Short- and Long-Term Functional Alterations of the Skeletal Muscle Calcium Release Channel (Ryanodine Receptor) by Suramin: Apparent Dissociation of Single Channel Current Recording and [³H]Ryanodine Binding

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ABSTRACT

The present study demonstrates the following characteristic suramin actions on the purified skeletal muscle calcium release channel in single-channel current recordings and [³H]ryanodine binding to HSR: 1) Suramin (0.3–0.9 mM) induced a concentration-dependent increase in the open probability ($P_o \cong 0.9$) at 20 to 100 μM Ca^{2+} and an almost fully open channel at 1 mM Ca^{2+} ($P_o = 0.95$) with a marked shift to longer open states ($\tau_o 3/\tau_o 4$). Suramin increased the apparent calcium affinity to the activating high-affinity calcium binding sites and reduced the apparent magnesium affinity to the inhibitory low affinity Ca^{2+}/Mg^{2+} binding sites. 2) Channel activation by suramin and sulfhydryl oxidation was additive. 3) Suramin (0.9 mM) reversed the Ca-calmodulin–induced channel inhibition at 0.1 or 1 to 5 μM Ca-calmodulin. 4) The open probability of the suramin activated channel was almost completely inhibited by 10 mM Mg^{2+} or Ca^{2+} on short suramin exposure. Prolonged suramin exposure (30–60 min) resulted in a time-dependent, slow increase in P_o, with long open states of low frequency in the presence of 10 to 20 mM Mg^{2+} or Ca^{2+}. 5) Magnesium induced inhibition of P_o ($IC_{50} = 0.38$ mM) and equilibrium [³H]ryanodine binding ($IC_{50} = 0.30$ mM) agreed well in control channels, but were dissociated in the presence of 0.9 to 1.0 mM suramin ($IC_{50} = 0.82$ mM versus 83 mM). [³H]ryanodine binding seemed to monitor predominantly the long-term alteration in channel function. 6) The multiple effects of suramin on channel function suggest an allosteric mechanism and no direct effects on binding of endogenous ligands involved in channel gating.

Calcium release in skeletal muscle occurs via the calcium release channel (ryanodine receptor) located in the terminal cisternae of the sarcoplasmic reticulum (Imagawa et al., 1987; Inui et al., 1987; Lai et al., 1988; Smith et al., 1988). The skeletal muscle calcium release channel (RyR-1) is a homotetramer with a molecular mass of 2260 kDa (Takeshima et al., 1989; Zorzato et al., 1990). Calcium release and the gating properties of the calcium release channel at a single channel level are regulated by endogenous effectors including calcium, magnesium, adenine nucleotides, the calcium binding proteins calmodulin and sorcin, the immunophillin FK506-binding protein, phosphorylation by protein kinases, sulfhydryl oxidation by nitric oxide, and various exogenous effectors (see reviews: Coronado et al., 1994; Meissner, 1994; Melzer et al., 1995; Zucchi and Ronca-Testoni, 1997).

The trypanocidal drug suramin, a polysulfonated napthy-lurea, is a potent activator of the ligand-gated calcium re-lease channel of sarcoplasmic reticulum. Suramin released calcium from passively loaded skeletal muscle sarcoplasmic reticulum vesicles (Emmick et al., 1994) as well as from cells containing the RyR-3 isoform, such as Jurkat T-lymphocytes (Hohenegger et al., 1999). The gating of the calcium release channel in skeletal (RyR-1) and cardiac (RyR-2) muscle is markedly influenced by suramin or suramin analogs. Characteristic for the activating effect of suramin in single-channel current recordings was an increase in longer open states, especially with the cardiac calcium release channel and the requirement of lower suramin concentrations to increase the open probability of the cardiac calcium release channel (Sitsapesan and Williams, 1996; Sitsapesan, 1999) compared with that from the skeletal muscle channel (Hohenegger et al., 1996; Sitsapesan and Williams, 1996; Klinger et al., 1999). Furthermore, [³H]ryanodine binding to the calcium release channel of heavy sarcoplasmic reticulum (HSR) of skeletal muscle was activated by suramin, but only margin-

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ally inhibited by very large concentrations of calcium or magnesium (Emmick et al., 1994; Hohenegger et al., 1996; Klinger et al., 1999). Suramin released the RyR-1 bound to a calmodulin Sepharose (Klinger et al., 1999) and inhibited [^{125}I]calmodulin binding to HSR (Suko et al., 2000).

Because of the effects of suramin on the skeletal muscle calcium release channel found in ligand binding studies to HSR (reduction in calmodulin binding, marginal inhibition of [^{3}H]ryanodine binding by high concentrations of magnesium or calcium), the present investigation was carried out to determine the effect of the inhibitory endogenous ligands (calcium-calmodulin, magnesium, and millimolar calcium) on the suramin-induced activation at a single channel level. Ryanodine is known as a very valuable probe to monitor functional states of the calcium release channel and a good correlation between open probability in single-channel current recordings and the amount of [^{3}H]ryanodine bound to the channel proteins was usually observed (Coronado et al., 1994; Meissner, 1994). Recent studies showed that a single point mutation of the mouse RyR-2 (alanine 4812 to glycine), the suggested pore-forming region of the calcium release channel (Zhao et al., 1999), or mutation of amino acids in the luminal loop linking the M3/M4 region of the RyR-1 (Gao et al., 1999) reduced single-channel current fluctuation and [^{3}H]ryanodine binding (Gao et al., 1999; Zhao et al., 1999). For the above reason, [^{3}H]ryanodine binding to HSR was also carried out in the absence or presence of suramin under similar conditions as used in single-channel current recordings.

The results demonstrate both short and long-term functional changes in single-channel current recordings, alterations in the gating of the calcium release channel by calcium and magnesium, and an apparent dissociation between single-channel current recordings and [^{3}H]ryanodine binding in the presence of suramin.

Experimental Procedures

Materials. Suramin, calmodulin, 4-(chloromercuri)phenyl-sulfonic acid (4-CMPS), MOPS, HEPES, Tris, histidine, CsCl (ultra pure), NaCl (ultra pure), ruthenium red, leupeptin, pepstatin, antipain, phenylmethylsulfonyl fluoride, tetracaine, and neomycin were purchased from Sigma-Aldrich GmbH (Vienna, Austria); [^{3}H]ryanodine was purchased from DuPont-New England Nuclear (Boston, MA); ryanodine was from Agrisystems International (Wind Gap, PA); phosphatidyl serine, phosphatidylethanolamine, and phosphatidylcholine were from Avanti Polar Lipids, Inc. (Alabaster, AL); Delrin bilayer chambers (CD22–200; CD13–200) were from Warner Instrument Corp. (Hamden, NJ). Aprotinin was a generous gift from Bayer Austria AG (Vienna, Austria). All reagents and agents (suramin, calmodulin, 4-CMPS, [^{3}H]ryanodine) were dissolved in MilliQ deionized water.

Preparation of Sarcoplasmic Reticulum. Heavy sarcoplasmic reticulum vesicles (HSR) from rabbit skeletal muscle were prepared as described previously (Suko and Hellmann, 1998). Briefly, white back muscle (fast twitch muscle) was homogenized in a Waring Blender for 1.5 min in a medium containing 10 mM histidine buffer, pH 7.0, and 100 mM NaCl, and centrifuged for 35 min at 4,000*g*. The supernatant was filtered through cheese cloth and centrifuged for 30 min at 30,000*g*. The pellet was resuspended in 10 mM histidine buffer, pH 7.0, 0.6 M KCl, and 250 mM sucrose and centrifuged for 35 min at 100,000*g*. The pellet was washed once in a medium containing 10 mM histidine buffer, pH 7.0, 100 mM NaCl, and 200 mM sucrose, centrifuged again for 35 min at 100,000*g* and stored at −80°C or used

immediately for the purification of the ryanodine receptor-calcium release channel. All buffers used for the preparation and resuspension of HSR contained 0.5 μg/ml leupeptin, 1 μg/ml antipain, 1.4 μg/ml aprotinin, 1 μM pepstatin, 0.1 mM PMSF, 1 mM benzamidine.

Preparation of Calcium Release Channel (Ryanodine Receptor). The calcium-release channel of the terminal cisternae of sarcoplasmic reticulum vesicles was prepared as described previously (Suko and Hellmann, 1998), which was a slight modification of the preparation used before that (Suko et al., 1993). Briefly, heavy sarcoplasmic reticulum vesicles from rabbit skeletal muscle (prepared as above) were solubilized with CHAPS (medium, 40 mM Mops/Tris, pH 7.0, 1 M NaCl, 2 mM DTT, 1% CHAPS, 0.25% or 0.5% phosphatidylcholine, 0.5 μg/ml leupeptin, 1 μg/ml antipain, 1.4 μg/ml aprotinin, 1 μM pepstatin, 0.1 mM PMSF, 1 mM benzamidine, 15 mg/ml HSR; incubation, 60 min at 3–4°C), followed by centrifugation twice for 35 min at 103,000*g* (Beckman 65 rotor). The supernatant was centrifuged through a linear 7.5 to 20% sucrose gradient equilibrated in 40 mM Mops/Tris, pH 7.0, 300 mM NaCl, 2 mM DTT, 0.5% CHAPS, 0.25% or 0.5% phosphatidyl-choline, 0.5 μg/ml leupeptin, 1 μg/ml antipain, 1.4 μg/ml aprotinin, 1 μM pepstatin, 0.1 mM PMSF, and 1 mM benzamidine for 14 h at 2°C (Beckman SW28 rotor; 38 ml tubes). Fractions containing the ryanodine receptor (determined by SDS-PAGE) were pooled and dialysed for 24 h in a medium containing 20 mM Mops/Tris, pH 7.0, 100 mM NaCl, 2 mM DTT, 0.15 mM CaCl$_2$, 0.1 mM EGTA, 0.5 μg/ml leupeptin, 1 μg/ml antipain, 1.4 μg/ml aprotinin, 1 μM pepstatin, 0.1 mM PMSF, and 1 mM benzamidine. Sucrose (200 mM final concentration) was added to the proteoliposomes before storage at −78°C. Preparation and dialysis were carried out at 2 to 4°C.

SDS-Polyacrylamide Gel Electrophoresis (PAGE). SDS-PAGE was performed in 5% polyacrylamide gels (0.75 mm thickness) with 3% stacking gels as described previously (Suko et al., 1993). Sucrose gradient fractions were added to a medium containing 10 mM Tris/HCl, pH 6.8, 2% SDS, 2% mercaptoethanol, and 10% glycerol and boiled for 2 min. Gels were stained with 0.05% Coomassie blue in 10% acetic acid. Molecular mass standards were run on two separate lanes of the same gel: Ferritin (440 kDa), thyroglobulin (330 kDa), and myosin (212 kDa). Gradient fractions with the highest content of ryanodine receptor were pooled and used for the preparation of proteoliposomes.

Single Channel Recordings. Single-channel recordings were carried out after incorporation of purified calcium release channels (ryanodine receptors) into planar lipid bilayers, essentially as described previously (Suko and Hellmann, 1998). Planar lipid bilayers were formed from phosphatidylserine (10 mg/ml) and phosphatidylethanolamine (10 mg/ml) in decane (Avanti Polar Lipids). The lipid solution was spread over a 200-μm diameter aperture in a Delrin cup (Warner Instrument Corp.) separating two aqueous compartments. The *cis* bath solution (2.6 ml) and the *trans* bath solution (4 ml) were connected to the head stage input of a model EPC-9 amplifier (Heka Elektronik, Lambrecht, Germany) via Ag/AgCl electrodes and CsCl-agar bridges. The *trans* bath was held at virtual ground. Cs$^+$ was used as the charge carrier through the calcium release channel to increase the conductance of the channel (Coronado et al., 1992). The *cis* solution was composed of 10 mM HEPES/Tris, pH 7.4, 480 mM CsCl, and 50 to 100 μM CaCl$_2$ or 100 μM CaCl$_2$ plus 80 μM EGTA (free calcium, 20 μM). The *trans* solution was composed of 10 mM HEPES/Tris, pH 7.4, and 50 mM CsCl without added calcium or plus calcium in concentrations as used in the *cis* bath. Unless stated otherwise, purified calcium release channels and other reagents were added to the cis chamber. Recordings were filtered at 4 kHz with a low-pass Bessel filter, digitized at 40 kHz (sampling rate 25 μs) and stored on the hard disc of a Apple Macintosh (Apple, Cupertino, CA). Single channel events were identified using TAC software (ver 2.5; Skalar Instruments, Inc., Seattle, WA). Mean open probability (P_o) of channels were identified by a 50% threshold analysis. The life times of open and closed events were

determined by the method of maximum likelihood (TACFit software; Skalar Instruments).

