehungen der Universität Wien" (M.M.).

<sup>1</sup> Permanent affiliation: Department of Physiology, University Medical School, H-4012 Debrecen, Hungary.

**ABBREVIATIONS:** AMP-PCP,  $\beta,\gamma$ -methylene-ATP; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonate; MOPS, 3-(N-morpholino)propanesulfonic acid; PIPES, piperazine-N,N'-bis(2-ethanesulfonic acid); oATP, sodium periodate-oxidized ATP;  $P_o$ , open probability; EGTA, ethylene glycol bis( $\beta$ -aminoethyl ether)-N,N,N',N'-tetraacetic acid.

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

The trypanocidal drug suramin is known to inhibit many ATP-dependent enzymes and to block some classes of ATP receptors ( $P_2$ -purinergic receptors). Despite its many other actions, suramin has been used extensively as a pharmacological tool compound (17). Recently, Emmick *et al.* (18) reported that similar to ATP and other adenine nucleotides, suramin promoted binding of [ $^3\text{H}$ ]ryanodine to and triggered  $\text{Ca}^{2+}$  release from sarcoplasmic reticulum vesicles. Given the affinity of suramin for ATP-binding sites, the authors proposed that suramin activated the ryanodine receptor directly via interaction with its adenine nucleotide-binding site. We tested this hypothesis by determining the effects of suramin on both the ryanodine receptor in the sarcoplasmic reticulum vesicles and the purified protein. Our observations show that suramin is a potent activator of the ryanodine receptor. However, this stimulation occurs via a site distinct from that mediating the effect of adenine nucleotides.

## Experimental Procedures

**Materials.** ATP and ADP were purchased from Boehringer-Mannheim Biochemica (Mannheim, Germany). AMP-PCP, ruthe-nium red, and low-molecular-mass protein standards were from Sigma Chemical (St. Louis, MO). The high-molecular-mass standard kit was from Pharmacia (Vienna, Austria). Lipids were obtained from Avanti Polar Lipids (Birmingham, AL). Ryanodine was from Calbiochem (San Diego, CA). [ $^3\text{H}$ ]Ryanodine was from Amersham Buchler (Braunschweig, Germany). [ $\alpha$ - $^{32}\text{P}$ ]ATP and [ $\gamma$ - $^{32}\text{P}$ ]ATP were from New England Nuclear Research Products (Boston, MA). All other reagents were of analytical grade.

Suramin was a generous gift from Bayer AG (Wuppertal, Germany) and alternatively obtained from Calbiochem (Bad Soden, Germany); suramin analogs were kindly provided by Dr. P. Nickel (Bonn, Germany). Their synthesis has been described previously (19): NF037 {8,8'-[carbonylbis(imino-3,1-phenylene)carbonylimino-(3,1-phenylene)]bis-(1,3,5-naphthalenetrisulfonic acid)}, NF018 {8-[3-(3-nitrobenzamido)benzamido]-1,3,5-naphthalenetrisulfonic acid}, NF023 {8,8'-[carbonylbis(imino-3,1-phenylene)]bis-(1,3,5-naphthalenetrisulfonic acid)}, and NF007 [8-(3-nitrobenzamido)-1,3,5-naphthalenetrisulfonic acid]. oATP was synthesized according to the method of Easterbrook-Smith *et al.* (20) and purified as previously described (21); ATP or [ $\alpha$ - $^{32}\text{P}$ ]ATP was used as the starting material.

**Membrane preparation and protein purification.** Heavy sarcoplasmic reticulum from rabbit white hind-leg and back muscle was prepared according to Wyskovsky *et al.* (22). In some experiments, cardiac sarcoplasmic reticulum was used that was prepared from calf hearts (10). The ryanodine receptor was purified by solubilization of heavy sarcoplasmic reticulum vesicles in CHAPS, followed by sucrose density gradient centrifugation in the presence of CHAPS and exogenous phospholipids as previously described (23).

**Affinity labeling with oATP.** Sarcoplasmic reticulum membranes (3 mg/ml) were sequentially pretreated with 1 mM oATP for 10 min, 80 mM NaCNBH<sub>3</sub> for 1 min, and 40 mM NaBH<sub>4</sub> for 30 min in labeling buffer consisting of 50 mM MOPS-NaOH, pH 7.2, 100 mM NaCl, 5 mM NaN<sub>3</sub>, and 100  $\mu\text{M}$  Pefabloc [4-(2-aminoethyl)-benzoyl-sulfonylfluoride]. The control reaction was performed in the absence of oATP. The incubation was carried out at room temperature, whereas the subsequent reduction with NaBH<sub>4</sub> was done on ice. To remove free oATP, NaCNBH<sub>3</sub>, NaBH<sub>4</sub>, and reduction products, the membranes were centrifuged at 100,000  $\times g$  for 30 min and washed three times in labeling buffer. The pellets were resuspended in labeling buffer to give a final protein concentration of 0.8–1.5 mg/ml.

Labeling of the adenine nucleotide-binding site of the purified

ryanodine receptor was done as previously described (16). Briefly, the ryanodine receptor (0.5–2.5  $\mu\text{g}$ ) was incubated in 20  $\mu\text{l}$  of solution containing 20 mM Tris-HCl, pH 7.4, 30  $\mu\text{M}$  CaCl<sub>2</sub>, 5 mM MgCl<sub>2</sub>, 10  $\mu\text{M}$  [ $\alpha$ - $^{32}\text{P}$ ]oATP (specific activity, 12–60 cpm/fmol), and suramin at the concentrations indicated in the figure legends. After 15 min, reduction was initiated by the addition of 100 mM NaCNBH<sub>3</sub> (for 1 min at 25°) followed by the addition of 30 mM NaBH<sub>4</sub> (for 1 hr on ice). Alternatively, [ $\gamma$ - $^{32}\text{P}$ ]ATP was incorporated into the ryanodine receptor after *in situ* oxidation according to the procedure described for labeling of G protein  $\alpha$  subunits with guanine nucleotides (24); briefly, 5  $\mu\text{g}$  of purified ryanodine receptor was incubated in 20  $\mu\text{l}$  of 50 mM MOPS-NaOH, pH 7.2, 100 mM KCl, 5 mM MgCl<sub>2</sub>, 0.5 mM [ $\gamma$ - $^{32}\text{P}$ ]ATP (specific activity, 2000 cpm/pmol), and 0.5 mM EGTA with 0.6 mM CaCl<sub>2</sub> or 2 mM EGTA for  $\text{Ca}^{2+}$ -containing and  $\text{Ca}^{2+}$ -free medium, respectively. After a 10-min incubation at room temperature, oxidation and reduction were achieved by the sequential addition of 4 mM NaO<sub>4</sub> for 1 min, followed by 80 mM NaCNBH<sub>3</sub> for 1 min. To remove residual oxidation products, 10 mM NaBH<sub>4</sub> was added and allowed to react for 1 hr on ice. The samples were directly diluted in 2× Laemmli sample buffer, to which 1% mercaptoethanol had been added. After the samples were heated at 95° for 2 min, the ryanodine receptor was resolved on discontinuous sodium dodecyl sulfate-polyacrylamide gels (5% stacking gel and 7% separating gel). Exposure time for autoradiography varied from 6 to 48 hr (Kodak-Xomat film). Molecular mass standards were 212,000 Da for myosin, 170,000 Da for  $\alpha_2$ -macroglobulin, 116,000 Da for  $\beta$ -galactosidase, 76,000 Da for transferrin, and 53,000 Da for glutamic dehydrogenase.

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

In association experiments, the reaction was started by the addition of the sarcoplasmic reticulum to a prewarmed buffer solution containing 5–100 nM [ $^3\text{H}$ ]ryanodine and suramin or adenine nucleotides at the concentrations indicated in the figure legends. At the time points indicated, aliquots (50- $\mu\text{l}$  aliquots containing 30  $\mu\text{g}$  of protein) were withdrawn, and the reaction was stopped by filtration. In dissociation experiments, [ $^3\text{H}$ ]ryanodine (10–100 nM) was preincubated for 60 min with the sarcoplasmic reticulum membranes. Thereafter, dissociation was initiated by diluting the reaction mixture 50-fold (20  $\mu\text{l}$  containing 25  $\mu\text{g}$  of protein/1 ml) with binding buffer containing or lacking suramin or adenine nucleotides. Alternatively, an excess of unlabeled ryanodine (10 or 100  $\mu\text{M}$ ) was added in the absence and presence of suramin or adenine nucleotides to the reaction mixture; aliquots containing 30  $\mu\text{g}$  of sarcoplasmic reticulum were withdrawn at the time points indicated in the figure legends, and the reaction was stopped by filtration. All association and dissociation experiments were performed at 37°.