[³H]Ryanodine Binding. [³H]Ryanodine binding was measured under equilibrium conditions as described previously (Suko and Hellmann, 1998). Unless stated otherwise, controls and test samples were assayed in duplicate or triplicate for 90 min at 37°C in 0.2 ml of solution containing 40 mM Mops/Tris, pH 7.0, 0.5 M CsCl (or 1 M NaCl), 0.1 mg HSR, 0.5 μg/ml leupeptin, 1.4 μg/ml aprotinin, 0.1 mM PMSF, and 10 nM [³H]ryanodine, 100 μM Ca^{2+} without or with suramin (0.1 to 10 mM). In a few experiments, the above control medium contained, in addition, 5 to 10 mM EGTA, 10 to 20 μM ruthenium red, 1 mM tetracaine, or 1 mM neomycin. In the calcium dependence experiments, free calcium was varied between 20 nM and 50 mM. In the magnesium inhibition experiments, magnesium was varied between 0.25 and 50 mM $MgCl_2$. Nonspecific [³H]ryanodine binding was measured in the presence of 100 μM unlabeled ryanodine. Samples were filtered on glass-fiber filters (presoaked in 1% polyethylene imine) and washed with 10 ml of 20 mM Mops/Tris, pH 7.0, 1 M NaCl.

Protein Assay. Protein was measured by the Folin method and in the presence of detergents plus phosphatidylcholine, according to Kaplan and Pedersen (1985), standardized against bovine serum albumin.

Calculations. Curve fitting was carried out using the standard Maquart-Levenberg algorithm provided by Sigma plot 2 (Jandel, San Rafael, CA). Statistical analysis was carried out by t test using Sigmastat 2 software (Jandel, San Rafael, CA) and for multiple comparisons by ANOVA and Scheffé post hoc comparisons. Averaged results are presented as means ± S.E.M.

Results

Single-Channel Current Recordings. In single-channel experiments, current fluctuations of a single purified and reconstituted skeletal muscle calcium release channel were recorded at 20 to 100 μM free calcium in a 50 to 480 mM CsCl gradient and 0 mV or +20 mV holding potential. Cs^+ was used as current carrier to increase the conductance of the calcium release channel. Calmodulin and the agents suramin and 4-CMPS were added to the *cis* chamber, which corresponded to the cytosolic face of the calcium release channel.

Effect of Suramin at Activating, Subactivating, and Inhibitory Ca^{2+}. The activation of the calcium release channel by suramin was strongly dependent on the concentration of activating free calcium. An example of the activation of a purified calcium release channel by suramin at 100 μM activating Ca^{2+} and 0 mV holding potential is shown in Fig. 1. Suramin (0.3 mM, 0.6 mM and 0.9 mM) increased the open probability in a concentration-dependent manner. In nine experiments, the mean open probability of purified calcium release channels increased from 0.47 to 0.72, 0.82, and 0.87 on addition of 0.3, 0.6, and 0.9 mM suramin, respectively, to the *cis* side (Table 1A). The channel activation by suramin was maximal at about 5 min after the addition of the compound and reversed by a reduction of free Ca^{2+} to subactivating concentrations. Suramin had no effect on the current amplitude, slope conductance, or reversal potential of the calcium release channel. The slope conductance of single calcium release channels with Cs^+ as permeant ion was 542 ± 16 pS and 529 ± 8 pS in the presence of 0.3 to 0.9 mM suramin ($n = 6$; means ± S.E.M.).

The suramin-induced activation of the calcium release channels was similar to the previously reported increase in the open probability by 0.3 mM suramin measured at 20 μM Ca^{2+} and 0 mV holding potential (control, $P_o = 0.40 \pm 0.05$,

suramin: $P_o = 0.67 \pm 0.06$; means ± S.E.M., $n = 5$; Klinger et al., 1999).

It is noted that the relative increase in the open probability by suramin was greater when the activation was started from a low open probability, similarily to previous reports for the activating effect of sulfhydryl oxidation of the calcium release channel (Suko et al., 2000). In five calcium release channels with an average P_o of 0.22 ± 0.04, 0.9 mM suramin increased P_o to 0.84 ± 0.02 (+20 mV holding potential, means ± S.E.M, $n = 5$), which represents an increase of ~4-fold.

Suramin shifted the calcium dependence of channel activation to lower free calcium concentrations, indicating an increase in the apparent calcium affinity to the activating high-affinity calcium binding sites (Table 1). The open probability of the calcium release channel was negligible at subactivating calcium concentrations of 0.6 μM or 0.05 μM free Ca^{2+} (Fig. 2; Table 1). Subsequent addition of 1.2 mM suramin resulted in a small but clearly demonstrable increase in the open probability (Fig. 2C). In five experiments, the mean open probability of the purified calcium release channels decreased from 0.51 (at 50–100 μM Ca^{2+}) to 0.003 (at 0.6 μM Ca^{2+}) and increased significantly to 0.05 after
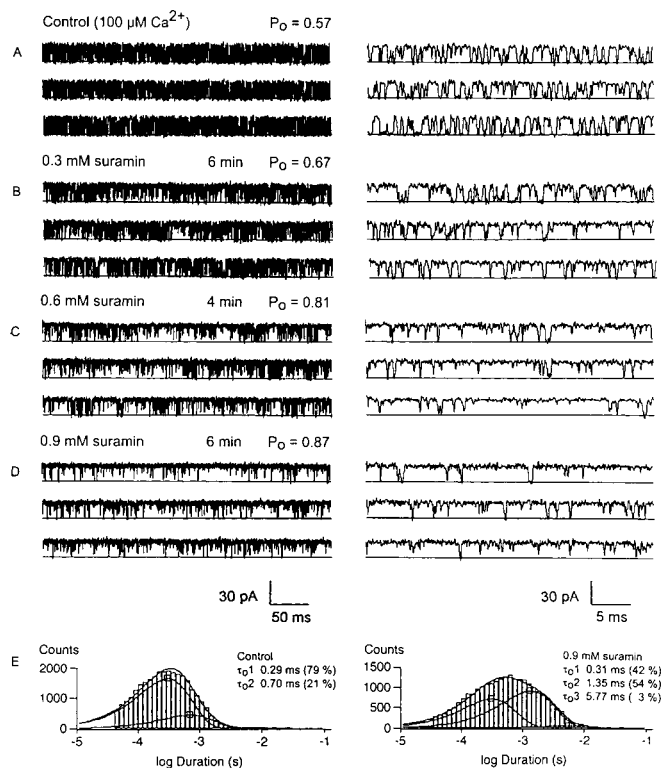

Fig. 1. Concentration dependence of activation of a single purified skeletal muscle calcium-release channel by suramin. Single-channel currents, shown as upward deflections, were recorded at 0 mV holding potential with 480 mM/50 mM CsCl (*cis/trans*). The solid lines indicate the baselines. Ca^{2+} and suramin were added to the *cis* side. Left, 400-ms recordings; right, the first 40 ms of each 400-ms recording. A, control, 100 μM Ca^{2+}. B, activation of the channel by 0.3 mM suramin. C, activation of the channel by 0.6 mM suramin. D, activation of the channel by 0.9 mM suramin. E, open lifetime histograms, cumulative mean open channel time constants (τ_o) and percentage areas for control and 0.9 mM suramin. Calibration bars represent 30 pA and 50 ms or 5 ms. Channel open probabilities (P_o) and τ_o were calculated from 56,000 events (control), 58,000 events (0.3 mM suramin), 51,000 events (0.6 mM suramin), 48,000 events (0.9 mM suramin), respectively.

addition of 1.2 mM suramin (Table 1B). In six experiments, the mean open probability of the purified calcium release channels decreased from 0.54 (at 50–100 μM Ca^{2+}) to 0.0008 (at 0.05 μM Ca^{2+}) and increased significantly to 0.018 after addition of 1.2 mM suramin (Fig. 2C; Table 1C). Judged from the relative increase of the open probability at 0.05 μM free Ca^{2+}, the activation with a high suramin concentration (1.2 mM) seems to be large (about 10-fold); however, the average P_o value was less than 0.02, which is about 25-fold lower than the average P_o value obtained at maximally activating calcium concentrations (i.e., channels remained predominantly in a closed state). In contrast to suramin, oxidation of sulfhydryls by 4-CMPS increased the channel open probability to 0.81 in the presence of 0.05 μM Ca^{2+} and 1.2 mM suramin (Fig. 2D; Table 1C).

In a few experiments, the effect of suramin was tested in the presence of <0.5 nM free calcium (10 μM total calcium plus 5 mM EGTA, pH 7.4; calcium was added before EGTA to assure the presence of an active calcium release channel). Suramin at 1.2 mM (5 min) and 2.4 mM (5 min) added sequentially after EGTA showed no clear channel activation [i.e., the channels remained practically closed (P_o <0.002; n = 3)] and there was no indication of a calcium-independent activation of the purified skeletal muscle calcium release channel at these high suramin concentrations.

The open probability of the calcium release channel activated by 20 to 100 μM Ca^{2+} in the absence of suramin (controls) was about 3-fold reduced on addition of 1 mM Ca^{2+} (from 0.54 $\pm$ 0.06 to 0.15 $\pm$ 0.01, n = 3; means $\pm$ S.E.M.; Suko and Hellmann, 1998). However, in the presence of 0.9 mM suramin, 1 mM Ca^{2+} had no inhibitory effect on P_o, and suramin induced an almost complete channel opening (Fig. 3). In five experiments, the calcium-activated (50 μM Ca^{2+}) calcium release channel (P_o = 0.38 $\pm$ 0.11) was further activated by 0.9 mM suramin (P_o = 0.86 $\pm$ 0.03) and subsequent addition of 1 mM Ca^{2+} increased P_o significantly to 0.95 $\pm$ 0.02 (means $\pm$ S.E.M., n = 5).

TABLE 1
Activation of single purified skeletal muscle calcium-release channels by suramin at 20 to 100 μM activating Ca^{2+} and subactivating calcium concentration (0.6 and 0.05 μM).

Single-channel currents were recorded at 0 mV holding potential (A) or +20 mV holding potential (B, C) with 480 mM/50 mM CsCl (*cis/trans*) and Ca^{2+} as indicated. Control and test records are from the same channel. Suramin was added to the *cis* side. A, activation of single channel current by 20 to 100 μM Ca^{2+} and 0.3, 0.6, and 0.9 mM suramin added sequentially to the *cis* side to give the indicated final concentration. Significantly different (P = 0.05), control vs. 0.3, 0.6, and 0.9 mM suramin; 0.3 mM suramin vs. 0.9 mM suramin (ANOVA). B, activation of single channel current by 1.2 mM suramin at 0.6 μM Ca^{2+} (*cis*). Control (0.6 μM Ca^{2+}) vs. suramin (at 0.6 μM Ca^{2+}) was significantly different (P = 0.03; t test). C, activation of single channel current by 1.2 mM suramin and 1.2 mM suramin plus 80 μM 4-CMPS at 0.05 μM Ca^{2+} (*cis*). Significantly different (P = 0.000001): Control (0.05 μM Ca) vs. suramin or suramin plus 4-CMPS (ANOVA). Values are presented as mean $\pm$ S.E.M. for the number of experiments (n).