[ $^3\text{H}$ ]Ryanodine binding of the purified ryanodine receptor was performed in 50  $\mu\text{l}$  containing 50 mM MOPS, pH 7.2, 1 M NaCl, 5% glycerol, 100 nM aprotinin, 1  $\mu\text{M}$  leupeptin, and 200  $\mu\text{M}$  Pefabloc and increasing concentrations of [ $^3\text{H}$ ]ryanodine in the absence and presence of suramin. The free  $\text{Ca}^{2+}$  concentration was adjusted with CaCl<sub>2</sub> and EGTA to give a concentration of 2  $\mu\text{M}$ . The binding was

started by the addition of 4  $\mu\text{g}$  of purified ryanodine receptor. After 2 hr at 30°, the samples were filtered over glass-fiber filters soaked with 5% polyethylenimine, and the radioactivity trapped on the filter was determined by liquid scintillation counting.

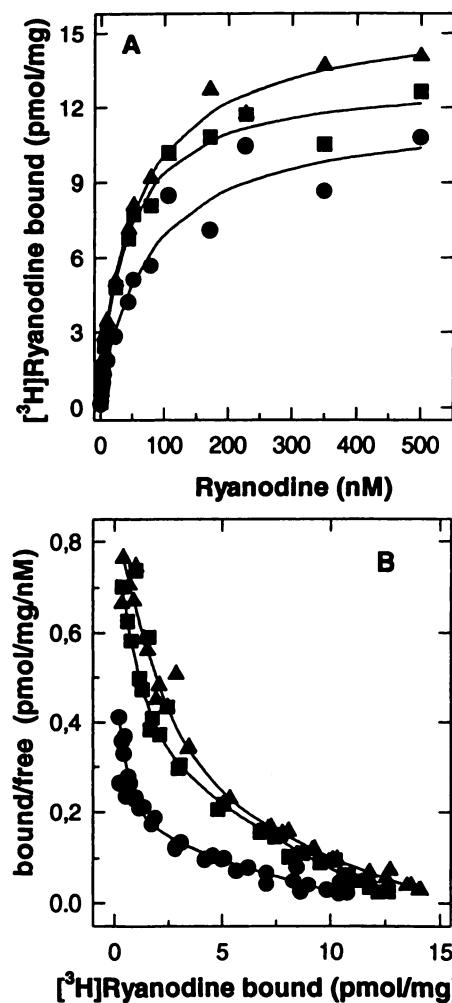
If not otherwise indicated, experiments were carried out in duplicate, and each experiment was repeated at least three times with different protein preparations.

**Planar lipid bilayer measurements.** The purified ryanodine receptor was incorporated into planar lipid bilayers according to Herrmann-Frank *et al.* (23). Briefly, reconstitution was started in symmetrical buffer solutions containing 250 mM KCl, 150  $\mu\text{M}$   $\text{Ca}^{2+}$ , 100  $\mu\text{M}$  EGTA, and 20 mM PIPES-KOH, pH 7.2. Small aliquots of the purified ryanodine receptor (<1  $\mu\text{g}$  of protein) were added to the *cis* side of the bilayer membrane defined as the cytoplasmic side of the channel. Successful incorporation of channels was detected as a steplike increase in current. Electrical signals were filtered at 1 kHz through an eight-pole, low-pass Bessel filter and digitized at 3 kHz.  $P_o$  values were calculated from representative data segments of 20–60-sec duration selected from total recording times of ~8 min for any condition tested. The indicated holding potentials were applied with reference to the *trans* chamber. Experiments were carried out at 22°.

**Miscellaneous procedures.** Protein concentration was measured by staining with amido black according to Kaplan and Petersen (25) or with the bicinchoninic acid assay (Micro-BCA; Pierce Chemical, Rockford, IL) using bovine serum albumin as the standard. Free  $\text{Ca}^{2+}$  concentrations were calculated by a computer program using the binding constants of Fabiato (26). Data were fit by nonlinear least-squares regression to the appropriate equations describing monoexponential and biexponential decay and association as well as saturation isotherms using the Gauss-Newton or Marquardt-Levenberg algorithm.

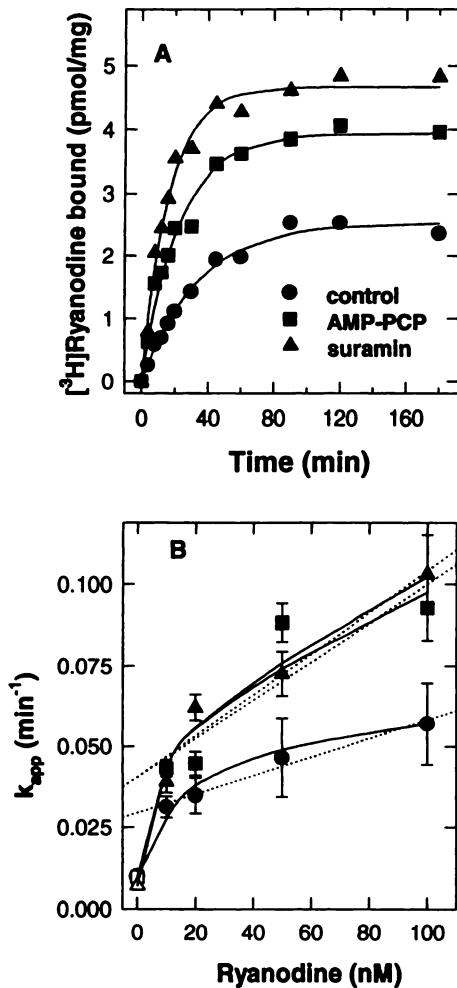
## Results

**Equilibrium binding of [ $^3\text{H}$ ]ryanodine in the presence of suramin.** When added to the incubation mixture, suramin and the nonhydrolyzable adenine nucleotide analog AMP-PCP stimulated the binding of [ $^3\text{H}$ ]ryanodine to sarcoplasmic reticulum membranes (Fig. 1). After Scatchard transformation, the data clearly exhibited two ryanodine-binding sites. Although the sum of the ryanodine-binding sites were constrained constant, in the presence of AMP-PCP or suramin, the  $B_{\max}$  value for the low affinity site shifted toward the high affinity site (parameters are given in the legend to Fig. 1A). The low affinity ryanodine-binding site in these saturation experiments was determined at  $\leq 500$  nM ryanodine. This concentration is close to the calculated  $K_D$  value for this site and therefore is only partially saturated. However, in the presence of suramin, the  $K_D$  value of the low affinity ryanodine binding site is decreased by a factor of 2. Similar effects were seen when suramin was tested on the purified skeletal muscle ryanodine receptor (data not shown). We conducted kinetic experiments to determine whether the ability of suramin to stimulate equilibrium binding of [ $^3\text{H}$ ]ryanodine resulted from an effect of suramin on the association or the dissociation rate. Both suramin and AMP-PCP increased the apparent association rate of [ $^3\text{H}$ ]ryanodine (Fig. 2A). Similarly, if the ryanodine concentrations were varied over a range of 10–100 nM, the apparent on-rate ( $k_{app}$ ) for [ $^3\text{H}$ ]ryanodine binding was stimulated at each concentration of the radioligand by both suramin and AMP-PCP to approximately equivalent extents (Fig. 2B). The  $k_{app}$  for [ $^3\text{H}$ ]ryanodine binding increased in a quasilinear manner if the concentration of [ $^3\text{H}$ ]ryanodine was raised from 10 to 100 nM