		P_o	n
A	Control (20–100 μM Ca^{2+})	0.47 $\pm$ 0.04	9
	0.3 mM Suramin	0.72 $\pm$ 0.03	9
	0.6 mM Suramin	0.82 $\pm$ 0.04	6
	0.9 mM Suramin	0.87 $\pm$ 0.01	6
B	Control (50–100 μM Ca^{2+})	0.51 $\pm$ 0.05	5
	Control (0.6 μM Ca^{2+})	0.003 $\pm$ 0.001	5
	1.2 mM Suramin	0.050 $\pm$ 0.020	4
C	Control (50–100 μM Ca^{2+})	0.54 $\pm$ 0.02	6
	Control (0.05 μM Ca^{2+})	0.0008 $\pm$ 0.0004	6
	1.2 mM Suramin	0.018 $\pm$ 0.004	6
	1.2 mM Suramin + 80 μM 4-CMPS	0.81 $\pm$ 0.03	3

Effect of Suramin on the Calcium-Calmodulin Induced Inhibition. Calcium-CaM inhibited the open probability of a purified calcium release channel in single-channel current recordings (Fuentes et al., 1994; Tripathy et al., 1995; Suko et al., 2000). The effects of suramin on the calmodulin-induced inhibition of the purified calcium release channel were determined at low (0.1 μM) and high (1–5 μM) calmodulin concentrations (Fig. 4). An example for the Ca-calmodulin induced inhibition and reactivation by suramin in the presence of 1 μM calmodulin is shown in Fig. 5. The percentage inhibition of the calcium release channel by 0.1 or 1 to 5 μM CaM was practically the same (about 27% of the controls). CaM (0.1 μM) significantly reduced the mean open duration (T_o), from 0.40 $\pm$ 0.03 ms to 0.18 $\pm$ 0.02 ms (n = 6); 1 to 5 μM CaM reduced the T_o from 0.39 $\pm$ 0.03 ms to 0.17 $\pm$
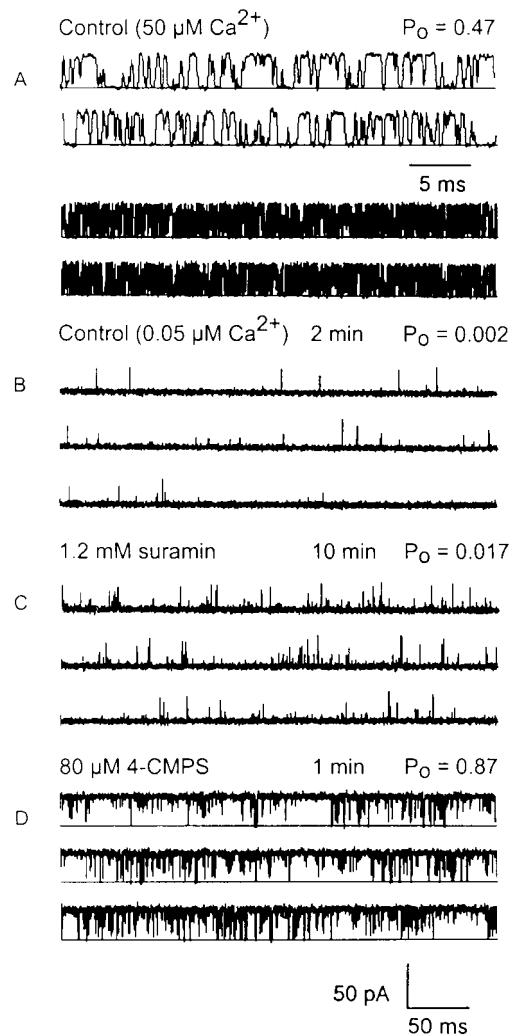

Fig. 2. Activation of a single purified skeletal muscle calcium-release channel by suramin at subactivating free calcium. Single-channel currents, shown as upward deflections, were recorded at +20 mV holding potential with 480 mM/50 mM CsCl (*cis/trans*). The solid lines indicate the baselines. Control and test records are from the same channel. Ca^{2+}, EGTA, suramin, and 4-CMPS were added sequentially to the *cis* side. A, control, 100 μM Ca^{2+}. B, control (0.05 μM Ca^{2+}). C, activation of the channel by 1.2 mM suramin. D, activation of the channel by 80 μM 4-CMPS in the presence of 1.2 mM suramin. Calibration bars represent 50 pA and 5 ms (A) or 50 ms (A-D). Channel open probabilities (P_o) were calculated from 42,000 events (control, 100 μM Ca), 1,200 events (control, 0.05 μM Ca), 9,600 events (suramin), and 19,000 events (suramin + 4-CMPS), respectively.

0.01 ms ($n = 5$; means $\pm$ S.E.M). The distribution of the open and closed lifetimes (Fig. 5) were similar in low and high calmodulin concentrations and identical to those reported previously (Suko et al., 2000).

In the presence of low concentrations of CaM (0.1 μM), the CaM-induced inhibition of the calcium release channel was completely reversed by suramin. In six experiments, 0.1 μM CaM reduced the open probability from 0.51 to 0.14, and 0.3, 0.6, and 0.9 mM suramin increased P_o to 0.58, 0.78, and 0.90, respectively (Fig. 4); i.e., 0.3 mM suramin increased the open probability to control levels seen in the absence of CaM, and 0.9 mM suramin induced a nearly maximal channel opening, similar to that observed in the absence of CaM (Fig. 1; Table 1). Statistical analysis of five experiments with suramin showed that 1 to 5 μM CaM reduced the open probability from 0.51 to 0.14 and 0.3, 0.6, and 0.9 mM suramin increased P_o to 0.50, 0.69, and 0.83, respectively (range, 0.68–0.88); i.e., 0.3 mM suramin increased the open probability to an extent similar to that observed at the low CaM concentration. There was a tendency that 0.6 mM and 0.9 mM suramin caused less activation in the presence of 1 to 5 μM CaM compared with 0.1 μM CaM (Fig. 4), but the difference was not statistically significant. Addition of 7 μM ruthenium red, the specific inhibitor of the calcium release channel, completely closed the calmodulin-inhibited and suramin-reactivated channels (data not shown). Suramin or calmodulin plus suramin had no effect on the current amplitude or conductance of the calcium release channel with Cs^+ as permeant ion (Fig. 5G).

The increase in the open probability of the suramin-stimulated channels in controls, starting with an open probability of about 0.5, was caused mainly by an increase in the dura-

tion of the open lifetimes. In control experiments, the open and closed lifetimes of channels recorded at 0 mV or +20 mV holding potential were best fitted by the sum of two exponential values. In the presence of suramin the best fit of the open lifetimes was obtained by the sum of three to four exponential values as illustrated in Figs. 1 and 3.

In the presence of CaM, which caused a reduction in the frequency of channel opening, a significant decrease of the channel open lifetimes (τ_o) and significantly prolonged closed lifetimes (Fig. 5; Suko et al., 2000), suramin increased the open probability at low concentrations by an increase in the frequency and duration of channel openings and at a higher P_o value, predominantly by an increase in the open lifetimes (Fig. 5). The shift to $\tau_o 2$ and $\tau_o 3$ open states by suramin was similar in the presence of 0.1 or 1 to 5 μM CaM.

Similar to the effect described in Fig. 2D, the submaximally activated calcium release channel (in the presence of 1 or 5 μM CaM and 0.9 mM suramin) was further activated by addition of the sulfhydryl oxidizing organic mercurial compound 4-CMPS. Subsequent addition of 100 μM 4-CMPS induced a fully open state ($P_o = 0.98$) within 45 s over a period of about 4 min followed by channel inhibition. 4-CMPS (100–200 μM) caused an almost complete closure of suramin-activated calcium release channels ($P_o < 0.005$; data not shown).

Effect of Magnesium in the Absence and Presence of Suramin. The inhibitory effect of magnesium on a purified calcium release channel activated by 40 to 100 μM Ca^{2+} (in
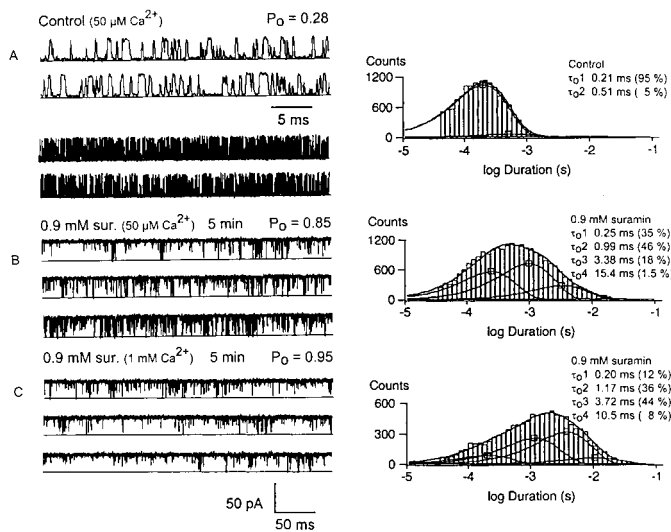