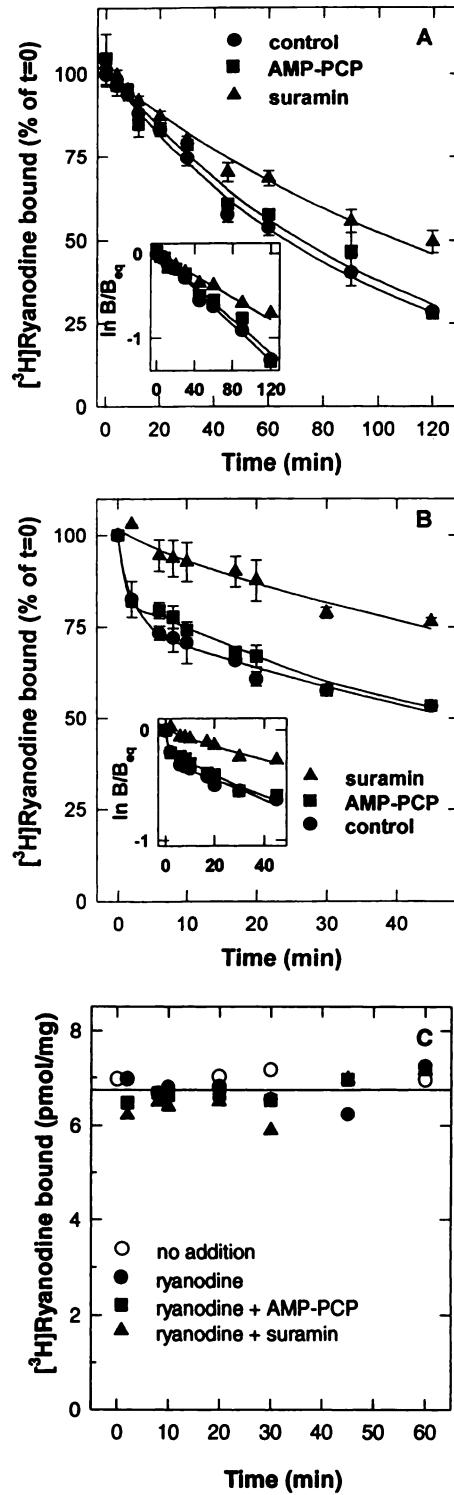
**Fig. 1.** Stimulation of [ $^3\text{H}$ ]ryanodine binding to heavy sarcoplasmic reticulum by suramin. A, Sarcoplasmic reticulum membranes (30  $\mu\text{g}$ ) were incubated with increasing concentrations of [ $^3\text{H}$ ]ryanodine for 120 min in the (○) absence and (■) presence of 1 mM AMP-PCP or (▲) 0.3 mM suramin at a free  $\text{Ca}^{2+}$  concentration of 2  $\mu\text{M}$ . Assay conditions were as given in Experimental Procedures. Data are the mean of duplicate determinations in a representative experiment that was repeated four times. Curvilinear regression to an equation assuming two independent binding sites gave a significantly better fit ( $F$  test) than that for binding to a single site and yielded the following parameters: control (○):  $K_{D1} = 10.9 \pm 2.1 \text{ nM}$ ,  $K_{D2} = 375 \pm 121 \text{ nM}$ ,  $B_{\max1} = 3.0 \pm 1.2 \text{ pmol/mg}$ , and  $B_{\max2} = 12.1 \pm 2.1 \text{ pmol/mg}$ ; 1 mM AMP-PCP (■):  $K_{D1} = 7.0 \pm 2.10 \text{ nM}$ ;  $K_{D2} = 317 \pm 145 \text{ nM}$ ;  $B_{\max1} = 4.2 \pm 0.9 \text{ pmol/mg}$ ; and  $B_{\max2} = 10.8 \pm 1.3 \text{ pmol/mg}$ ; 0.3 mM suramin (▲):  $K_{D1} = 4.9 \pm 1.4 \text{ nM}$ ;  $K_{D2} = 184 \pm 98 \text{ nM}$ ;  $B_{\max1} = 4.9 \pm 0.7 \text{ pmol/mg}$ ; and  $B_{\max2} = 10.2 \pm 0.8 \text{ pmol/mg}$ . The data were fit by constraining the sum of  $B_{\max1}$  and  $B_{\max2}$  as constant and represent the mean  $\pm$  standard deviation of duplicate determinations in a single experiment that was reproduced three times. B, Scatchard transformations of the data.

(Fig. 2B, dotted lines). However, the intercept of the regression lines with the  $y$ -axis yielded  $k_{off}$  values of  $0.0275 \text{ min}^{-1}$  without the addition and  $0.0375 \text{ min}^{-1}$  with the addition of suramin or AMP-PCP. These values did not correspond with the off-rates determined directly in dissociation experiments (Fig. 3A). The apparent dissociation rate constant  $k_{off}$  values have been added to the plot at the concentration of 0 nM ryanodine (Fig. 2B, open symbols) to illustrate this anomaly, which is consistent with the fact that binding of ryanodine to its receptor deviates from a simple bimolecular reaction (27, 28).



**Fig. 2.** Association of  $[^3\text{H}]$ ryanodine binding to sarcoplasmic reticulum membranes in the absence and presence of suramin or AMP-PCP. **A**, Sarcoplasmic reticulum membranes were added to prewarmed buffer containing 20 nM  $[^3\text{H}]$ ryanodine and a free  $\text{Ca}^{2+}$  concentration of 2  $\mu\text{M}$  in the absence and presence of 2 mM AMP-PCP or 0.3 mM suramin. At the time points indicated, aliquots (50  $\mu\text{l}$  containing 30  $\mu\text{g}$  of sarcoplasmic reticulum membrane protein) were withdrawn and immediately filtered as described in Experimental Procedures. Data are the mean of duplicate determinations in a representative experiment. **B**, Apparent on-rates ( $k_{app}$ ) for binding of increasing  $[^3\text{H}]$ ryanodine concentrations were determined in the absence or presence of 2 mM AMP-PCP or 0.3 mM suramin as in **A** and plotted as a function of the radioligand concentration. Data are the mean  $\pm$  standard deviation of three independent experiments carried out in duplicate. Dotted lines, linear regression through the three sets of  $k_{app}$  values based on the relation  $k_{app} = k_{on} * L + k_{off}$ . Data points (open symbols) at 0 nM ryanodine represent the  $k_{off}$  rates determined from the experiments in Fig. 3A.

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



**Fig. 3.** Dissociation of  $[^3\text{H}]$ ryanodine from sarcoplasmic reticulum membranes in the absence and presence of suramin or AMP-PCP. **A**, Sarcoplasmic reticulum membranes were allowed to bind 10 nM  $[^3\text{H}]$ ryanodine at a free  $\text{Ca}^{2+}$  concentration of 2  $\mu\text{M}$ . After 1 hr (time = 0), the incubation was split and diluted 50-fold into ryanodine-free incubation buffer, buffer supplemented with 2 mM AMP-PCP, or buffer supplemented with 0.3 mM suramin. Aliquots of the diluted samples (1 ml containing 25  $\mu\text{g}$  of sarcoplasmic reticulum membrane protein) were filtered at the indicated time points. Data represent the mean  $\pm$  standard deviation of four independent experiments. Equilibrium binding determined before dilution amounted to  $1.9 \pm 0.2 \text{ pmol}/\text{mg}$  and was set 100% to normalize for interassay variation. **B**, Sarcoplasmic reticulum membranes were allowed to bind 100 nM  $[^3\text{H}]$ ryanodine at a free  $\text{Ca}^{2+}$

ciation ( $0.0065 \pm 0.0003 \text{ min}^{-1}$ ), whereas AMP-PCP had no effect ( $0.010 \pm 0.0007 \text{ min}^{-1}$ ). Although the difference in the rates was modest, the effect was statistically significant ( $p = 0.02$ ; paired *t* test; four experiments). Under all three conditions, the dissociation kinetics followed a monoexponential decay, resulting in linear plots on logarithmic transformation (Fig. 3A, inset). We cannot rule out that a deviation from this monoexponential dissociation may have been detected if the dissociation reaction had been observed over a longer incubation period. However, under our experimental conditions, [ $^3\text{H}$ ]ryanodine binding progressively declined with incubation times of  $>3$  hr. This made it impossible to differentiate between loss of binding due to dissociation of the ligand and denaturation/degradation of the channel protein.