Fig. 3. Activation of a single purified skeletal muscle calcium-release channel by suramin at 50 μM and 1 mM Ca^{2+}. Single-channel currents, shown as upward deflections, were recorded at +20 mV holding potential with 480 mM/50 mM CsCl (*cis/trans*). The solid lines indicate the baselines. Control and test records are from the same channel. Left, 40- and 400-ms recordings. Suramin and 1 mM Ca^{2+} were added sequentially to the *cis* side. A, control, 50 μM Ca^{2+}. B, activation of the channel by 0.9 mM suramin. C, activation by addition of 1 mM Ca^{2+}. Calibration bars represent 50 pA and 50 ms (A-C) or 5 ms (A). Right, open lifetime histograms, cumulative mean open channel time constants (τ_o), and percentage areas. The solid lines represent a fit according to two (control) and four exponential values (suramin). Channel open probabilities (P_o) and τ_o were calculated from 28,000 events (control), 45,000 events (0.9 mM suramin), 21,000 events (0.9 mM suramin plus 1 mM Ca^{2+}), respectively.
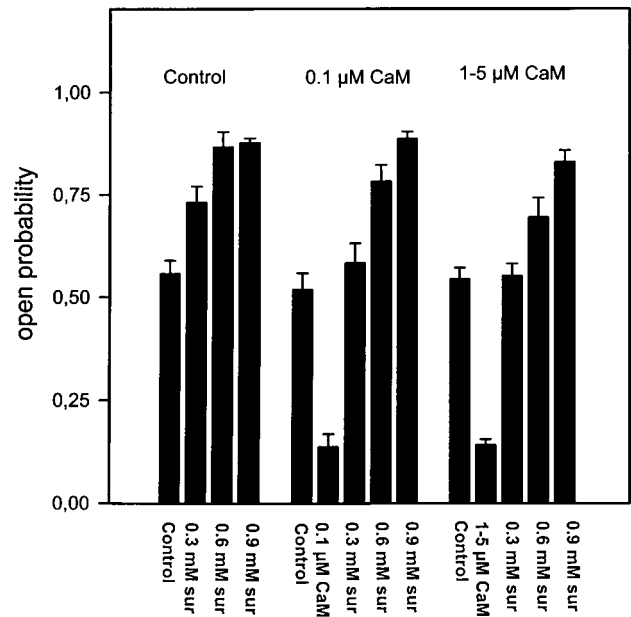
Fig. 4. Inhibition of a single purified skeletal muscle calcium-release channel by 0.1 to 5 μM calmodulin and reactivation by suramin. Single-channel currents were recorded at 0 mV holding potential with 480 mM/50 mM CsCl (*cis/trans*) at 50 to 100 μM Ca^{2+}. CaM (0.1 μM CaM, 1 μM or 5 μM) and suramin (0.3, 0.6, and 0.9 mM) were added sequentially to the *cis* side. Control and test recordings are from the same channel. Values are means $\pm$ S.E.M. from four to six experiments in each group. Channel open probabilities (P_o) were calculated from 30,000 to 77,000 events. Significantly different ($P = 0.05$): control versus 0.1 μM CaM; 0.1 μM CaM versus 0.3, 0.6, and 0.9 mM suramin; 0.1 μM CaM + 0.3 mM suramin versus 0.1 μMCaM + 0.9 mM suramin. Control versus 1 to 5 μM CaM; 1 to 5 μM CaM versus 0.3, 0.6, and 0.9 mM suramin; 1 to 5 μM CaM + 0.3 mM suramin versus 1 to 5 μM CaM + 0.6 mM or 0.9 mM suramin (ANOVA).
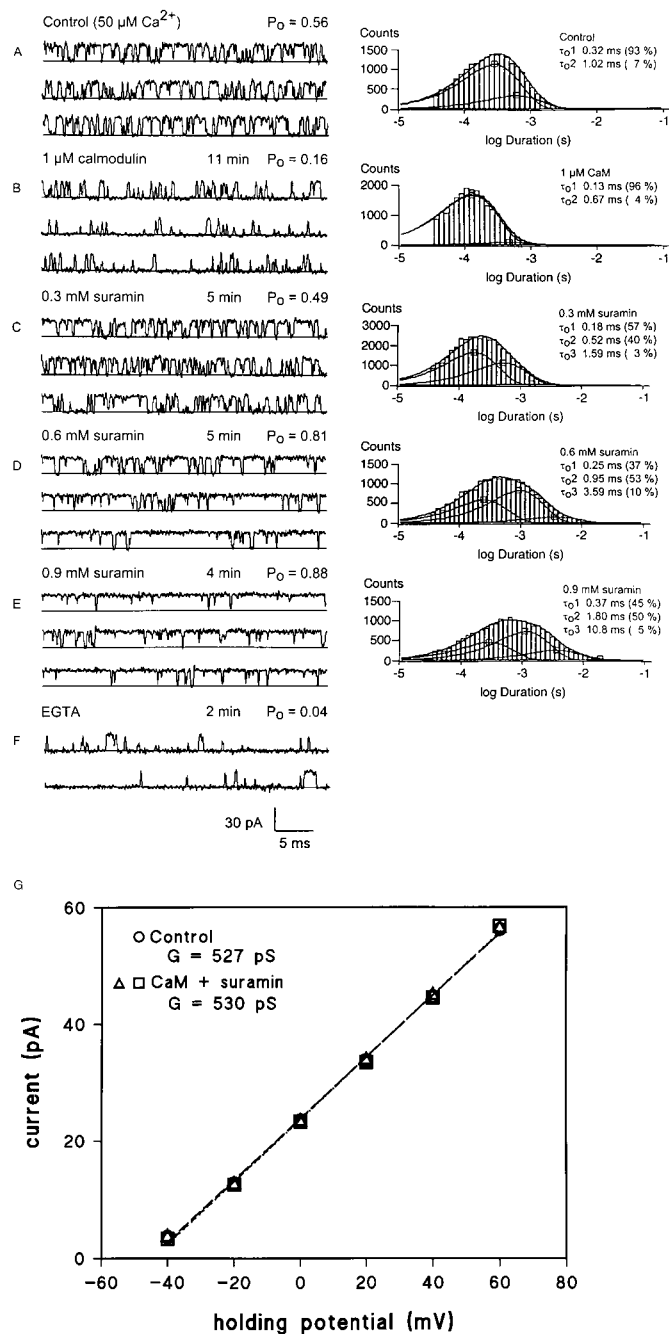
Fig. 5. Inhibition of a single purified skeletal muscle calcium-release channel by 1 μM calmodulin and reactivation by suramin. Single-channel currents, shown as upward deflections, were recorded at 0 mV holding potential with 480 mM/50 mM CsCl (*cis/trans*). The solid lines indicate the baselines. Ca^{2+} (50 μM) and 1 μM CaM and suramin were added sequentially to the *cis* side. Left, 40-ms recordings. Right, open lifetime histograms, cumulative mean open channel time constants (τ_o) and percentage areas. The solid lines represent a fit according to two to four exponential values. A, control, 50 μM Ca^{2+}. B, inhibition of the channel by 1 μM CaM. C, activation of the channel by 0.3 mM suramin. D, activation of the channel by 0.6 mM suramin. E, activation of the channel by 0.9 mM suramin. F, addition of EGTA (free Ca^{2+} 0.05 μM). Calibration bars represent 30 pA and 5 ms. Channel open probabilities (P_o) and τ_o were calculated from 41,000 events (control), 42,000 (CaM), 77,000 events (0.3 mM suramin), 44,000 events (0.6 mM suramin), 42,000 events (0.9 mM suramin), respectively. G, single-channel current-voltage relationship for control and CaM plus suramin. Slope conductance: control, 527 pS (reversal potential, −45 mV); 1 μM CaM plus 0.6 mM suramin ($\triangle$), 530 pS (reversal potential, −44.4 mV); 1 μM CaM plus 0.9 mM suramin ($\square$), 524 pS (reversal potential, −44.6 mV).

the absence of suramin) is shown in Fig. 6. Half-maximum inhibition of the open probability was obtained at 0.38 mM Mg^{2+}. Mean channel open and closed duration and open and closed time constants in the presence of 1 mM magnesium are shown in Table 2.

With calcium as the sole channel activator, magnesium affected the open probability predominantly by a reduction in the frequency of channel opening. The mean closed channel duration (T_c) was significantly prolonged and the closed time constants were significantly shifted to longer closed times (τ_c2, τ_c3) in the presence of 1 mM Mg^{2+} (Table 2). In addition, the magnesium-inhibited channels showed only short openings. The mean open channel duration (T_o) and the τ_o1 open time constant were significantly shortened (Table 2). These effects of magnesium on the open and closed time distribution were similar to those seen in the presence of calcium-calmodulin (present study and Suko et al., 2000).

The effect of suramin on the calcium release channel in the presence of magnesium was determined under several experimental conditions (Table 3), because of the lack of inhibition of high magnesium concentrations on [3H]ryanodine binding to HSR as shown below. 1) The calcium activated (50 μM Ca^{2+}) calcium release channel was further activated by 0.9 mM suramin and inhibited by sequential addition of 4 and 10 mM Mg^2 (Fig. 7; Table 3A). 2) Activation of the calcium release channel by suramin and inhibition by magnesium were repeated with higher concentrations of suramin and magnesium (Fig. 8; Table 3B). Suramin-activated, magnesium-inhibited calcium release channels could be reactivated by higher suramin concentrations and again inhibited by increasing the magnesium concentration. In six experiments, 0.9 mM suramin increased the open probability of the calcium release channel from 0.56 to 0.87 and subsequent ad-
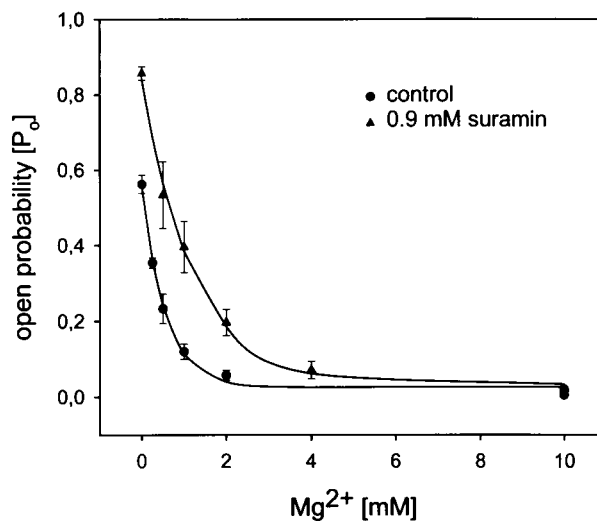

Fig. 6. Inhibition of the open probability of single purified skeletal muscle calcium-release channels by Mg^{2+} in the absence and presence of suramin. Single-channel currents were recorded at +20 mV holding potential with 480 mM/50 mM CsCl (*cis/trans*). Open probabilities for inhibition of the suramin-activated channels by Mg^{2+} include data from Table 3, A to C. Magnesium inhibition of controls includes data derived in experiments given in Table 3C. Also see text. Data points are means ± S.E.M. The mean values for controls and suramin in the absence and presence of 0.5, 1, and 2 mM Mg^{2+} were significantly different ($P < 0.05$). The solid lines represent a fit according to a single exponential decay of P_o. Half-maximal inhibition of P_o by magnesium occurred at 0.38 mM (control) and 0.83 mM (suramin).

dition of 1 mM Mg^{2+} reduced P_o to 0.40; subsequent addition of suramin (2.4 mM total) increased P_o to 0.68 and 4 mM and 10 mM Mg^{2+} (added sequentially) reduced P_o again to 0.15 and 0.017, respectively (the evaluation of P_o was derived from recordings 2 to 5 min after addition of magnesium), (Fig. 8; Table 3B). 3) The calcium release channel activated by 50 to 100 μM Ca^{2+} was inhibited by 2 mM magnesium and subsequently reactivated by 0.9 to 1.5 mM suramin (Table 3C). Channel open probability was reduced more than 10-fold by addition of 2 mM magnesium to the *cis* side, and sequential addition of 0.9 and 1.5 mM suramin to the *cis* bath resulted in a concentration-dependent activation of the calcium release channel (P_o increased significantly from 0.045

to 0.35 with 1.5 mM suramin); i.e., the open probability remained below the control values observed in the absence of magnesium (Table. 3C). 4) In a few experiments, the magnesium inhibition of the suramin activated calcium release channel was measured by sequential addition of magnesium ranging from 0.5 to 10 mM (similar to effects observed in control samples in the absence of suramin; Fig. 6).

Suramin shifted the magnesium inhibition of the open probability of the calcium release channel to higher magnesium concentrations (Figs. 6 and 8). Half-maximum inhibition of the suramin-induced channel activation (0.9 mM) was obtained at 0.82 mM Mg^{2+} (Fig. 6). Furthermore, 10 mM Mg^{2+} inhibited the open probability of the suramin (0.9 mM

TABLE 2
Mean open probability, mean current amplitude, and mean open and closed lifetimes in controls and in the presence of 1 mM Mg^{2+}

Single-channel currents were recorded at +20 mV voltage holding potential with 480 mM/50 mM CsCl (*cis/trans*). Control and test records are from the same channel. Channel open probabilities (P_o), mean current amplitude (pA), mean channel open (T_o) and closed (T_c) duration (ms), cumulative mean open and closed channel time constants (τ_o, τ_c) and percentage of area represented by a time constant for purified calcium release channels activated by 50 to 100 μM *cis* Ca^{2+} and in the presence of 1 mM Mg^{2+} added to the *cis* chamber. Values are presented as means $\pm$ S.E.M. for the indicated number (n) of channels included in the analysis.

	Control $n = 10$	1 mM Mg^{2+} $n = 10$	
		area %	
P_o	0.51 $\pm$ 0.04	0.10 $\pm$ 0.02[d]	
Amplitude (pA)	31.1 $\pm$ 0.7	29.1 $\pm$ 1.0 N.S.	
Mean T_o (ms)	0.41 $\pm$ 0.03	0.21 $\pm$ 0.03[c]	
Mean T_c (ms)	0.40 $\pm$ 0.05	2.74 $\pm$ 0.58[c]	
$\tau_o 1$ (ms)	0.31 $\pm$ 0.02 (82 $\pm$ 2)	0.15 $\pm$ 0.02[d]	(94 $\pm$ 2)[b]
$\tau_o 2$ (ms)	0.71 $\pm$ 0.05 (18 $\pm$ 2)	0.54 $\pm$ 0.05[a]	(6 $\pm$ 2)[b]
$\tau_c 1$ (ms)	0.28 $\pm$ 0.02 (84 $\pm$ 9)	0.72 $\pm$ 0.20[a]	(17 $\pm$ 4)[d]
$\tau_c 2$ (ms)	1.27 $\pm$ 0.50 (19 $\pm$ 10)	2.80 $\pm$ 0.59 N.S.	(60 $\pm$ 8)[b]
$\tau_c 3$ (ms)		6.81 $\pm$ 1.34	(23 $\pm$ 9)

N.S., not significant.
[a] $P < 0.05$.
[b] $P < 0.01$.
[c] $P < 0.001$.
[d] $P < 0.0001$.

TABLE 3
Effect of magnesium on the suramin-induced activation of the purified calcium release channel.

Single-channel currents were recorded at +20 mV holding potential with 480 mM/50 mM CsCl (*cis/trans*) in the presence of 50 to 100 μM Ca^{2+} (*cis*). Control and test records are from the same channel. Suramin or magnesium was added sequentially to the *cis* bath to give the indicated final concentrations. A, activation of single purified calcium release channel by 0.9 mM suramin and inhibition by 4 mM and 10 mM Mg^{2+}. Significantly different ($P = 0.05$): 0.9 mM suramin vs. 0.9 mM suramin plus 4 mM Mg^{2+} or 10 mM Mg^{2+}; 0.9 mM suramin plus 4 mM Mg^{2+} vs. 0.9 mM suramin plus 10 mM Mg^{2+} (ANOVA). B, activation of a single purified calcium release channel by 0.9 mM suramin, inhibition by 1 mM Mg^{2+}, reactivation by 2.4 mM suramin followed by inhibition with 4 and 10 mM Mg^{2+}. C, inhibition of a single calcium release channel by 2 mM Mg^{2+} and reactivation by 0.9 to 1.5 mM suramin. Significantly different ($P = 0.05$): 2 mM Mg^{2+} vs. 2 mM Mg^{2+} plus 1.5 mM suramin (ANOVA). Values are presented as means $\pm$ S.E.M. for the number of experiments (n).

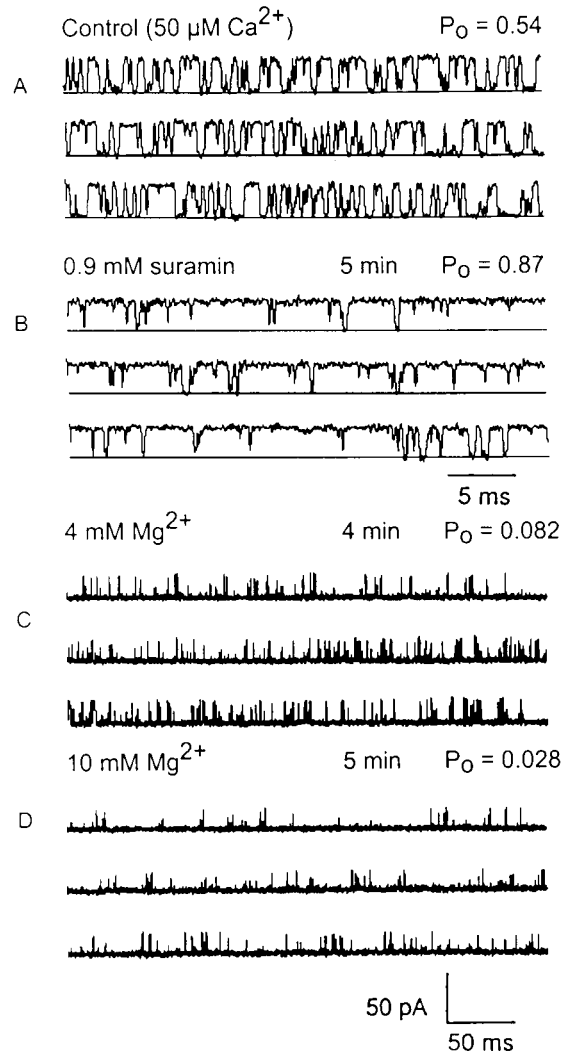|		P_o	n
A	Control (50 μM Ca^{2+})	0.48 $\pm$ 0.06	7
	0.9 mM Suramin	0.84 $\pm$ 0.03	7
	4 mM Mg^{2+}	0.064 $\pm$ 0.020	7
	10 mM Mg^{2+}	0.019 $\pm$ 0.004	5
B	Control (50 μM Ca^{2+})	0.56 $\pm$ 0.04	6
	0.9 mM Suramin	0.87 $\pm$ 0.02	6
	1 mM Mg^{2+}	0.40 $\pm$ 0.07	6
	2.4 mM Suramin	0.68 $\pm$ 0.09	4
	4 mM Mg^{2+}	0.15 $\pm$ 0.05	4
	10 mM Mg^{2+}	0.017 $\pm$ 0.009	2
C	Control (50–100 μM Ca^{2+})	0.56 $\pm$ 0.05	5
	2 mM Mg^{2+}	0.045 $\pm$ 0.010	5
	0.9 mM Suramin	0.18 $\pm$ 0.08	5
	1.5 mM Suramin	0.33 $\pm$ 0.02	3

Fig. 7. Inhibition of a suramin-activated single purified skeletal muscle calcium-release channel by 4 and 10 mM Mg^{2+}. Single-channel currents, shown as upward deflections, were recorded at +20 mV holding potential with 480 mM/50 mM CsCl (*cis/trans*). The solid lines indicate the base-lines. Controls and test records are from the same channel. Ca^{2+} (50 μM), 0.9 mM suramin, 4 and 10 mM Mg^{2+} were added sequentially to the *cis* side; 40-ms (control, suramin) and 400-ms (magnesium) recordings. A, control, 50 μM Ca^{2+}. B, activation of the channel by 0.9 mM suramin. C, inhibition of the channel by 4 mM Mg^{2+}. D, inhibition by 10 mM Mg^{2+} (P_o was 0.013 with 20 mM Mg^{2+}, not shown). Calibration bars represent 50 pA and 5 ms (A, B) or 50 ms (C, D). Channel open probabilities (P_o) were calculated from 42,000 events (control), 33,000 events (suramin), and 12,000 to 33,000 events (magnesium), respectively.

or 2.4 mM) -activated calcium release channels to about 0.02 (i.e., the channels were predominantly closed).

Suramin activation of calcium release channels was characterized by an increase in the frequency of channel opening and an increase in the mean open channel duration and a shift from $\tau_o 1/\tau_o 2$ to $\tau_o 2/\tau_o 3/\tau_o 4$ open states (Table 4). Characteristic of the inhibition of suramin activated channels by magnesium was a decline in the mean channel open duration (T_o) and a decline in long open states [with only $\tau_o 1$ to $\tau_o 3$
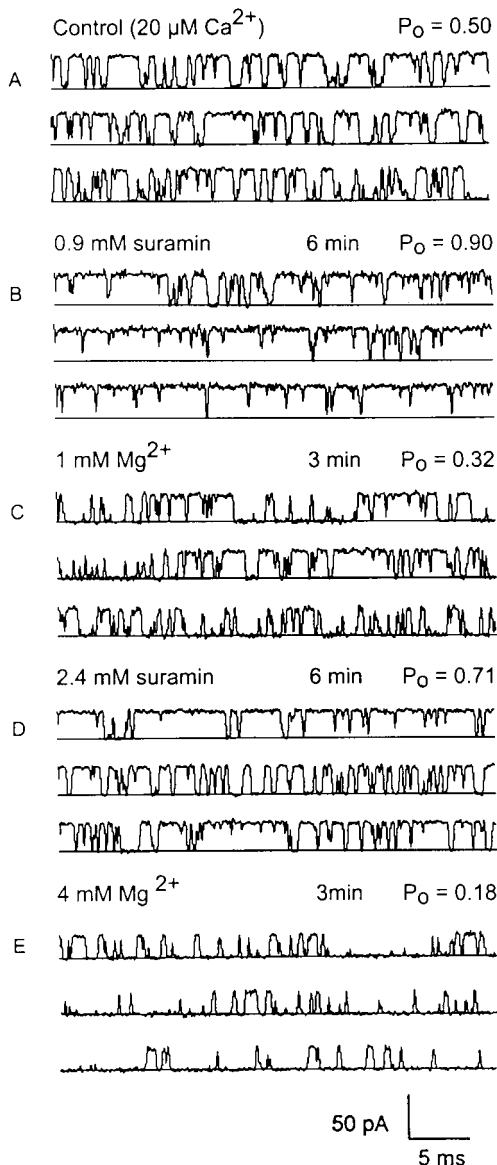

Fig. 8. Inhibition of a suramin-activated single purified skeletal muscle calcium-release channel by 1 mM Mg^{2+}, reactivation by 2.4 mM suramin and inhibition by 4 mM Mg^{2+}. Single-channel currents, shown as upward deflections, were recorded at +20 mV holding potential with 480 mM/50 mM CsCl (*cis/trans*). The solid lines indicate the baselines. Control and test records are from the same channel. 400-ms recordings. Ca^{2+} (20 μM), 0.9 mM suramin, 1 mM Mg^{2+}, 2.4 mM suramin (total), and 4 mM Mg^{2+} (total) were added sequentially to the *cis* side. A, control, 20 μM Ca^{2+}. B, activation of the channel by 0.9 mM suramin. C, inhibition by 1 mM Mg^{2+}. D, reactivation of the channel by 2.4 mM suramin (total). E, inhibition of the channel by 4 mM Mg^{2+}. Calibration bars represent 50 pA and 5 ms. Channel open probabilities (P_o) were calculated from 40,000 events (control), 24,000 to 37,000 (suramin), 39,000 to 61,000 events (magnesium), respectively.

open states at 1 mM and 4 mM Mg^{2+} and only $\tau_o 1$ and $\tau_o 2$ open states at 10 mM Mg^{2+} (Table 4)]. In only one of seven experiments with channel activation by 0.9 mM ($n = 5$) or 2.4 mM ($n = 2$) suramin followed by inhibition with 10 mM Mg^{2+} could a $\tau_o 3$ open state be fitted within 3 to 5 min after addition of magnesium ('short-term experiments'). The reduction of the current amplitude by magnesium in suramin activated channels was not affected.

[³H]Ryanodine Binding. [³H]Ryanodine binding to HSR vesicles was carried out at equilibrium conditions in 90-min assays at 37°C in the presence of 40 mM Mops/Tris, pH 7.0, 1 M NaCl or 0.5 M CsCl, 10 nM [³H]ryanodine, 100 μM Ca^{2+}, and protease inhibitors or at free calcium concentrations ranging from 0.02 μM to 50 mM or in the presence of 0.001 to 50 mM Mg^{2+}. Cs^+ (0.5 M) was used in the calcium dependence and magnesium inhibition assays to keep the conditions in [³H]ryanodine binding as close as possible to the conditions used in single-channel current experiments (0.48 M Cs^+ *cis*).

The concentration dependence of suramin on specific [³H]ryanodine binding (performed with 10 nM [³H]ryanodine, 1 M NaCl, 100 μM Ca^{2+}) in the presence of three different free calcium concentrations is illustrated in Fig. 9. The activation of [³H]ryanodine binding by suramin was strongly dependent on the free calcium concentration. A reduction of the free calcium concentration from 100 μM to 4 μM and 0.5 μM shifted the binding curves to the right. The EC_{50} for suramin increased from 0.11 mM to 0.24 mM and 2.22 mM suramin at 100 μM Ca^{2+}, 4 μM Ca^{2+}, and 0.5 μM Ca^{2+}, respectively ($n = 3$). The maximum [³H]ryanodine binding to HSR in the presence of 100 μM Ca^{2+} was observed at about 1 mM suramin, which was very similar to the maximally stimulatory effect in single-channel current recordings. When the suramin concentrations were increased to 5 to 10 mM, the stimulatory effect on [³H]ryanodine binding declined in the presence of 100 μM and 4 μM Ca^{2+} (Fig. 9).

Fig. 10 shows the effect of suramin on [³H]ryanodine binding in the presence of 100 μM activating Ca^{2+} and increasing magnesium concentrations carried out in the presence of 0.5 M CsCl. Ca^{2+} (100 μM) and the indicated magnesium concentrations were present from the start of the incubation. The inhibitory effect of magnesium on [³H]ryanodine binding was very markedly reduced in the presence of 1 mM suramin. With 0.5 M Cs^+, half-maximum inhibition of [³H]ryanodine binding by magnesium occurred at 0.30 mM Mg^{2+} in the absence of suramin and 83 mM Mg^{2+} in the presence of suramin ($n = 3$).