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

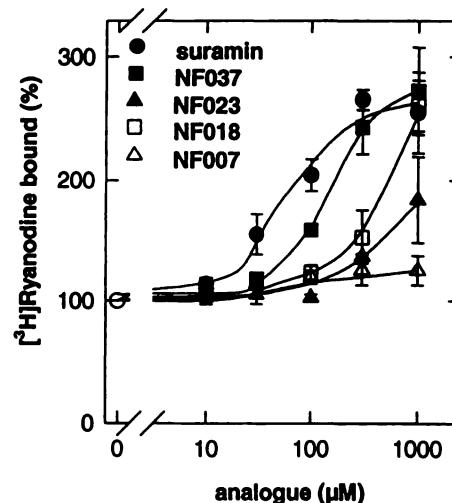
To calculate the  $K_D$  value from the kinetics parameters, we used the apparent on-rates of  $0.0312 \pm 0.0032 \text{ min}^{-1}$  for the control,  $0.0432 \pm 0.0026 \text{ min}^{-1}$  in the presence of AMP-PCP, and  $0.0390 \pm 0.0034 \text{ min}^{-1}$  in the presence of suramin, as

concentration of 2  $\mu\text{M}$ . After 1 hr (time = 0), the incubation was split and diluted 50-fold into ryanodine-free incubation buffer, buffer supplemented with 1 mM AMP-PCP, or buffer supplemented with 0.3 mM suramin. Aliquots (25  $\mu\text{g}$  of protein) of the diluted samples were filtered at the indicated time points. Data represent the mean  $\pm$  standard deviation of two independent experiments in duplicate. Equilibrium binding determined before dilution amounted to  $6.5 \pm 0.4 \text{ pmol}/\text{mg}$  and was set 100% to normalize for interassay variation. C, Sarcoplasmic reticulum membranes (25  $\mu\text{g}$ ) were allowed to bind 100 nM [ $^3\text{H}$ ]ryanodine for 1 hr at 2  $\mu\text{M}$  free  $\text{Ca}^{2+}$ . Subsequently, dissociation was induced by the addition of buffer containing 100  $\mu\text{M}$  unlabeled ryanodine, the combination of 100  $\mu\text{M}$  ryanodine and 2 mM AMP-PCP, or the combination of 100  $\mu\text{M}$  ryanodine and 0.3 mM suramin. Aliquots of the original incubation were withdrawn after the indicated time points without any addition. Data are from a single experiment carried out in duplicate that was reproduced twice.

derived from measurements with 10 nM [ $^3\text{H}$ ]ryanodine (Fig. 2B). Based on calculations with the experimentally determined  $k_{\text{on}}$  parameters, we obtained  $K_D$  values of 5, 3, and 2 nM for control conditions, AMP-PCP, and suramin, respectively. These parameters are in agreement with the directly determined  $K_D$  values given in the legend to Fig. 1A. In contrast, use of the extrapolated  $k_{\text{off}}$  values determined graphically (see Fig. 2B) resulted in  $K_D$  values that were 15–125-fold higher.

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

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



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

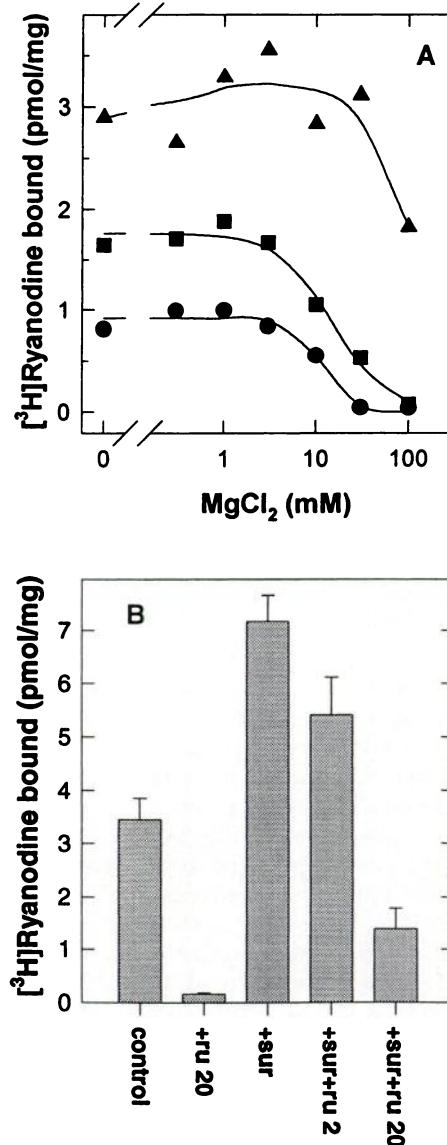
ments were carried out under nonequilibrium conditions [i.e., the incubation lasted 45 min (see Fig. 2A)], which gave a more favorable signal-to-noise ratio to detect effects of weakly active analogs. However, the EC<sub>50</sub> value for suramin was identical if measured under equilibrium conditions (EC<sub>50</sub> = 63 ± 21 μM; three experiments).

We also determined the EC<sub>50</sub> value for suramin on the purified ryanodine receptor and obtained a value of 43 ± 23 μM (three experiments; data not shown). In addition, the effect of suramin was investigated on ryanodine binding to cardiac sarcoplasmic reticulum membranes prepared from calf hearts. When the vesicles were incubated in the presence of 10 nM [<sup>3</sup>H]ryanodine and a free Ca<sup>2+</sup> concentration of 2 μM, suramin stimulated binding by a factor of ~2 (0.52 ± 0.25 and 1.1 ± 0.3 pmol/mg in the absence and presence of 0.3 mM suramin, respectively). Therefore, the effect of suramin is not restricted to the skeletal isoform of the ryanodine receptor.

**Effect of regulatory ligands in the presence of suramin.** The dissociation experiment (see Fig. 3A) indicated that suramin and AMP-PCP induced different effects on the binding characteristics of the ryanodine receptor. We therefore tested whether suramin affected the interaction of other regulatory ligands with the ryanodine receptor. At Mg<sup>2+</sup> concentrations of >1 mM, channel gating and ryanodine binding are progressively suppressed (12, 29) (Fig. 5A). Half-maximal inhibition of [<sup>3</sup>H]ryanodine binding was observed at concentrations of 13.2 ± 2.4 and 16.3 ± 2.0 mM Mg<sup>2+</sup> in the absence and presence of AMP-PCP, respectively. However, in the presence of suramin, this Mg<sup>2+</sup>-dependent inhibition was relieved so that at a concentration of 100 mM, Mg<sup>2+</sup> caused only a modest reduction in [<sup>3</sup>H]ryanodine binding of ~30% from the stimulated value (Fig. 5A). In contrast to Mg<sup>2+</sup>, the inhibition of the ryanodine binding by ruthenium red was not appreciably affected by suramin (Fig. 5B).

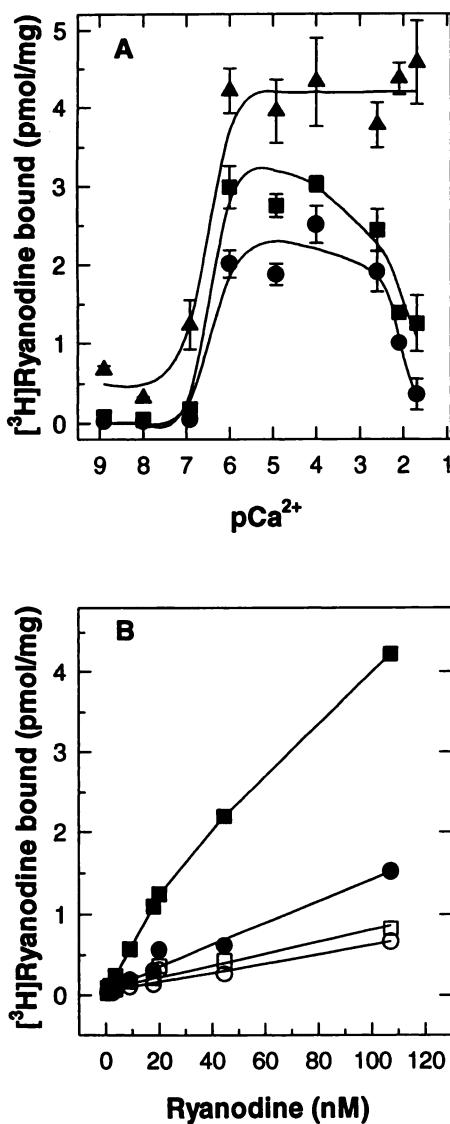
Channel gating of the Ca<sup>2+</sup>-release channel and ryanodine binding are highly dependent on the free Ca<sup>2+</sup> concentration. Under the assay conditions that we used, essentially no high affinity ryanodine binding was observed unless the Ca<sup>2+</sup> concentrations were >0.5 μM. The concentration-response curve for Ca<sup>2+</sup> was steep and bell shaped; at concentrations of ≥1 mM, Ca<sup>2+</sup> inhibited [<sup>3</sup>H]ryanodine binding in the absence and presence of 1 mM AMP-PCP (Fig. 6A, ● and ■). The Ca<sup>2+</sup> dependency of [<sup>3</sup>H]ryanodine binding was not altered by the ATP analog (Fig. 6A) (31). In contrast, suramin had two effects on the concentration-response curve for Ca<sup>2+</sup> (Fig. 6A, ▲). First, the inhibition at high Ca<sup>2+</sup> concentrations was absent; second, ryanodine binding was promoted at low Ca<sup>2+</sup> concentrations, whereas the Ca<sup>2+</sup>-stimulated component of ryanodine binding was only modestly affected. This phenomenon is exemplified in Fig. 6B. High affinity binding of [<sup>3</sup>H]ryanodine was observed in a nominally Ca<sup>2+</sup>-free solution (0 mM CaCl<sub>2</sub> and 0.5 mM EGTA) in the presence of suramin (■), whereas it was virtually undetectable in its absence (●). Furthermore, suramin had no effect on the nonspecific [<sup>3</sup>H]ryanodine binding under these conditions (Fig. 6B, open symbols).