Fig. 11 shows the calcium dependence of [³H]ryanodine binding to HSR vesicles in the absence and presence of 1 mM suramin (performed with 10 nM [³H]ryanodine in the presence of 0.5 M CsCl). Suramin (1 mM) shifted the calcium activation curve to the left. The apparent K_a values in the presence of suramin (0.46 μM) were significantly lower than in controls (1.60 μM), indicating that suramin increased the apparent affinity of the high-affinity calcium activation sites (Fig. 11). Furthermore, the activation curve in the presence of suramin was steeper (Hill coefficient, 3.76) than in the absence of suramin (Hill coefficient, 2.86). Suramin (1 mM) shifted the calcium inhibition curve to the right. The apparent K_i value for suramin (48,153 μM) was markedly higher than for controls (1,175 μM), indicating that suramin decreased the apparent affinity of the calcium inactivation sites

(Fig. 11). The apparent K_a and K_i values in controls determined in the presence of 0.5 M CsCl in the present study were somewhat lower than those obtained previously in the presence of 1 M NaCl (4.5 μM and 7,356 μM; Suko and Hellmann, 1998), which is most probably because of the lower ionic strength and less inhibition with CsCl than with NaCl (Meissner et al., 1997).

Inhibition of total [³H]ryanodine binding to HSR in the absence or presence of 1 mM suramin (performed with 100 μM CaCl₂, 0.5 M CsCl, and 10 nM [³H]ryanodine) by various agents is given in Table 5. Nonspecific [³H]ryanodine binding (ryanodine binding in the presence of excess unlabeled ryanodine) was not significantly different in the presence and absence of suramin and about 0.5 to 1% of total [³H]ryanodine binding. In controls, zero free calcium and 10 μM ruthenium red reduced [³H]ryanodine binding to the level of nonspecific binding; 50 mM Ca^{2+} or 50 mM Mg^{2+} reduced [³H]ryanodine binding by 99.5%, 200 μM 4-CMPS reduced binding by 95%, and 1 mM neomycin or 1 mM tetracaine by more than 90%. Suramin (1 mM) approximately doubled the amount of [³H]ryanodine bound to HSR. The inhibition of the suramin-stimulated ryanodine binding was greatest by 4-CMPS (98%); the inhibition with ruthenium red was incomplete (94%), whereas 50 mM Ca^{2+} or 50 mM Mg^{2+} caused a reduction of only about 40 to 47%. Tetracaine or neomycin (1 mM) had only a small inhibitory effect in the presence of suramin (Table 5).

Long-Term Effect of Suramin. Long-term effects of suramin (0.9 mM or 2.4 mM) were determined in the presence of high concentrations of magnesium or calcium (10 to 20 mM) as illustrated in Figs. 12 to 14. Conditions were similar to those in Fig. 7; alternatively, the calcium-activated calcium release channel was inhibited by 10 mM Mg^{2+}, reactivated by suramin about 3 min later, and the time course of activation was observed over a period of 30 to 60 min (Fig. 12). These latter conditions were similar to those used in [³H]ryanodine binding, when magnesium and suramin were simultaneously present at the start of the incubation with HSR.

When the calcium-activated release channel was inhibited by 10 mM Mg^{2+} and reactivated by 2.4 mM suramin, the open probability increased from 0.008 ± 0.005 (10 mM Mg^{2+}) to 0.016 ± 0.050 within about 3 min and to 0.208 ± 0.053 within 30 to 45 min (means ± S.E.M., $n = 3$) with the appearance of long open states (τ_o3/τ_o4 states) (Fig. 12). Long time exposure to 2.4 mM suramin plus 20 mM Mg^2 (Fig. 13) resulted in a similar time-dependent increase in the open probability within about 30 min, but the open probability was less than 10%; the reappearance of τ_o3/τ_o4 open states is shown in Fig. 13D. These experiments show that suramin caused a slow, time-dependent alteration in channel gating in the presence of very large inhibitory magnesium concentrations. Notably, in these long-term experiments, a marked fluctuation in the open probability occurred (determined from several 20-s periods) when long open states appeared. Long incubation periods with 0.9 mM suramin in the presence of 10 to 15 mM calcium gave results similar to those obtained with high magnesium concentrations (Fig. 14).

Discussion

The main points of the suramin-induced functional alterations of the skeletal muscle calcium release channel shown in the present study (and not reported in previous studies with suramin) are: the slowly developing channel activation in the presence of inhibiting concentrations of magnesium and calcium ('long-term effect' of suramin); the apparent dissociation of inhibition of the open probability in single-channel current recordings and [³H]ryanodine binding to HSR in the presence millimolar concentrations of magnesium or calcium; the reversal of the calcium-calmodulin induced inhibition by suramin in single-channel current recordings; and the additive activation of P_o by suramin and sulfhydryl oxidation.

Short- and Long-Term Suramin-Induced Channel Activation. At calcium concentrations that cause maximal channel activation (20–100 μM Ca^{2+}) or in the presence of 1 mM Ca^{2+} (which inhibits the calcium release channel in the absence of suramin), 0.9 to 1.0 mM suramin caused a marked activation of the purified skeletal muscle calcium release channel (P_o up to 0.9) or a nearly full open state of the channel ($P_o = 0.95$) within a few minutes after addition of

TABLE 4
Mean open probability, mean current amplitude, and mean open and closed lifetimes of controls, 0.9 mM suramin, and 0.9 mM suramin plus 1, 4, and 10 mM Mg^{2+}

Single-channel currents were recorded at +20 mV voltage holding potential with 480 mM/50 mM CsCl *cis/trans*. Channel open probabilities (P_o), mean current amplitude (pA), mean channel open (T_o) and closed (T_c) duration (ms), cumulative mean open and closed channel time constants (τ_o, τ_c) and percentage of area represented by a time constant for purified calcium release channels activated by 50 to 100 μM *cis* Ca^{2+} and 0.9 mM suramin and inhibited by 1, 4, and 10 mM Mg^{2+} (*cis*). Values are presented as means ± S.E.M. Values in parentheses are area %.

	Control ($n = 13$)	0.9 mM Suramin ($n = 13$)	0.9 mM Suramin + 1 mM Mg^{2+} ($n = 6$)	0.9 mM Suramin + 4 mM Mg^{2+} ($n = 7$)	0.9 mM Suramin + 10 mM Mg^{2+} ($n = 5$)
			area %		
P_o	0.53 ± 0.03[a]	0.86 ± 0.01[a,b]	0.40 ± 0.07[b,c]	0.065 ± 0.020[a,b,c]	0.019 ± 0.04[a,b,c]
Amplitude (pA)	31.6 ± 0.6[a]	30.7 ± 0.9[b]	27.2 ± 0.7[a,c]	20.8 ± 0.6[a,b,c]	18.9 ± 1.7[a,b,c]
Mean T_o (ms)	0.419 ± 0.026[a]	1.53 ± 0.12[a,b]	0.50 ± 0.07[b]	0.27 ± 0.05[b]	0.26 ± 0.02[b]
Mean T_c (ms)	0.383 ± 0.039[a]	0.25 ± 0.02[b]	0.84 ± 0.17[a,b]	5.03 ± 0.86[a,b,c]	11.5 ± 1.7[a,b,c]
τ_o1 (ms)	0.32 ± 0.02 (85 ± 2)	0.26 ± 0.01 (21 ± 3)*	0.19 ± 0.01 (60 ± 3)	0.12 ± 0.01 (77 ± 5)	0.07 ± 0.03 (74 ± 8)
τ_o2 (ms)	0.85 ± 0.09 (15 ± 2)	1.05 ± 0.14 (41 ± 3)*	0.65 ± 0.07 (37 ± 2)	0.48 ± 0.07 (21 ± 3)	0.31 ± 0.12 (24 ± 7)
τ_o3 (ms)		3.35 ± 0.50 (30 ± 4)*	2.59 ± 0.26 (3 ± 1)	2.10 ± 0.79 (3 ± 1)	
τ_o4 (ms)		11.4 ± 2.3 (4 ± 0.9)*			
τ_c1 (ms)	0.27 ± 0.02 (87 ± 7)	0.15 ± 0.01 (91 ± 2)	0.54 ± 0.30 (47 ± 4)	0.36 ± 0.07 (25 ± 2)	0.37 ± 0.11 (24 ± 6)
τ_c2 (ms)	1.28 ± 0.50 (19 ± 10)	0.88 ± 0.19 (9 ± 2)	0.94 ± 0.20 (47 ± 3)	3.00 ± 0.32 (53 ± 2)	6.6 ± 1.9 (47 ± 5)
τ_c3 (ms)		3.44 ± 0.82 (6 ± 2)	12.6 ± 1.8 (23 ± 2)	42.5 ± 14 (29 ± 3)	

Superscript letters indicate significant differences between the groups ($P = 0.05$; ANOVA).
* A four-exponential fit with τ_o4 >1% was obtained in 10 of 13 experiments [three-exponential fit ($n = 13$): $\tau_o1 = 0.35 ± 0.04$ (41 ± 2), $\tau_o2 = 1.53 ± 0.15$ (50 ± 2), $\tau_o3 = 6.19 ± 0.76$ (9 ± 1%)].

suramin. Characteristic of these increases in P_o was a 3.5-fold increase in the mean open duration ($T_o = 0.41$ ms versus 1.46 ms) with a shift to τ_o3/τ_o4 open states (Table 4). At a P_o of 0.95 (1 mM Ca^{2+}), the percentage of τ_o3/τ_o4 states increased (Fig. 3). Inhibition of the suramin-activated channels with 10 mM magnesium reduced P_o, T_o, and eliminated τ_o3/τ_o4 open states (Table 4). The activating effect of suramin on the skeletal and cardiac calcium release channel of HSR was shown to be reversed when suramin was removed from the *cis* chamber by perfusion (Sitsapesan and Williams, 1996).

A time-dependent, slowly developing activation of the calcium-release channel by suramin was observed in the presence of strongly inhibitory magnesium or calcium concentrations of 10 to 20 mM on suramin exposure over long time periods (Figs. 12–14). Characteristic of this so-called 'long-term effect' of suramin was: 1) The appearance of long open states of low frequency (τ_o3/τ_o4 open states) at an overall low open probability (Figs. 13 and 14). 2) Channel current fluctuations between higher and lower open probability caused by changes in the frequency of the long open states. The cause of the fluctuation in P_o under these conditions is unknown; it may be caused by an instability in channel gating and/or pore formation. The best characterization of the long-term effects of suramin is therefore obtained from the distribution of the open lifetimes (a slight fluctuation in P_o was

also observed in short time experiments) because P_o represents an average of short and long open events, but gives no information on the appearance of long open states. 3) No effect on the reduction in the current amplitude by high Mg^{2+} or Ca^{2+}. 4) In all 'long-term experiments' carried out for up to 60 min, a continuous channel fluctuation was observed over the tested time period (i.e., pauses in channel activity were not observed). A functional alteration of the calcium release channel with τ_o3/τ_o4 open states at a low open probability (below 0.4) has not been described for any of the known exogenous activators of the calcium release channel and appears to be unique for suramin. These suramin effects on the open probability and lifetime distribution contrast sharply with the effects of magnesium (or inhibitory calcium) in the absence of suramin, which caused the known reduction in P_o by decreasing the frequency of channel opening, a shift to τ_o1 open states and τ_c2/τ_c3 closed states (Table 2).

Apparent Dissociation between Single-Channel Current Fluctuation and [³H]Ryanodine Binding to HSR in the Presence of Suramin. Ryanodine binding is thought to occur only to the open calcium release channel (Pessah et al., 1987; Coronado et al., 1994; Meissner, 1994; Hasselbach and Migala, 1998). This interpretation is based on its dependence on channel-activating calcium and complete inhibition by ruthenium red (Table 5). Activation or inhibition of single-channel current fluctuations of the purified calcium release channel and [³H]ryanodine binding to HSR are usually in good agreement; i.e., an increase or decrease in P_o associated
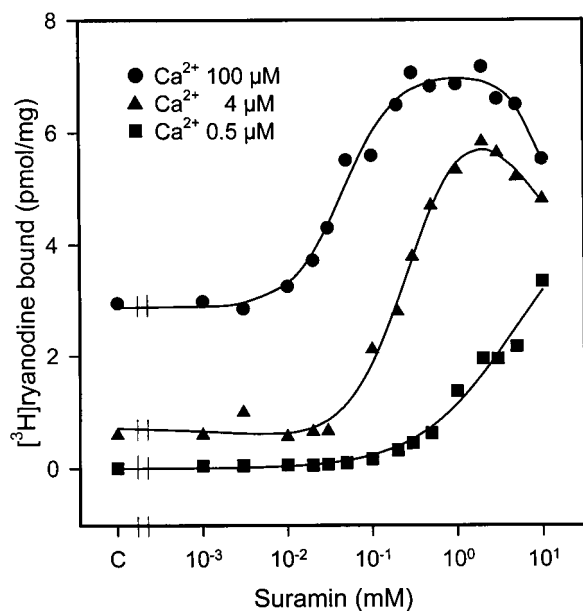

Fig. 9. Concentration dependence of suramin on specific [³H]ryanodine binding to HSR in the presence of 1 M NaCl at 100 μM, 4 μM, and 0.5 μM Ca^{2+}. Specific [³H]ryanodine binding was performed with 40 mM Mops/Tris, pH 7.0, 10 nM [³H]ryanodine, 1 M NaCl and protease inhibitors (see *Experimental Procedures*) at 37°C for 90 min in the presence of the indicated concentrations of suramin and 100 μM Ca^{2+} (○), 4 μM Ca^{2+} (△), or 0.5 μM Ca^{2+} (□). Data points are means of duplicate determinations of a representative experiment that was repeated twice. The solid line represents a fit of the data according to the Hill equation. The calculated parameters are means ± S.E.M. from three experiments: B_{max} = maximum [³H]ryanodine binding; EC_{50} = Ca^{2+} concentration giving half-maximum activation; n_H = Hill coefficient. Suramin independent binding (pmol/mg HSR): 2.23 ± 0.35 (100 μM Ca^{2+}), 0.40 ± 0.16 (4 μM Ca^{2+}), 0.006 ± 0.005 (0.5 μM Ca^{2+}). Suramin-dependent binding: B_{max} = 3.61 ± 0.78 pmol/mg, EC_{50} = 0.11 ± 0.04 mM, n_H = 2.15 ± 0.52 (100 μM Ca^{2+}). B_{max} = 6.06 ± 1.29 pmol/mg, EC_{50} = 0.24 ± 0.03 mM, n_H = 1.37 ± 0.13 (4 μM Ca^{2+}). B_{max} = 2.74 ± 1.18 pmol/mg, EC_{50} = 2.22 ± 1.32 mM, n_H = 1.12 ± 0.22 (0.5 μM Ca^{2+}).
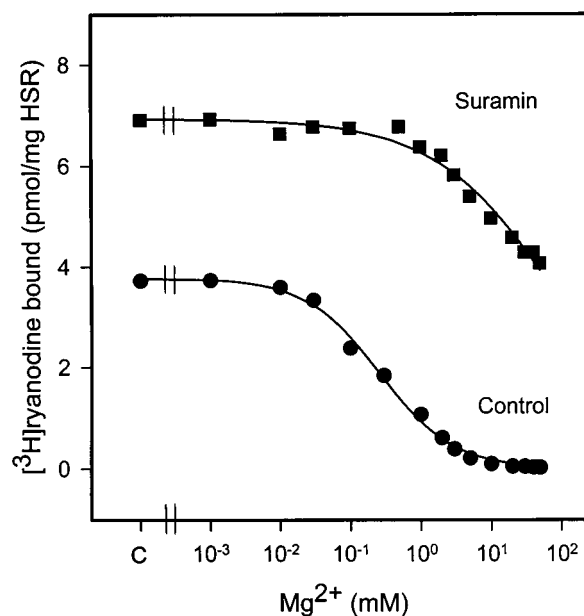

Fig. 10. Inhibition of specific [³H]ryanodine binding to HSR by magnesium in the absence and presence of 1 mM suramin. Specific [³H]ryanodine binding was performed in the absence (●) or presence of 1 mM suramin (■) with 40 mM Mops/Tris, pH 7.0, 10 nM [³H]ryanodine, 0.5 M CsCl, and protease inhibitors (see *Experimental Procedures*) at 37°C for 90 min. Data points are means from three experiments in the absence or presence of 1 mM suramin. Solid lines represent a fit of the data according to the Hill equation. Calculated parameters are means ± S.E.M. (n = 3); B_{max} = (maximum [³H]ryanodine binding); control, 3.80 ± 1.19 pmol/mg HSR; suramin, 6.92 ± 0.93 pmol/mg HSR. Mg^{2+} concentration giving half-maximum inhibition: control, 0.30 ± 0.12 mM; suramin, 83.4 ± 31.7 mM; the Hill coefficients were 0.81 ± 0.09 (control) and 0.52 ± 0.03 (suramin).

with an increase or decrease in [³H]ryanodine binding was shown with calmodulin (Tripathy et al., 1995, Suko et al., 2000), after oxidation of sulfhydryls of cysteines of the cal-
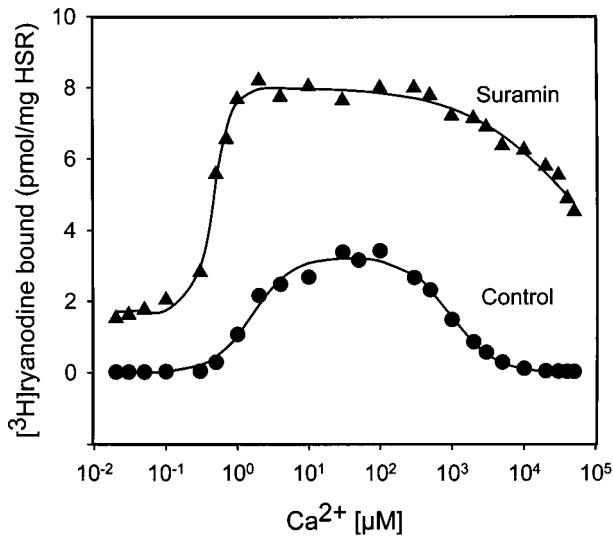

Fig. 11. Calcium dependence of control and suramin-stimulated [³H]ryanodine binding to HSR in the presence of 0.5 M CsCl. Specific [³H]ryanodine binding was performed in the absence (●) or presence of 1 mM suramin (▲) with 40 mM Mops/Tris, pH 7.0, 10 nM [³H]ryanodine, 0.5 M CsCl, and protease inhibitors (see *Experimental Procedures*) at 37°C for 90 min. Data points are means from three control experiments (absence of suramin) and three experiments in the presence of 1 mM suramin. The solid lines represent a fit of the data according to the sum of two Hill equations as described previously (Meissner et al., 1997; Suko and Hellmann, 1998). Calculated parameters are means ± S.E.M. (control, $n = 4$; suramin, $n = 3$): B_{max} = maximum [³H]ryanodine binding; K_a, Ca^{2+} concentration giving half-maximum activation; K_I, Ca^{2+} concentration giving half-maximum inhibition; n_a and n_i, number of activating or inhibiting calcium species; c_res, calcium-independent binding. Control, B_{max} = 3.16 ± 0.24 pmol/mg HSR; K_a = 1.60 ± 0.61 μM; n_a = 2.86 ± 0.48; K_i = 1175 ± 145 μM, n_i = 2.86 ± 0.48, c_res = 0.025 ± 0.006. Suramin, 1 mM, B_{max} = 6.60 ± 0.32 pmol/mg HSR; K_a = 0.46 ± 0.03 μM; n_a = 3.76 ± 0.64; K_i = 48153 ± 10719 μM; n_i = 0.51 ± 0.08; c_res = 1.78 ± 0.01.

TABLE 5

Inhibition of suramin-stimulated [³H]ryanodine binding by various agents.

[³H]Ryanodine binding was performed in the absence or presence of 1 mM suramin with 40 mM Mops/Tris, pH 7.0, 10 nM [³H]ryanodine, 0.5 M CsCl, protease inhibitors (see *Experimental Procedures*) and the indicated agents at 37°C for 90 min. Values are presented as means ± S.E.M. of total [³H]ryanodine binding from five experiments with different HSR preparations.

		Total [³H]Ryanodine Bound		n
		Control	1 mM Suramin	
		pmol/mg HSR		
None		3.38 ± 0.35	7.55 ± 0.69	5
Cold ryanodine	100 μM	0.051 ± 0.010	0.09 ± 0.02	5
Ruthenium red	10 μM	0.058 ± 0.011	0.53 ± 0.03	5
	20 μM	0.045 ± 0.004	0.39 ± 0.02	3
EGTA	5 mM	0.046 ± 0.005	1.11 ± 0.05	3
	10 mM	0.044 ± 0.002	1.16 ± 0.01	2
MgCl2	10 mM	0.132 ± 0.025	5.38 ± 0.46	5
	30 mM	0.077 ± 0.013	4.69 ± 0.45	5
	50 mM	0.066 ± 0.012	4.50 ± 0.44	5
CaCl2	10 mM	0.161 ± 0.030	5.64 ± 0.47	5
	30 mM	0.084 ± 0.007	4.93 ± 0.46	5
	50 mM	0.061 ± 0.008	4.02 ± 0.41	5
4-CMPS	100 μM	0.262 ± 0.062	0.31 ± 0.08	5
	200 μM	0.204 ± 0.042	0.22 ± 0.05	5
Neomycin	1 mM	0.680 ± 0.012	7.07 ± 0.56	5
Tetracaine	1 mM	0.265 ± 0.046	5.46 ± 0.41	3

cium release channel (Suko and Hellmann, 1998; Suko et al., 2000) and for suramin or the suramin analog NF307 (present study; Hohenegger et al., 1996; Klinger et al., 1999).

In the present study, inhibition of single-channel current recordings and [³H]ryanodine binding by magnesium in controls (absence of suramin) showed a close correlation (half-maximum inhibition of P_o, 0.38 mM Mg^{2+}; [³H]ryanodine binding, 0.30 mM Mg^{2+}; Table 2, Fig. 10). However, in the presence of suramin half-maximum inhibition of P_o occurred at magnesium concentrations (0.82 mM Mg^{2+}) 2-fold higher than in controls, but at 50- to 100-fold higher magnesium concentrations (83 mM Mg^{2+}) in [³H]ryanodine binding to HSR (Table 4; Fig. 10). One possible reason for the apparent discrepancy between single-channel current recordings and equilibrium [³H]ryanodine binding might have originated from the different time periods of suramin exposure. In contrast to the single-channel current recordings, [³H]ryanodine binding measures channel alterations on a low time scale. The dissociation between single-channel current measurements in the presence of suramin and equilibrium [³H]ryano-
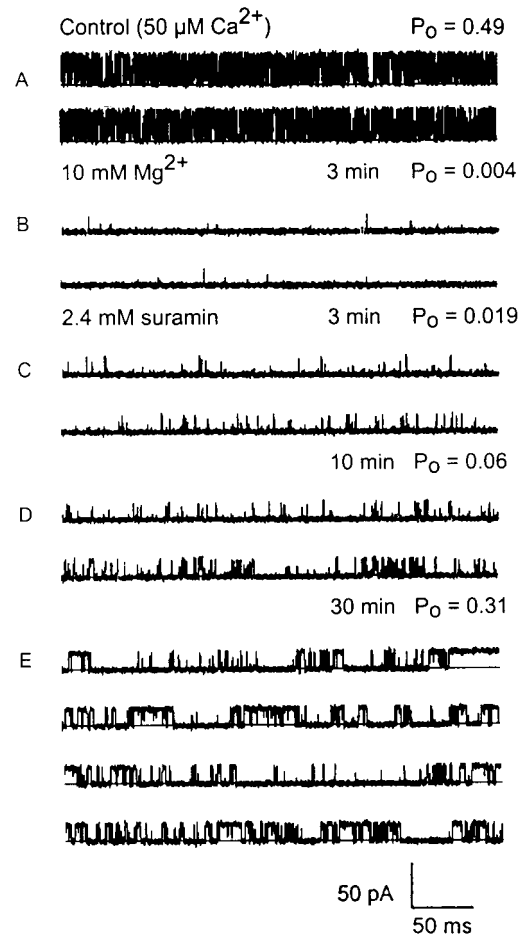