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



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

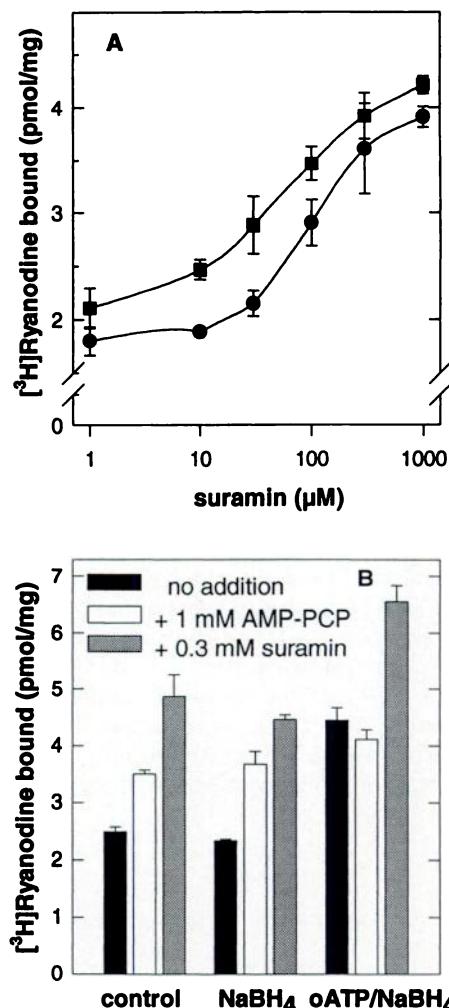
the presence of 0.3 mM AMP-PCP, a concentration corresponding to the EC<sub>50</sub>, the concentration-response curve for suramin was shifted only upward, and the AMP-PCP-induced increment remained constant. Analogous results were also obtained at 1 mM AMP-PCP (data not shown). We stress that these experiments were carried out under equilibrium conditions. Therefore, these findings indicated that AMP-PCP and suramin acted independently and that the effects of both compounds were additive. Analogous experiments were also carried out in the presence of caffeine; a saturating



**Fig. 6.** Effect of  $\text{Ca}^{2+}$  on  $[^3\text{H}]$ ryanodine binding in the absence and presence of suramin. A, Sarcoplasmic reticulum membranes ( $50 \mu\text{g}$ ) were incubated with  $20 \text{nM}$   $[^3\text{H}]$ ryanodine for 90 min. The indicated free  $\text{Ca}^{2+}$  concentrations were adjusted with EGTA and  $\text{CaCl}_2$ .  $\text{Ca}^{2+}$  concentrations exceeding  $100 \mu\text{M}$  were adjusted only by the addition of  $\text{CaCl}_2$ . The incubation was performed in the (●) absence and presence of (■)  $1 \text{ mM}$  AMP-PCP or (▲)  $0.3 \text{ mM}$  suramin. Data represent the mean of two experiments carried out in duplicate. Error bars, standard deviation. B,  $[^3\text{H}]$ ryanodine binding was performed in buffer containing  $0.5 \text{ mM}$  EGTA and no added  $\text{CaCl}_2$ . Sarcoplasmic reticulum membranes ( $30 \mu\text{g}$ ) were incubated for 90 min in the (●, ○) absence and (■, □) presence of  $0.3 \text{ mM}$  suramin. ● and ■, Total binding. ○ and □, Non-specific binding. Data represent the mean of duplicate determinations in a single experiment that was reproduced twice.

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

We have previously shown that the ATP analog oATP binds to the ryanodine receptor in a quasi-irreversible manner via Schiff's base formation with lysine residues in the adenine nucleotide-binding pocket. Subsequent reduction with  $\text{NaCNBH}_3/\text{NaBH}_4$  results in covalent incorporation of oATP (16). We exploited this property of oATP through an



**Fig. 7.** Additivity of adenine nucleotide- and suramin-dependent stimulation of  $[^3\text{H}]$ ryanodine binding. A, Sarcoplasmic reticulum membranes ( $30 \mu\text{g}$ ) were incubated at a free  $\text{Ca}^{2+}$  concentration of  $2 \mu\text{M}$  with  $10 \text{nM}$   $[^3\text{H}]$ ryanodine for 90 min. Increasing concentrations of suramin were added in the (●) absence and (■) presence of  $0.3 \text{ mM}$  AMP-PCP. B, Thirty micrograms of sarcoplasmic reticulum membranes was incubated with  $10 \text{nM}$   $[^3\text{H}]$ ryanodine at a free  $\text{Ca}^{2+}$  concentration of  $2 \mu\text{M}$  for 90 min (control). Alternatively, sarcoplasmic reticulum membranes were pretreated with  $\text{NaCNBH}_3$  and  $\text{NaBH}_4$  or with oATP and  $\text{NaCNBH}_3$  and  $\text{NaBH}_4$  (oATP/ $\text{NaBH}_4$ ) as described in Experimental Procedures. The control binding (no addition) was stimulated with  $1 \text{ mM}$  AMP-PCP or  $0.3 \text{ mM}$  suramin. Data are mean  $\pm$  standard deviation from three independent experiments carried out in duplicate.

alternative approach to show that suramin and adenine nucleotides acted independently (Fig. 7B). The sarcoplasmic reticulum was preincubated in the absence and presence of oATP followed by the addition of  $\text{NaCNBH}_3/\text{NaBH}_4$ . The reaction products were subsequently removed through centrifugation. When the preincubation was done in the absence of oATP, both AMP-PCP and suramin stimulated  $[^3\text{H}]$ ryanodine binding (Fig. 7B, control); this stimulation was not affected by the sole addition of  $\text{NaCNBH}_3/\text{NaBH}_4$  (Fig. 7B,  $\text{NaBH}_4$ ). In contrast, when the preincubation was carried out with oATP and the reducing agents  $\text{NaCNBH}_3/\text{NaBH}_4$  were subsequently added,  $[^3\text{H}]$ ryanodine binding was enhanced and no additional stimulation was observed in the presence of AMP-PCP (Fig. 7B, oATP/ $\text{NaBH}_4$ ). This was to be expected because the adenine nucleotide-binding site was occu-

pied by covalently incorporated oATP. More importantly, this incorporation did not block the stimulation by suramin.

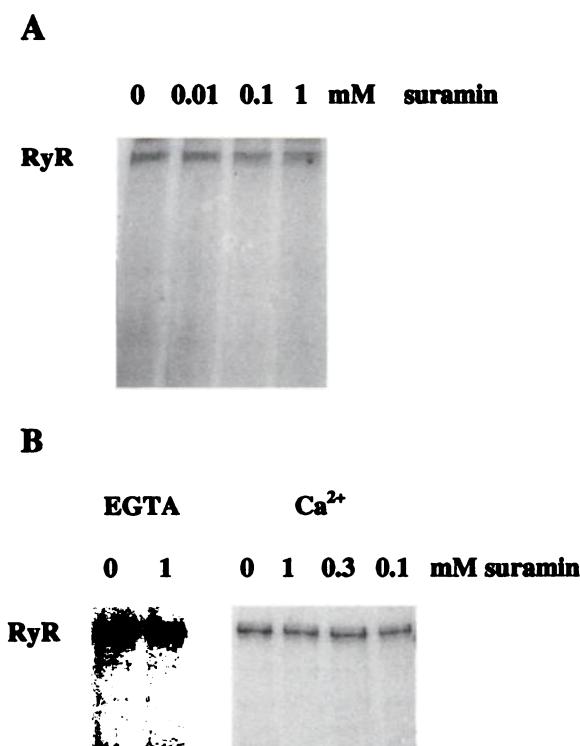
**Affinity labeling of the ryanodine receptor.** Based on their functional effects on [<sup>3</sup>H]ryanodine binding, adenine nucleotides and suramin do not seem to share a common binding site on the Ca<sup>2+</sup>-release channel protein. To obtain more direct evidence for this interpretation, we labeled the purified ryanodine receptor with radioactive oATP in the absence and presence of suramin. Two methods were used. The purified protein was incubated in the presence of 10 μM [α-<sup>32</sup>P]oATP followed by reduction with NaBH<sub>4</sub> (Fig. 8A); we have previously shown that this labeling is specific because it is blocked by unlabeled ATP in a competitive manner (16). Alternatively, the incubation was carried out in the presence of 0.5 mM [γ-<sup>32</sup>P]ATP, and *in situ* oxidation and reduction were achieved by the sequential addition of NaIO<sub>4</sub>, NaCNBH<sub>3</sub>, and NaBH<sub>4</sub> (Fig. 8B). We rule out that labeling under the latter condition was due to phosphorylation by a contaminating protein kinase because radioactive labeling was not observed when NaIO<sub>4</sub> and the reducing agents were omitted (not shown). The gel slides corresponding to the ryanodine receptor were also excised, and the radioactivity was determined by liquid scintillation counting. Radioactive labeling determined in this manner remained unaffected by the addition of suramin. Therefore, regardless of the method used, suramin did not affect the incorporation of oATP into the ryanodine receptor. This confirms that suramin does not

block the interaction of adenine nucleotide with the binding site on the Ca<sup>2+</sup>-release channel protein. A minor band is visible in the autoradiogram of Fig. 8A; this corresponds to the top of the separating gel, where some of the radioactivity is retained. It is unclear whether this is due to retention of labeled protein or simply due to interaction of the radioactive substrate with the gel matrix.

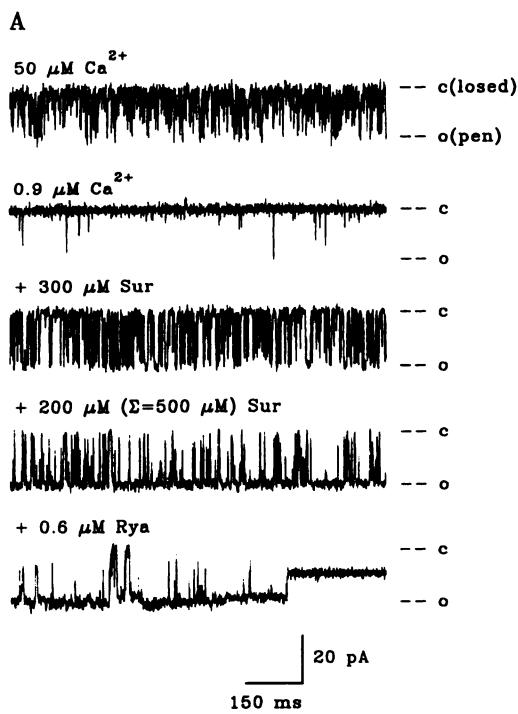
**Activation of the isolated Ca<sup>2+</sup>-release channel/ryanodine receptor complex.** To investigate a possible direct interaction of suramin with the ryanodine receptor, the purified protein was incorporated into planar lipid bilayers. The effect of suramin on the gating properties of the reconstituted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

The analysis of the gating kinetics revealed that the activating effect of suramin is accompanied by an increase in the duration of channel open events. Representative open-time histograms in the presence of Ca<sup>2+</sup> and suramin are shown in Fig. 10. At a low Ca<sup>2+</sup> concentration, the corresponding open-time histograms could be adequately fit by a single exponential function with a time constant of 0.3 msec. When the channel was activated by suramin, a second exponential component had to be introduced (τ<sub>1</sub> = 1.3 msec; τ<sub>2</sub> = 4.6 msec). Compared with the activation in the presence of 50 μM Ca<sup>2+</sup>, both time constants were increased 2–3-fold. The addition of ATP also resulted in an increase of the two open-time constants but did not induce long periods of high and low activity as were observed in the presence of suramin (11).

At concentrations in the low micromolar range, ryanodine

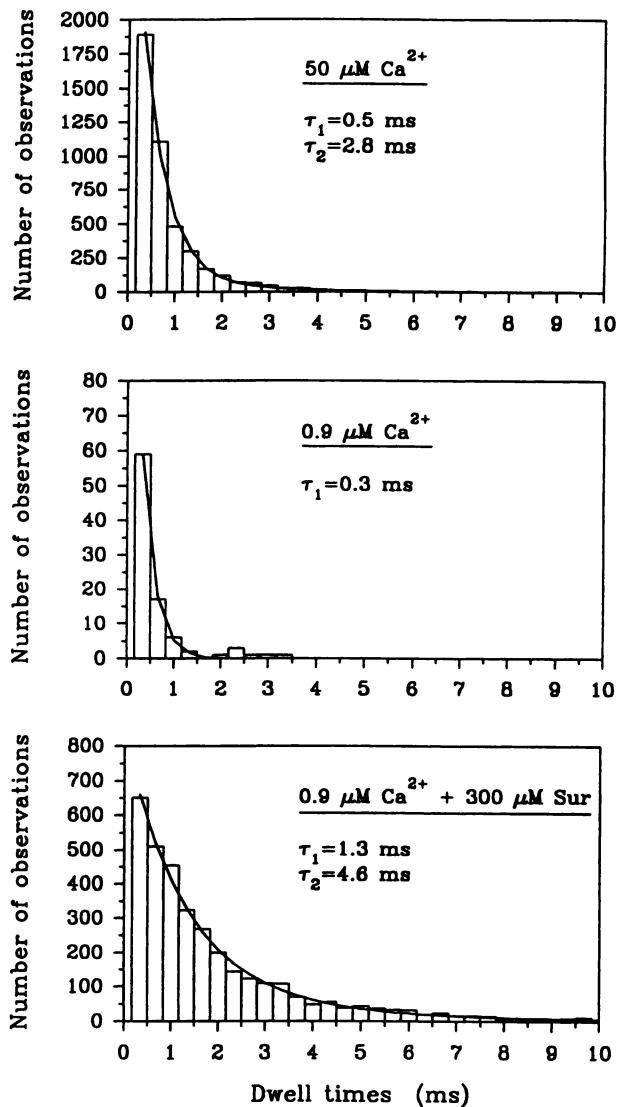


**Fig. 8.** Covalent labeling of the purified ryanodine receptor (RyR) with (A) [α-<sup>32</sup>P]oATP or (B) *in situ* oxidized [<sup>32</sup>P]ATP in the absence and presence of suramin. Purified ryanodine receptor (A, 2 μg; B, 5 μg) was incubated with (A) 10 μM [α-<sup>32</sup>P]oATP or (B) 0.5 mM [<sup>32</sup>P]ATP in Ca<sup>2+</sup>-free medium (EGTA) or Ca<sup>2+</sup>-containing medium (Ca<sup>2+</sup>) as described in Experimental Procedures. Suramin was added to give the indicated concentrations. The samples were electrophoretically resolved on a 7% sodium dodecyl sulfate-polyacrylamide gel; the autoradiograms shown were obtained after exposure of the film for (A) 2 days or (B) 12 hr at room temperature with an intensifying screen.