Fig. 12. Long-term effect of suramin on a single purified skeletal muscle calcium-release channel in the presence of 10 Mg^{2+}. Single-channel currents, shown as upward deflections, were recorded at +20 mV holding potential with 480 mM/50 mM CsCl (*cis/trans*). The solid lines indicate the baselines. Control and test records are from the same channel; 400-ms recordings. Mg^{2+} and suramin were added sequentially to the *cis* side. A, control, 50 μM Mg^{2+}. B, inhibition of the channel by 10 mM Mg^{2+}. C, suramin (2.4 mM; 3 min). D, suramin (2.4 mM; 10 min). E, suramin (2.4 mM; 30 min). Calibration bars represent 50 pA and 50 ms. Channel open probabilities (P_o) were calculated from 2250 events (10 mM Mg^{2+}), 24,000 events (10 mM Mg^{2+} plus 2.4 mM suramin), respectively.

dine binding data may be caused in part by the fact that [^{3}H]ryanodine binding (carried out at 37°C over 90 min) predominantly monitored the long-term suramin-induced alteration of the calcium release channel. In line with the
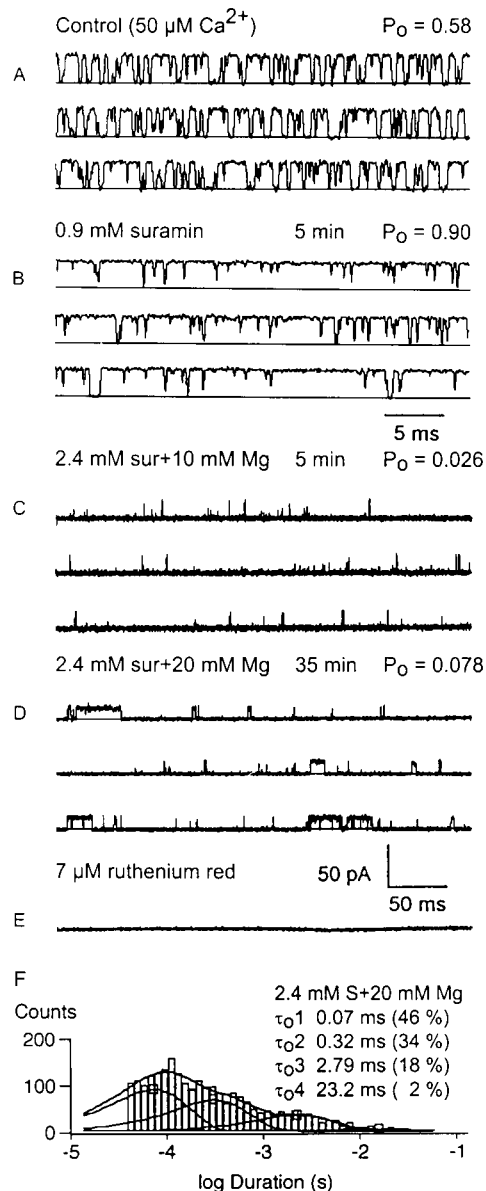
above argument is the observation that agents, such as 4-CMPS (100 μM), showed a fast calcium release from HSR vesicles, a transient maximal channel opening ($P_o \geq 0.9$) followed by a marked channel inhibition within a few minutes in single-channel current recordings, whereas equilibrium [^{3}H]ryanodine binding solely detected channel inhibition (Suko and Hellmann, 1998). On the other hand, additional effects on [^{3}H]ryanodine binding in the presence of suramin might have occurred as judged from the incomplete reduction in [^{3}H]ryanodine binding by ruthenium red, the
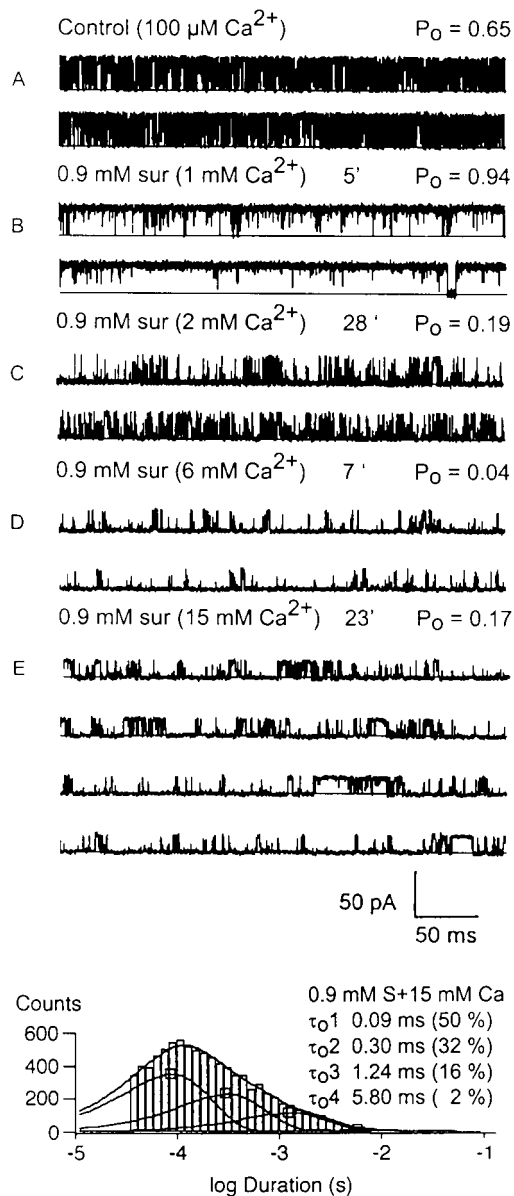

Fig. 13. Long-term effect of suramin on a single purified skeletal muscle calcium-release channel in the presence of 20 mM Mg^{2+}. Single-channel currents, shown as upward deflections, were recorded at +20 mV holding potential with 480 mM/50 mM CsCl (*cis/trans*). The solid lines indicate the baselines. Control and test records are from the same channel; 40-ms recordings (A, B), 400-ms recordings (C-E). Ca^{2+}, suramin and Mg^{2+} were added sequentially to the *cis* side. A, control, 50 μM Ca^{2+}. Activation of the channel by 0.9 mM suramin. C, suramin (2.4 mM) plus 10 mM Mg^{2+} (5 min). D, suramin (2.4 mM) plus 20 mM Mg^{2+} (35 min); note the appearance of long open states. E, ruthenium red (7 μM), closed the channel. Calibration bars represent 50 pA and 5 ms (A, B) or 50 ms (C-E). F, open lifetime histograms, cumulative mean open channel time constants (τ_o) and percentage areas in the presence of 20 mM Mg^{2+} plus 2.4 mM suramin (D). The solid lines represent a fit according to four exponential values. Channel open probabilities (P_o) and (τ_o) were calculated from 40,200 events (control), 30,000 events (0.9 mM suramin), 2,300 events (2.4 mM suramin plus 10 mM Mg^{2+}), and 4,542 events (2.4 mM suramin plus 20 mM Mg^{2+}; evaluated from 200 400-ms recordings), respectively.

Fig. 14. Long-term effect of suramin on a single purified skeletal muscle calcium-release channel in the presence of 10 to 15 mM Ca^{2+}. Single-channel currents, shown as upward deflections, were recorded at +20 mV holding potential with 480 mM/50 mM CsCl (*cis/trans*). The solid lines indicate the baselines. Control and test records are from the same channel; 400-ms recordings. A, control, 100 μM Ca^{2+}. B, activation with 0.9 mM suramin plus 1 mM Ca^{2+} (5 min). C, inhibition of the channel by 2 mM Ca^{2+} (28 min). D, inhibition of the channel by 6 mM Ca^{2+} (7 min). E, activation of the channel by 10 mM Ca^{2+}(15 min) and 15 mM Ca^{2+} (8 min). τ_o are given for 0.9 mM suramin plus 15 mM Ca^{2+}. Calibration bars represent 50 pA and 50 ms. Channel open probabilities (P_o) at 15 mM Ca^{2+} were calculated from 13,000 events.

negligible effect of tetracaine or neomycin, and the presence of an apparently calcium-independent [3H]ryanodine binding (Table 5). In single-channel current recordings all tested suramin-activated channels were completely closed by 7 to 14 μM ruthenium red.

Additive Channel Activation by Suramin and Sulfhydryl Oxidation. It has been shown with cardiac HSR that suramin does not act via oxidation of sulfhydryls of the calcium release channel (Sitsapesan and Williams, 1996). Sulfhydryl oxidation of the skeletal muscle calcium release channel by 4-CMPS (Oba et al., 1996; Suko and Hellman, 1998; Suko et al., 1999, 2000) or thiomersal (Abramson et al., 1995; Marengo et al., 1998) increased the apparent calcium affinity of the high-affinity calcium binding sites (Marengo et al., 1998; Suko and Hellmann, 1998). The suramin-induced small increase in P_o at subactivating free calcium of 0.05 μM was markedly increased by addition of 4-CMPS, indicating that the suramin and sulfhydryl-induced activation of the calcium release are additive (Fig. 2D; Table 1C). On the other hand, the 4-CMPS–induced inhibition of the calcium release channel eliminated the suramin-induced channel activation. Notably, 100 μM 4-CMPS inhibited [3H]ryanodine binding to a greater extent than 20 μM ruthenium red (Table 5).

Mechanism of Suramin-Induced Channel Activation. The mechanism of the suramin-induced activation of the calcium release channel is not clear. Suramin induced several alterations of channel function: 1) An increase in the apparent calcium affinity to the activating high-affinity calcium binding sites (Table 1; Fig. 11). 2) A reduction of the apparent magnesium affinity to the inhibitory low affinity calcium/magnesium binding site(s) (Tables 2 and 4; Meissner, 1994; Laver et al., 1997). 3) Suramin released the calcium release channel bound to calmodulin Sepharose (Klinger et al., 1999) and inhibited 125I-calmodulin binding to HSR (Suko et al., 2000). These multiple effects exclude a direct interaction with the above ligand binding sites. These data suggest that suramin binding to the calcium release channel, affects the affinity of calcium, magnesium, and calmodulin indirectly by an allosteric mechanism caused by alteration in subunit interactions.

In the case of the calcium binding protein calmodulin (Klee and Vanaman, 1982; Babu et al., 1985; Suko et al., 1986) suramin reversed completely the inhibition of the calcium release channel by Ca-calmodulin (0.1–5 μM), similar to that reported for sulfhydryl oxidation, which did not influence 125I-calmodulin binding to HSR (Suko et al., 2000). In several experiments, calcium release channels were first inhibited by low CaM concentrations (0.1 μM) and reactivated by suramin (0.3 or 0.6 mM) followed by addition of high calmodulin concentrations (1–5 μM); these high concentrations of CaM were unable to reduce the suramin-induced channel activation, which supports an indirect effect on Ca-CaM binding site(s). The location of the suramin binding site(s) has not been identified yet, and it may be identical to the DIDS binding site as postulated by Sitsapesan (1999) from a similar increase in current amplitude (about 20%) and conductance of the cardiac calcium release channel by suramin and DIDS, measured in the presence of 50 mM/10 μM Ca^{2+} *trans*/*cis*.

In summary, suramin induced multiple functional alterations of the skeletal muscle calcium release channel. The most prominent effect was the time-dependent, slowly devel-oping activation of the calcium release channel with long open states at an overall low open probability in the presence of high, inhibitory concentrations of magnesium or calcium. This effect can at least partly explain the apparent dissociation between single-channel current recordings and [3H]ryanodine binding.

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