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

transferred the skeletal and cardiac  $\text{Ca}^{2+}$ -release channel into a characteristic long open state of reduced conductance (30, 32, 33). This property was retained by the suramin-activated channel (Fig. 9A). At  $\sim 5$  min after the addition of 0.6  $\mu\text{M}$  ryanodine, the suramin-activated channel was transferred into the typical long open half-conductance state (Fig. 9A, bottom trace), which was not further modified by other known modulators of the ryanodine receptor. The applied



**Fig. 10.** Gating kinetics of the  $\text{Ca}^{2+}$ -release channel. Representative open-time histograms in the presence of  $\text{Ca}^{2+}$  and suramin. The histograms were fit by a single exponential function in the presence of 0.9  $\mu\text{M}$   $\text{Ca}^{2+}$  or by the sum of two exponential functions at 50  $\mu\text{M}$   $\text{Ca}^{2+}$  and 300  $\mu\text{M}$  suramin (Sur) with the indicated time constants.

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

## Discussion

We characterized the interaction of suramin with the ryanodine receptor. Our observations show that suramin is a direct activator of the channel protein. This conclusion is based on two sets of observations. First, suramin promoted binding of [<sup>3</sup>H]ryanodine to both the channel protein in sarcoplasmic reticulum membranes of skeletal and cardiac muscle and the protein purified from skeletal muscle. Second,

suramin increased the  $P_o$  value of the purified channel protein after insertion into a planar lipid bilayer. These findings confirm and extend those of a previous report (18). More importantly, we designed a series of experiments to test the working hypothesis put forth previously (18): that suramin and adenine nucleotides exert similar effects on the ryanodine receptor because they interact with a common binding site.

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

The binding of [ $^3H$ ]ryanodine is complex, and the apparent affinity constants derived from equilibrium binding experiments depend on the assay conditions. In general, two affinity binding sites can be derived in saturation experiments with sarcoplasmic reticulum membranes: a high affinity site with a  $K_D$  value of ~5 nM and a low affinity site with a  $K_D$  value in the submicromolar range (34). Affinity estimates consistent with these values were also calculated from the current saturation experiments. However, a detailed kinetic analysis has revealed as many as four interdependent affinity states/binding sites (27), and our observations are in line with these findings. First, the increment in the apparent on-rate with increasing concentrations of ligand suggests a quasilinear relationship between [ $^3H$ ]ryanodine (10–100 nM) and  $k_{app}$ ; however, if binding occurred via a simple bimolecular reaction, extrapolation to zero ligand concentration ( $L$ ) should yield  $k_{off}$  based on the following relation:  $k_{app} = k_{on} * L + k_{off}$ . This clearly was not the case. The kinetically determined dissociation constant  $K_D$  thus depends on which ligand concentration is used to derive this parameter from the association and dissociation rate constants. In addition, the fact that the addition of an excess of unlabeled ryanodine failed to displace the radioligand bound at equilibrium (Fig. 3C and Refs. 30 and 35) demonstrates that multiple non-equivalent ryanodine-binding sites exist within the tetrameric protein and that these are linked in a cooperative manner. Finally, high affinity binding of [ $^3H$ ]ryanodine is too slow (by 3–4 orders of magnitude) to be a simple diffusion-limited reaction. It is therefore plausible to postulate that the rate-limiting step is the access of ryanodine to the binding site and that this reaction requires the open-channel conformation (36, 37); under these assumptions, suramin and other agonists (e.g., adenine nucleotides and  $Ca^{2+}$  at micromolar concentrations) promote binding of ryanodine primarily by accelerating the association rate via channel opening. This

interpretation is consistent with the increased  $P_o$  value observed in single-channel recordings in the presence of suramin. We have no mechanistic explanation for the modest effect of suramin on the dissociation rate of ryanodine.

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

Suramin has pleiotropic effects and binds to many receptors and enzymes (17). It is popular among pharmacologists because it is used to subclassify  $P_2$ -purinergic receptors; suramin is a competitive antagonist for ATP at the  $P_{2x}$ - and  $P_{2y}$ -purinoceptors (38). It is clear that binding of suramin requires positive charges on the surface of the proteins (39, 40). Nevertheless, differences in the structure-activity relation for the suramin analogs that we used are readily apparent if their affinity for  $P_2$ -purinergic receptors and ecto-ATPases (19) as well as G proteins (41, 42) is compared with their ability to promote [ $^3H$ ]ryanodine binding. This is best exemplified with NF023; this compound is a high affinity inhibitor of G proteins of the  $G_{i\alpha}/G_{o\alpha}$  group (41) and a potent antagonist at  $P_2$ -purinergic receptors (19). However, NF023 (with six negative charges) has only a very modest effect on [ $^3H$ ]ryanodine binding, and NF018 (the half-molecule of NF037 with only three negative charges) is more active. In addition, NF037 (demethylated of suramin) has a 3-fold-lower apparent affinity than suramin. This indicates that the negative charges are not the sole determinants of activation of the ryanodine receptor.

We conclude from our findings that the suramin-binding site is distinct from that for other regulatory ligands, such as ATP, ryanodine, and activating  $\text{Ca}^{2+}$ . Ryanodine receptors play a crucial role as molecular switches in the phasic release of  $\text{Ca}^{2+}$  and in the amplification of  $\text{Ca}^{2+}$ -induced  $\text{Ca}^{2+}$  release. We therefore believe that this class of intracellular channels may represent a potential target for drug action, in particular, in pathophysiological situations associated with cellular  $\text{Ca}^{2+}$  overload (43). A search for inhibitory ligands that target the suramin-binding site on the various ryanodine receptor isoforms may yield therapeutically useful drugs.

#### Acknowledgments

We thank Dr. P. Nickel for the generous gift of the suramin analogs. We are indebted to E. Tuisl for the artwork.

#### References

- Sorrentino, V., and P. Volpe. Ryanodine receptors: how many, where, and why? *Trends Pharmacol. Sci.* **14**:98–103 (1993).
- Melzer, W., A. Herrmann-Frank, and H. C. Lüttgau. The role of  $\text{Ca}^{2+}$  ions in excitation-contraction coupling of skeletal muscle fibers. *Biochim. Biophys. Acta* **1241**:59–116 (1995).
- Flucher, B. E., S. B. Andrews, S. Fleischer, A. R. Marks, A. Caswell, and J. A. Powell. Triad formation: organization and function of the sarcoplasmic reticulum calcium release channel and triadin in normal and dysgenic muscle in vitro. *J. Cell Biol.* **123**:1161–1174 (1993).
- Lu, X., L. Xu, and G. Meissner. Phosphorylation of dihydropyridine receptor II-III loop peptide regulates skeletal muscle calcium release channel function: evidence for an essential role of the  $\beta$ -OH group of Ser687. *J. Biol. Chem.* **270**:18459–18464 (1995).
- El-Hayek, R., B. Antoniu, J. Wang, S. L. Hamilton, and N. Ikemoto. Identification of calcium-release triggering and blocking regions of the II-III loop of the skeletal muscle dihydropyridine receptor. *J. Biol. Chem.* **270**:22116–22118 (1995).
- Palade, P., C. Dettbarn, D. Brunder, P. Stein, and G. Hals. Pharmacology of calcium release from sarcoplasmic reticulum. *J. Bioenerg. Biomembr.* **21**:295–320 (1989).
- Witcher, D. R., R. J. Kovacs, H. Schulmann, D. C. Cefali, and L. R. Jones. Unique phosphorylation site on the cardiac ryanodine receptor regulates calcium channel activity. *J. Biol. Chem.* **266**:11144–11152 (1991).
- Wang, J., and P. M. Best. Inactivation of the sarcoplasmic reticulum calcium channel by protein kinase. *Nature (Lond.)* **359**:739–741 (1992).
- Herrmann-Frank, A., and M. Varsányi. Enhancement of  $\text{Ca}^{2+}$  release channel activity by phosphorylation of skeletal muscle ryanodine receptor. *FEBS Lett.* **332**:237–242 (1993).
- Hohenegger, M., and J. Suko. Phosphorylation of the purified cardiac ryanodine receptor by exogenous and endogenous protein kinases. *Biochem. J.* **296**:303–308 (1993).
- Smith, J. S., R. Coronado, and G. Meissner. Sarcoplasmic reticulum contains adenine nucleotide-activated channels. *Nature (Lond.)* **316**:446–449 (1985).
- Smith, J. S., R. Coronado, and G. Meissner. Single channel measurements of the calcium release channel from skeletal muscle reticulum. *J. Gen. Physiol.* **88**:573–588 (1986).
- Smith, J. S., T. Imagawa, J. Ma, M. Fill, K. P. Campbell, and R. Coronado. Purified ryanodine receptor rabbit skeletal muscle is the  $\text{Ca}^{2+}$  release channel of sarcoplasmic reticulum. *J. Gen. Physiol.* **92**:1–26 (1988).
- Lai, F. A., H. P. Erikson, E. Rousseau, Q. Y. Liu, and G. Meissner. Purification and reconstitution of the calcium release channel from skeletal muscle. *Nature (Lond.)* **331**:315–319 (1988).
- Zarka, A., and V. Shoshan-Barmatz. Characterization and photoaffinity labeling of the ATP binding site of the ryanodine receptor from skeletal muscle. *Eur. J. Biochem.* **213**:147–154 (1993).
- Hohenegger, M., A. Herrmann-Frank, M. Richter, and F. Lehmann-Horn. Activation and labeling of the purified skeletal muscle ryanodine receptor by an oxidized ATP analogue. *Biochem. J.* **308**:119–125 (1995).
- Voogd, T. E., E. L. M. Vansterkenburg, J. Wilting, and L. H. M. Janssen. Recent research on the biological activity of suramin. *Pharmacol. Rev.* **45**:177–203 (1993).
- Emmick, J. T., Kwon, S., Bidasee, K. R., Mesch, K. T., and Besch, H. R. Dual effect of suramin on calcium fluxes across sarcoplasmic reticulum vesicle membranes. *J. Pharmacol. Exp. Ther.* **269**:717–724 (1994).
- van Rhee, A. M., M. P. A. van der Heijden, M. W. Beukers, A. P. IJzerman, W. Soudjin, and P. Nickel. Novel competitive antagonists for P2 purinoreceptors. *Eur. J. Pharmacol.* **268**:1–7 (1994).
- Easterbrook-Smith, S. B., J. C. Wallace, and D. B. Keech. Pyruvate carboxylase: affinity labeling of the magnesium adenosine triphosphate binding site. *Eur. J. Biochem.* **62**:125–130 (1976).
- Hohenegger, M., and M. Makinose. 2',3'-Dialdehyde ATP analog labels the  $\text{Ca}^{2+}$  ATPase of sarcoplasmic reticulum via the catalytic adenosine nucleotide binding site. *Eur. J. Biochem.* **205**:173–179 (1992).
- Wyskovsky, W., M. Hohenegger, B. Plank, G. Hellmann, S. Klein, and J. Suko. Activation and inhibition of the calcium-release channel of isolated skeletal muscle heavy sarcoplasmic reticulum: models of the calcium-release channels. *Eur. J. Biochem.* **194**:549–559 (1990).
- Herrmann-Frank, A., M. Richter, S. Sarközi, U. Mohr, and F. Lehmann-Horn. 4-Chloro-m-cresol, a potent and specific activator of the skeletal muscle ryanodine receptor. *Biochim. Biophys. Acta* **1289**:31–40 (1996).
- Hohenegger, M., C. Nanoff, H. Ahorn, and M. Freissmuth. Structural and functional characterization of the interaction between 2',3'-dialdehyde guanine nucleotide analogues and the stimulatory G protein  $\alpha$ -subunit. *J. Biol. Chem.* **269**:32008–32015 (1994).
- Kaplan, R. S., and P. L. Pedersen. Determination of microgram quantities of protein in the presence of milligram levels of lipid with amido black 10B. *Anal. Biochem.* **150**:97–104 (1985).
- Fabiato, A. Computer programs for calculating total free or free from specified total ionic concentration in aqueous solution containing multiple metals and ligands. *Methods Enzymol.* **157**:378–417 (1988).
- Buck, E., I. Zimanyi, J. J. Abramson, and I. N. Pessah. Ryanodine stabilizes multiple conformational states of the skeletal muscle calcium release channel. *J. Biol. Chem.* **267**:23560–23567 (1992).
- Pessah, I. N., and I. Zimanyi. Characterization of multiple [ $^3\text{H}$ ]ryanodine binding sites on the  $\text{Ca}^{2+}$  release channel of sarcoplasmic reticulum from skeletal and cardiac muscle: evidence for a sequential mechanism in ryanodine action. *Mol. Pharmacol.* **39**:679–689 (1991).
- Pessah, I. N., R. A. Stambuk, and J. E. Casida.  $\text{Ca}^{2+}$ -activated ryanodine binding: mechanisms of sensitivity and intensity modulation by  $\text{Mg}^{2+}$ , caffeine, and adenine nucleotides. *Mol. Pharmacol.* **31**:232–238 (1987).
- Lai, F. A. M. Misra, L. Xu, H. A. Smith, and G. Meissner. The ryanodine receptor  $\text{Ca}^{2+}$  release channel complex of skeletal muscle sarcoplasmic reticulum: evidence for a cooperatively coupled, negatively charged homotetramer. *J. Biol. Chem.* **264**:16776–16785 (1989).
- Ogawa, Y., and H. Harafuji. Effect of temperature on [ $^3\text{H}$ ]ryanodine binding to sarcoplasmic reticulum from bullfrog skeletal muscle. *J. Biochem.* **107**:887–893 (1990).
- Rousseau, E., J. S. Smith, and G. Meissner. Ryanodine modifies conductance and gating behavior of single  $\text{Ca}^{2+}$  release channel. *Am. J. Physiol.* **253**:C364–C368 (1987).
- Nagasaki, K., and S. Fleischer. Ryanodine sensitivity of the calcium release channel of sarcoplasmic reticulum. *Cell Calcium* **9**:1–7 (1988).
- McGrew, S. G., C. Wolleben, M. Inui, and S. Fleischer. Positive cooperativity of ryanodine binding to the calcium release channel of sarcoplasmic reticulum from heart and skeletal muscle. *Biochemistry* **28**:1686–1691 (1989).
- Ogawa, Y., and H. Harafuji. Osmolarity dependent characteristics of [ $^3\text{H}$ ]ryanodine binding to sarcoplasmic reticulum. *J. Biochem.* **107**:894–898 (1990).
- Imagawa, T., J. Smith, R. Coronado, and K. Campbell. Purified ryanodine receptor from muscle sarcoplasmic reticulum is the  $\text{Ca}^{2+}$ -permeable pore of the calcium release channel. *J. Biol. Chem.* **262**:16636–16643 (1987).
- Pessah, I. N., A. Francini, D. Scales, A. Waterhouse, and J. E. Casida. Calcium-ryanodine receptor complex: solubilization and partial characterization from skeletal muscle junctional sarcoplasmic reticulum vesicles. *J. Biol. Chem.* **261**:8643–8648 (1986).
- Hoyle, C. H. V., G. E. Knoght, and G. Burnstock. Suramin antagonizes responses to P<sub>2</sub>-purinoreceptor agonists and purinergic nerve stimulation in the guinea-pig urinary bladder and taenia coli. *Br. J. Pharmacol.* **90**:617–621 (1990).
- Wieringa, R. K., B. Swinkels, P. A. M. Michels, K. Osinga, O. Missel, J. Van Beeumen, W. C. Gibson, J. P. M. Postma, F. R. Oppenhoes, and W. G. J. Hol. Common elements on the surface of glycolytic enzymes from *Trypanosoma brucei* may serve as topogenic signals for import into glycosomes. *EMBO J.* **6**:215–221 (1987).
- Missel, O., and F. R. Oppenhoes. The phosphoglycerate kinase from *Trypanosoma brucei*: a comparison of the glycosomal and cytosolic isoenzymes and their selectivity towards suramin. *Eur. J. Biochem.* **162**:493–500 (1987).
- Freissmuth, M., S. Boehm, W. Beindl, P. Nickel, A. P. IJzerman, M. Hohenegger, and C. Nanoff. Suramin analogues as subtype-selective G protein inhibitors. *Mol. Pharmacol.* **49**:602–611 (1996).
- Beindl, W., T. Mitterauer, M. Hohenegger, A. P. IJzerman, C. Nanoff, M. Freissmuth. Inhibition of receptor/G protein coupling by suramin analogues. *Mol. Pharmacol.* **50**:415–423 (1996).
- Mack, M. M., I. Zimanyi, and I. N. Pessah. Discrimination of multiple binding sites for antagonists of the calcium release channel complex of the skeletal and cardiac sarcoplasmic reticulum. *J. Pharmacol. Exp. Ther.* **262**:1028–1037 (1992).